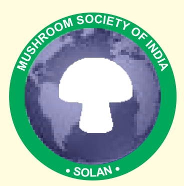


# Proceedings of 8th International Conference on Mushroom Biology and Mushroom Products

## Volume I

World Society of Mushroom Biology and Mushroom Products
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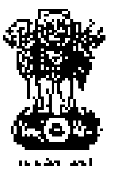

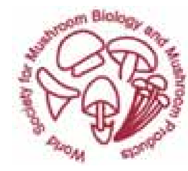

# Proceedings of 8th International Conference on Mushroom Biology and Mushroom Products

# Volume I

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Chairman Steering Committee, 8th ICMBMP

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# PREFACE

The eighth International Conference on Mushroom Biology and Mushroom Products is a continuum of the conferences organized by World Society of Mushroom Biology and Mushroom Products since 1993 after every three years in one or other part of the world. The seventh conference was held in Arcachon, France in October 2011 where we agreed to hold the eighth conference in India. Considering the changes taking place all over the globe and impetus towards growth of mushroom research and development in India, this was an apt decision and the interactions during the event will help all of us to promote the research in various facets of mushroom biology and mushroom products.

We thank our colleagues from all parts of the world for their overwhelming response to the call for presentations and participation in eighth International Conference on Mushroom Biology and Mushroom Products jointly organized by World Society of Mushroom Biology and Mushroom Products (WSMBMP), ICAR-Directorate of Mushroom Research, Solan (ICAR-DMR) and Mushroom Society of India (MSI) at New Delhi from 19-22 November 2014.

In this conference there were 231 contributions as abstracts that were grouped into 10 sessions. Full papers were received for 90 presentations. The presentations were grouped into 10 sessions that are: (i) Biodiversity and taxonomy, (ii) Genomics, genetics and breeding, (iii) Bioinformatics and nanotechnology, (iv) Biology, biochemistry, physiology and development, (v) Waste conversion & utilization, substrates, casing and crop management, (vi) Myco-molecules, medicinal, nutritional and nutraceutical properties, (vii) Mycorrhizal, entomopathic and other novel mushrooms, (viii) Pests and diseases, (ix) Value addition and mushroom products and (x) Economics, social, IT and marketing issues. Volume I contains the papers included in sessions I to V.

I would like to thank all members of Scientific Advisory Committee and Organizing Committee for their help and support. I particularly thank my colleagues at ICAR-DMR who have been working for this conference since the inception of the concept to organize the conference in India. We thank Indian Council of Agricultural Research and Department of Agriculture and Cooperation, Government of India for their financial support for this conference. I thank Mrs. Sunila Thakur for her secretarial support.

(Manjit Singh)
Chairman, Steering Committee

# CONTENTS (Volume I)


| Sr. No. | Title | Author(s) | Page No. |
|---|---|---|---|
| | **THEME LECTURE** | | |
| 1. | A global perspective on the high five: *Agaricus, Pleurotus, Lentinula*, *Auricularia* & *Flammulina* | Daniel J Royse | 1 |
| | **SESSION-I: BIODIVERSITY AND TAXONOMY** | | |
| | **Keynote Presentation** | | |
| 2. | Mushroom biodiversity in India-prospects and potential | TN Lakhanpal | 7 |
| | **Oral Presentations** | | |
| 3. | Collection, identification and morphological characterization of indigenous mushrooms in coastal Kenya | Gateri MW, Ndung UB, Muriuki AW, Rauwl V and Kabacia S | 17 |
| 4. | Diversity of resupinate, non-poroid agaricomycetous fungi in the Himalaya and adjoining areas | Gurpaul Singh Dhingra | 24 |
| 5. | Conservation and characterization of *Pleurotus* variability of India | Meera Pandey, Rajesh Babu D, Satisha G, Shivshankara KS, Shamina Azeez, Tapas Roy, Mazhar Jameel, Nandini R, Punita Kumari and P Swarnlatha | 42 |
| 6. | Medicinal mushrooms in Italy and their *ex situ* conservation through culture collection | E Savino, C Girometta, S Chinaglia, M Guglielminetti, M Rodolfi, A Bernicchia, C Perini, E Salerni and AM Picco | 50 |
| 7. | Family *Pluteaceae* in North West India | Munruchi Kaur and Yadwinder Singh | 55 |
| 8. | Diversity of macrofungal communities in Chikmagalur district of Western Ghats, India | Krishnappa M, Swapna S and Syed Abrar | 71 |
| 9. | Diversity of poroid mushrooms in Punjab: family Hymenochaetaceae | Avneetpal Singh, Gurpreet Kaur and Gurpaul Singh Dhingra | 83 |
| | **Poster Presentations** | | |
| 10. | Diversity of wild mushroom flora from Indian Thar desert | Praveen Gehlot, Ruchika Sharma and Kamna Sharma | 92 |
| 11. | Ethnomycology in the “Tacaná volcano biosphere reserve”, Chiapas, Mexico | Medina Arias FG, Andrade Gallegos RH and Sánchez JE | 98 |
| | **SESSION-II: GENOMICS, GENETICS AND BREEDING** | | |
| | **Oral Presentations** | | |
| 12. | Spawn cryopreservation of *Agaricus bisporus* and *A. subrufescens* strains | Gerardo Mata, Rosalía Pérez Merlo, Jean Michel Savoie and Dulce Salmones | 108 |
| 13. | Phylogenetic relationships of *Ganoderma* species based on mitochondrial and nuclear DNA sequences from Tamil Nadu, India | K Malarvizhi | 113 |

| Sr. No. | Title | Author(s) | Page No. |
|---|---|---|---|
| 14. | DNA markers reveal genome-wide variations in the strains of button mushroom (*Agaricus bisporus*) | Mahesh C Yadav | 122 |
| 15. | Genetic variability in strains of *Volvariella volvacea* collected from the state of Odisha, India | OP Ahlawat, KB Mohapatra, Harleen Kaur and Manjit Singh | 135 |
| | **Poster Presentations** | | |
| 16. | Genetic characterization of single spore isolates of *Agaricus bisporus* (Lange) Imbach | Geeta Sharma, SK Mishra and KPS Kushwaha | 145 |
| 17. | Sequence analysis of partial *tef1*α and *rpb2* genes of different *Pleurotus eryngii* isolates by means of PCR-RFLP | József Szarvas, Károly Pál and Andras Geösel | 151 |
| | **SESSION-III: BIOINFORMATICS AND NANOTECHNOLOGY** | | |
| | **Oral Presentations** | | |
| 18. | The whole genome sequence of *Volvariella volvacea* will facilitate resolution of problems limiting its commercial exploitation | Dapeng Bao, Mingjie Chen, Hong Wang and Qi Tan | 160 |
| 19. | Identification of the WRKY transcription factors in *Agaricus bisporus* (white button mushroom) | Shwet Kamal, Manjit Singh, RC Upadhyay, VP Sharma, Mamta Gupta, V Arunachalam and Pat Heslop Harrison | 167 |
| | **SESSION-IV: BIOLOGY, BIOCHEMISTRY, PHYSIOLOGY AND DEVELOPMENT** | | |
| | **Keynote Presentations** | | |
| 20. | Fungal unspecific peroxygenases : a new generation of oxygen-transferring biocatalysts | Martin Hofrichter, René Ullrich, Harald Kellner, Ramesh C Upadhyay and Katrin Scheibner | 172 |
| | **Oral Presentations** | | |
| 21. | A study on bioactive fluorescent compounds from selected mushrooms and its antimicrobial activity | Sadasivam Mona, D Priya Tharsani, Subramanian Chandra Sekarenthiran and Karuppan Perumal | 182 |
| 22. | Anti-fatigue effects of *Agaricus bisporus* extract in rats | Naveen S and Anilakumar KR | 185 |
| 23. | Comparative study on the production, purification and characterization of exopolysaccharides from oyster mushrooms *Pleurotus florida* and *Hypsizygus ulmarius* and their applications | Karunagaran Latha and Ramakrishnan Baskar | 192 |
| 24. | Purification and characterization of an *N*-acetyl-D-glucosamine specific lectin from the Australian mushroom *Psathyrella asperospora* | Mohamed Ali Abol Hassan, Razina Rouf , Joao P Ribeiro, Fabia Jäggin, Christopher J Day, Annabelle Varrot, Evelin Tiralongo, Tom W May, Anne Imberty and Joe Tiralongo | 199 |
| 25. | Antioxidant and functional properties of β–glucan extracted from edible mushrooms *Agaricus bisporus, Pleurotus ostreatus* and *Coprinus atramentarius* | Asma Ashraf Khan, Adil Gani, FA Masoodi, Shaheen Kousar and Mudasir Ahmad | 210 |
| 26. | Qualitative phytochemical screening, total phenolic content and *in vitro* antioxidant activity in methanolic extracts of *Morchella esculenta* Fr. | Monika Thakur and TN Lakhanpal | 215 |

| Sr. No. | Title | Author(s) | Page No. |
|---|---|---|---|
| 27. | SEM study of exosporial ornamentation of basidiospores in genus *Russula*, *Lactarius* and *Lactifluus* from North-West Himalaya | NS Atri, Munruchi Kaur and Samidha Sharma | 221 |
| 28. | Lignolytic enzymes of *Calocybe indica* | RC Upadhyay and Tripathi Astha | 225 |
| | **Poster Presentations** | | |
| 29. | Growth of the edible mushroom *Pleurotus ostreatus* on different concentrations of di (2-ethyl hexyl) phthalate in solid and in liquid media | Gabriela Córdoba-Sosa, Angel González-Márquez, José Luis Torres, Miriam Ahuactzin -Perez, Gerardo Díaz-Godínez, Rubén Díaz Godinez and Carmen Sánchez | 231 |
| 30. | Laccase gene expression of *Pleurotus ostreatus* grown at different pH of the liquid culture medium | Díaz Rubén Godinez, Sánchez Carmen and Díaz-Godínez Gerardo | 238 |
| 31. | Laccases and manganese peroxidases of *Pleurotus ostreatus* grown in solid-state fermentation | Varela-Floriano V, Díaz R, Tellez-Tellez M, Sanchez C and Diaz-Godinez G | 243 |
| 32. | Identification of ergosterol in mushrooms | Shiveta Raina, Harpreet Singh Sodhi and Shikha Sethi | 247 |
| | **SESSION-V: WASTE CONVERSION & UTILIZATION, SUBSTRATES, CASING AND CROP MANAGEMENT** | | |
| | **Keynote Presentations** | | |
| 33. | Biochemical features influencing mushroom-substrate compatibility | John Buswell | 252 |
| 34. | Production of spawn for lignicolous mushroom in a clean room- environment: risk analysis and rules of conduct | Magda Verfaillie and Kasper Moreaux | 258 |
| 35. | Biotechnological potential of ten *Pleurotus djamor* strains | Lilia Moreno Ruiz, René H Andrade, José E Sánchez and El Colegio De La Frontera Sur | 262 |
| 36. | *Lentinula edodes* cultivation techniques and models in China | Qi Tan, Xiaodong Shang, Lujun Zhang, Yu Li, Hailong Yu, Feng Zhou, Meiyan Zhang and Chunyan Song | 271 |
| | **Oral Presentations** | | |
| 37. | Biological efficiency of wild type and commercial *Agaricus bisporus* strains | Johan Baars, Patrick Hendrix and Anton Sonnenberg | 274 |
| 38. | Vegetative growth of different strains of *Pleurotus* and *Lentinula* species on cassava (*Manihot esculenta*) and yam (*Dioscorea rotundata*) wastes in Ghana | Dzomeku M, Narh Mensah DL, Obodai M, Prempeh J, Takli RK, Komlaga GA, Dziedzoave N and Sonnenberg A | 280 |
| 39. | Cultivation of oyster mushrooms on cassava waste | Anton SM Sonnenberg, Johan JP Baars, Mary Obodai and Agnes Asagbra | 286 |
| 40. | Exploitation of thermophilic fungi in compost production for white button mushroom (*Agaricus bisporus*) cultivation- A review | B Vijay and Ashutosh Pathak | 292 |

| Sr. No. | Title | Author(s) | Page No. |
|---|---|---|---|
| 41. | Investigation into the microbial community changes that occur in the casing layer during cropping of the white button mushroom, *Agaricus bisporus* | John Pecchia, Rachel Cortese and Istvan Albert | 309 |
| 42. | Performance of straw mushroom (*Volvariella volvacea*) raised as an intercrop in coconut plantations of coastal Odisha | KB Mohapatra and Niranjan Chinara | 314 |
| 43. | The research, development and application of the fermentation accelerator, a new material for edible mushroom cultivation on unsterilized substrate | Han Shenghua and Ye Caiyun | 318 |
| 44. | Effect of *Pleurotus ostreatus* colonized substrate on broiler chicken growth | Mateus Santos, Luiz Alberton, Juliana Valle, Giani Linde and Nelson Colauto | 323 |
| 45. | Drip irrigation, a new way of watering *Agaricus bisporus* cultivation: increased production and lower carbon footprint | Ofer Danay, Dov Raz, Nirit Ezoz, Ilana Barski and Dan Levanon | 325 |
| | **Poster Presentations** | | |
| 46. | An innovative method of preparation of healthy grain spawn | Arvind Kumar Paswan and RN Verma | 330 |
| 47. | Comparative cultivation experiments of *Pleurotus eryngii* isolates | József Szarvas, András Geösel and Anna Szabó | 337 |
| 48. | Comparative evaluation of casing mixtures on the yield potential of button mushroom (*Agaricus bisporus*) | Ram Chandra, VN Pandey and HB Singh | 345 |
| 49. | Evaluation of different strains of oyster mushroom for their cultural, morphological and yield attributes | Rakesh Kumar and KPS Kushwaha | 351 |
| 50. | Optimizing mushroom spawn production in Uganda | Pradeep Malakar, Qi Tan, Mingjie Chen, Zengtao Xing, John Buswell, Qui Sun, MR Xiaoping Wei, Charles Kwesiga, Arthur Tugume, Peace Byandusya, Magda Verfaillie, Janiffer Mayambala, Perpetua Ipulet and James Akanyijuka | 356 |
| 51. | Quality improvement of casing material and yield in milky mushroom (C*alocybe indica*) by using biofertilizers and different substrates | Ashok C Jadhav, Dattatray B Shinde, Santosh B Nadre and Darshan S Deore | 359 |
| 52. | Yield performance and element profiling of different strains of *Lentinula edodes* (Berk.) Pegler | Neelam, KPS Kushwaha and SK Mishra | 365 |
| 53. | Use of *Trichoderma* enriched button mushroom spent substrate (TEBMSS) for enhancing yield and quality of Kinnow mandarin | Chandra Bhanu, JP Singh and B Gangwar | 369 |

# A GLOBAL PERSPECTIVE ON THE HIGH FIVE: *AGARICUS*, *PLEUROTUS*, *LENTINULA*, *AURICULARIA* & *FLAMMULINA*

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## ABSTRACT

World production and consumption of mushrooms has increased at a rapid rate, especially since the mid 1990s. Not only has production and consumption increased as the world's population has increased, but *per capita* consumption of mushrooms has increased as well. Over a 15-year period (1997 to 2012), *per capita* consumption of mushrooms increased from about 1 kg/year to over 4 kg/year. China is the main producer and consumer of mushrooms. The demand for mushrooms has been phenomenal – production to meet the growing demand is a performance seldom duplicated in agriculture today.

**Keywords:** world mushroom production, *per capita* consumption

## INTRODUCTION

The worldwide mushroom industry has grown at a rapid rate since the late 1990s (Fig.1). World mushroom production has increased more than 25-fold during the last 35 years (from about 1 billion kg in 1978 to about 27 billion kg in 2012). This is a remarkable accomplishment, especially considering the human population has increased 1.7-fold during that same period (from about 4.2 billion in 1978 to about 7 billion in 2012). Thus, *per capita* consumption of mushrooms has increased at a relatively rapid rate and now exceeds 4 kg/person compared to only about 1 kg in 1997. In 2012, nearly all consumption of mushrooms in China, the EU and India was supplied from domestic sources. Alternatively, nearly all mushroom consumption in Russia was supplied from imports while consumption in the United States, Canada, Japan and Australia was supplied mostly by domestic sources but also by substantial amounts of imports (USITC 2010).

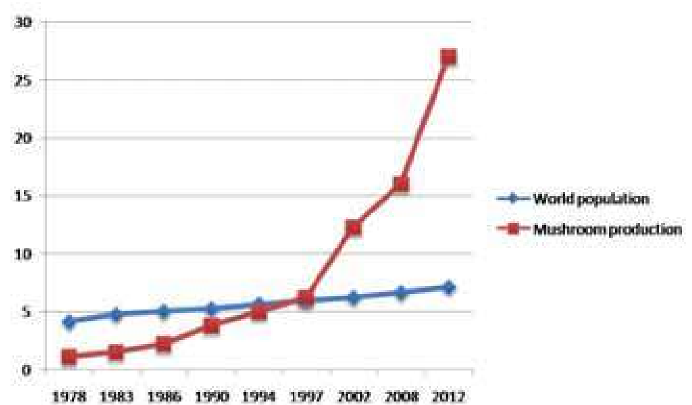


**Figure 1.** World population (billion) vs. total world mushroom production (billion kg)

Five main genera constitute ca. 85% of the world's mushroom supply (Fig. 2). *Agaricus* (primarily *A. bisporus* with some *A. brasilensis*) is the major genus, contributing about 30% of the world's cultivated mushrooms. *Pleurotus*, a close second, with 5 to 6 cultivated species, constitutes about 27% of the world's output while *Lentinula edodes* (shiitake), contributes ca. 17%. The other two genera, *Auricularia* and *Flammulina* are responsible for 6% and 5% of the volume, respectively.

China is the main producer of edible mushrooms. The Chinese national government has increasingly encouraged Chinese growers to shift their agricultural production out of traditional crops to value-added crops like mushrooms for export (USITC 2010). Estimates of mushroom production in China from 2002 to 2010 vary considerably (Fig. 3). For example, FAOSTAT (2014) estimates mushroom and truffle production in China in 2002 at 2.8 million t while the Chinese Edible Fungi Association (CEFA) estimates production at 9 million t – a 3-fold difference. This conflict is even more pronounced in 2010 where FAOSTAT estimates production at 4.8 million t while CEFA estimates volume at 21.52 million t – a 4.5-fold difference. While these discrepancies remain unexplained, they may be related to the consideration of primarily *A. bisporus*

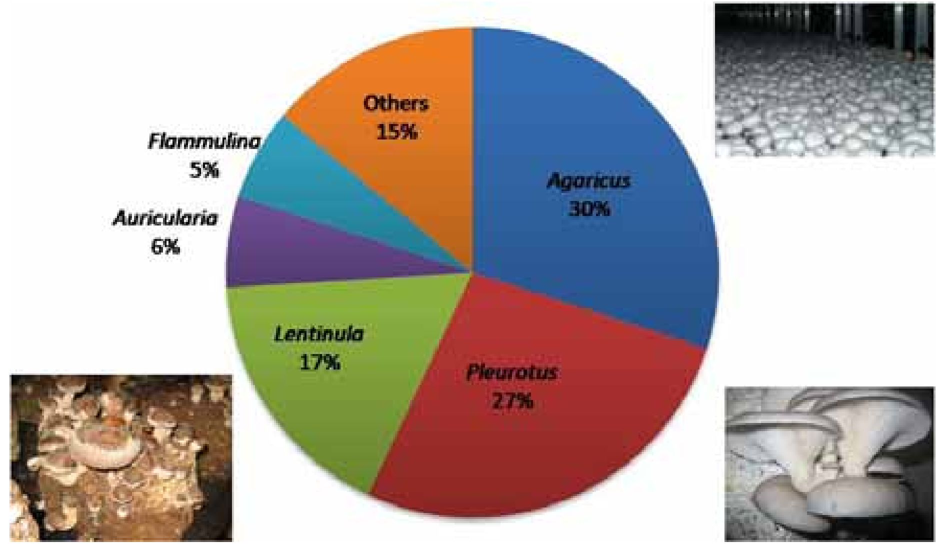


**Figure 2.** Estimated percentage of world production of edible mushrooms by genus in 2010

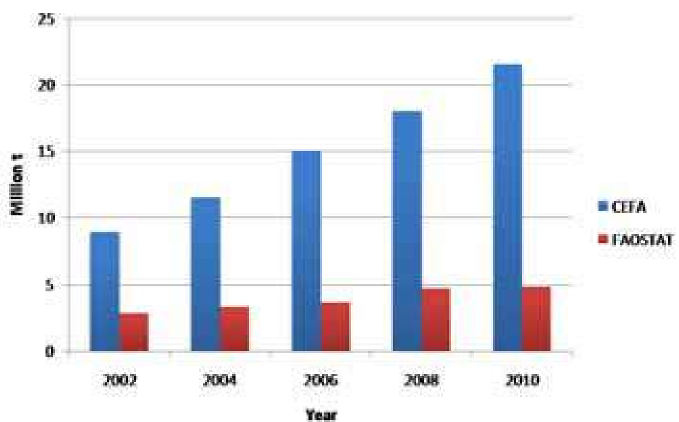


**Figure 3.** Conflicting estimates of mushroom production in China (2002-2010): Chinese Edible Fungi Association (CEFA, Li 2012) and Food and Agriculture Organization of the United Nations (FAOSTAT, 2014)

in some figures supplied by FAOSTAT and to some degree of over estimation by CEFA. Regardless, China is a very large producer of mushrooms with as many as 30 million growers, processors, suppliers and merchandizers involved in the industry (Chang, 2005).

## *AGARICUS BISPORUS*

Production of *A. bisporus* has continued to increase worldwide especially since the 1950s. Beginning in about 1998, China became the world's leading producer of this species (Chang, 2005). According estimates provided by the CEFA (Li, 2012), China produced over 2.18 million t in 2010, which is about 6 times the volume (350 thousand t) in the United States, the second largest producer of *A. bisporus*.

In the last few years, production of mushrooms in China has gradually moved northward as climatic conditions in the northern provinces are more conducive for mushroom production and raw materials are more readily available compared to southern provinces. Li (2012) expects this trend to continue for the foreseeable future. In the United States, production of *A. bisporus* has increased only about 4.6% over the last 10 years. All of the growth in production of this species has occurred in the brown varieties (portabella and crimini) with the white variety showing a slight decline (-3%) over this period (Royse, 2013).

In the Netherlands, the third largest producer of *A. bisporus*, over 90% of production is in the southeastern part of the country, *i.e.*, in the provinces of Limburg, Brabant and Gelderland (Baars, 2012). Approximately 90% of the crop is exported either as canned or frozen (60%) while nearly 30% is exported as fresh mushrooms. The UK purchases about 41% of the fresh supply while Germany, France, Norway, Belgium and Sweden bought most of the remainder of the fresh mushrooms.

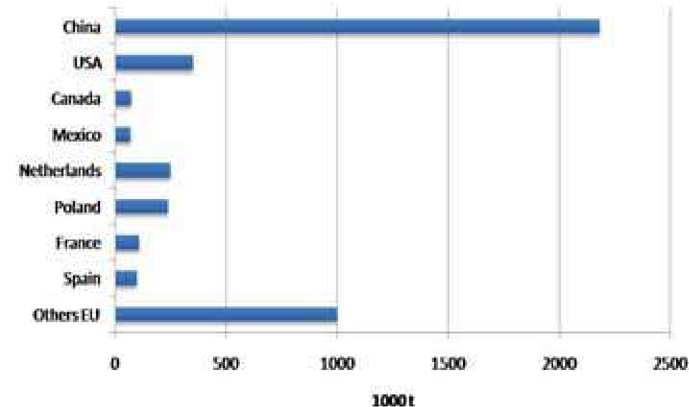


**Figure 4.** Estimated production of *Agaricus bisporus* in selected countries in 2010

Production of *A. bisporus* in Europe continues to move eastward (Royse, 2013). Poland has become a major producer of mushrooms and now nearly equals the output of the Netherlands. Many Dutch-style farms have been constructed recently in Poland – especially in the eastern part of the country (Bieniecka and Dreve, 2012). Nearly 80% of Poland's mushrooms are destined for the fresh market, with Russia and countries in Western Europe importing substantial quantities. Currently, about 90% of the Russian market is supplied by Poland but it is anticipated that the Ukraine will capture a considerable portion of that market in the not-too-distant future (Rozendaal, 2012).

## *PLEUROTUS* SPP.

Oyster mushroom production has increased at a rapid rate worldwide in the last few years (Table 1). From 1997 to 2010, *Pleurotus* spp. production increased from 876 t to 6,288 t (618%). China was responsible for most of the production increase and accounted for over 85% of the world's total output in 2010. Approximately 25% of China's mushroom production in 2010 was from two species of *Pleurotus*: *P. ostreatus* and *P. cornucopiae*. In the last five years or so, however, substantial increases in production of *P. eryngii* and *P. nebrodensis* have occurred. In China, administrative and professional agencies are developing plans to guide farmers in their selection of regions where production and utilization of resources may be optimized for mushroom production. The northeast of China, where the climate is cool and sawdust readily available, is particularly suitable for production of *P. ostreatus*, *P. eryngii* and *P. nebrodensis* (Li, 2012).

In Japan, production of *Pleurotus* spp. increased 198% from 1997 (13,300 t) to 2010 (39,600 t) (Table 1). *Pleurotus eryngii* experienced the largest gains in production, in terms of percentage (+452.8%), increasing from 6,734 t in 2000 to over 37,000 t in 2009 (Yamanaka, 2011). Most *P. eryngii* is cultivated on sawdust of Japanese cedar, or ground corncobs supplemented with bran, and contained in polypropylene bottles.

Other countries in Asia, particularly South Korea, Taiwan, Thailand, Vietnam and India are major producers of mushrooms. Oyster mushroom production in these and other Asian countries collectively have risen nearly 800% from 1997 to 2010 (Table 1).

**Table 1.** Comparison of production of *Pleurotus* spp. in various countries in 1997 and 2010

| Country | Production (x1,000 t) | | | | % increase 1997-2010 |
|---|---|---|---|---|---|
| | 1997 | % | 2010 | % | |
| China | 760.0 | 86.8 | 5,391.0 | 85.7 | 609 |
| Japan | 13.3 | 1.5 | 39.6[a] | 0.6 | 198 |
| Rest of Asia | 88.4 | 10.1 | 786 | 12.5 | 789 |
| North America | 1.5 | 0.2 | 8.6 | 0.1 | 473 |
| Central & South America | 0.2 | - | 1.5 | - | 650 |
| EU | 6.2 | 0.7 | 32.0 | 0.5 | 416 |
| Rest of Europe | 5.8 | 0.7 | 28.0 | 0.5 | 383 |
| Africa | 0.2 | - | 1.3 | - | 550 |
| **Total** | **875.6** | **100** | **6,288** | **99.9** | **618** |

Sources: Chang 1999, Li 2012, USDA 2014, Sanchez and Mata 2012, Royse 2013; [a]2009 production (Yamanaka 2011)

## *LENTINULA EDODES*

Until the late 1980s, Japan was the world's main producer of *L. edodes* (shiitake) (Fig. 5). Shiitake was traditionally cultivated in Japan on natural logs of the shii tree – thus the derivation of the name shiitake. Using sawdust-based techniques that reduces crop cycle time and increases production efficiency, however, China became the major producer of shiitake

by 1990. From 1995 to 2000, Chinese farmers increased shiitake production from about 0.5 million t to over 2 million t – a huge increase by most standards of measuring change. In 2012, it is estimated that China produced over 4 million t of shiitake and now accounts for more than 90% of total production. Entire communities in China have been lifted from poverty because of the economic opportunity afforded to them by growing shiitake (Chang, 2005).

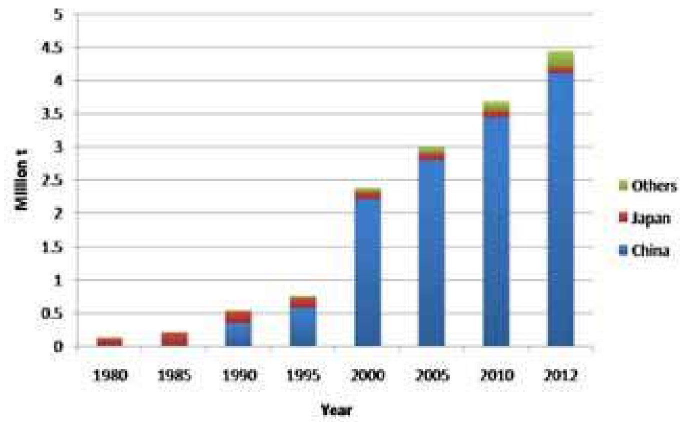


**Figure 5.** Growth in world shiitake production, 1980-2012 (Chang 2005, Li 2012, USDA 2014, Yamanaka 2011). Author's own estimate for 2012

Production of dried shiitake in Japan has been decreasing steadily since 1984 (Yamanaka, 2011). During the 10-year period 2000-2009, dried *L. edodes* production declined by 37% while fresh *L. edodes* production increased by 11.6%. Production increases for fresh *L. edodes* were due mainly to growers increasing production to fulfill consumer demand left by a decrease in imports in fresh *L. edodes* from China. Total production of *L. edodes* (based on fresh *L. edodes* and dried *L. edodes* converted to fresh weight) was 101,392 t in 2009, which ranked third with 22% of total production of edible mushrooms in Japan.

In the United States, most shiitake production is on nutrient supplemented, sawdust-based substrates (Royse, 2009). Many growers use a 17 to 20-day spawn run then remove the bag for browning of the exterior surface of the "log" while other growers conduct spawn run and browning inside the bag. In general, a longer production period is required when logs are browned inside the bag compared to logs browned outside the bag. In addition, higher rates of nutrient supplement may be used when logs are browned outside the bag resulting in higher yield potential compared to logs browned inside the bag. Over the last 10 years, shiitake production in the United States has remained relatively steady (USDA, 2014).

## *AURICULARIA* SPP.

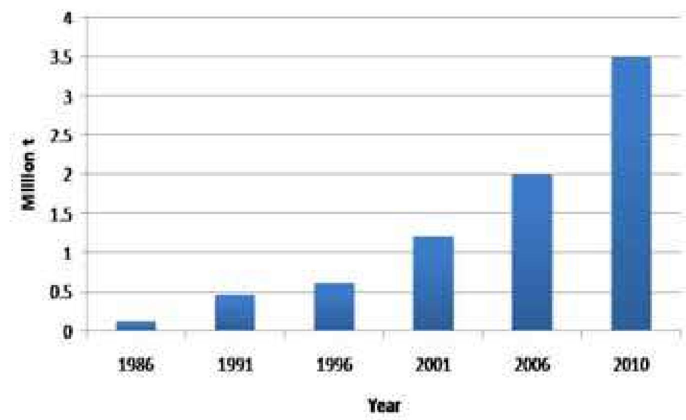


**Figure 6.** Growth in world production of *Auricularia* spp., 1986-2010 (Royse 1997, Chang 2005, Li 2012)

Now widely cultivated in China, Taiwan, Thailand, Philippines, Indonesia and Malaysia, black fungus or wood ear mushrooms (*Auricularia auricula* and *A. polytricha*) are widely considered to be the earliest cultivated mushrooms (Tang *et al.,* 2010). Production of wood ear accounts for about 6% of the world's total output of mushrooms (Fig. 6). Annual production of *Auricularia* spp. in China alone reached nearly 3.6 million t in 2010 making them the second most widely cultivated mushrooms in that country (Li, 2012). Chinese growers in the two major production regions, i.e., Changbaishan and Shennongjia have been able to domesticate wild-type strains using selections over an extended period (Tang *et al.,* 2010). Some of these selections now have been introduced to new cultivation regions located in the Northern and Southeastern regions of China. Success of the wood ear industry in China may be attributed, in part, to the genetic diversity of cultivars adapted to the prevailing differences in climate, cultivation methods and cultivation seasons (Tang *et al.,* 2010).

**Figure 7.** Clockwise from top left: 1) *Auricularia auricula* production in bottles (Hawaii, USA), 2) close-up of *A. auricula* emerging from bottles (Hawaii, USA), 3) sun drying *A. auricula* on large mats (China), and 4) sliced *A. auricula* (China)

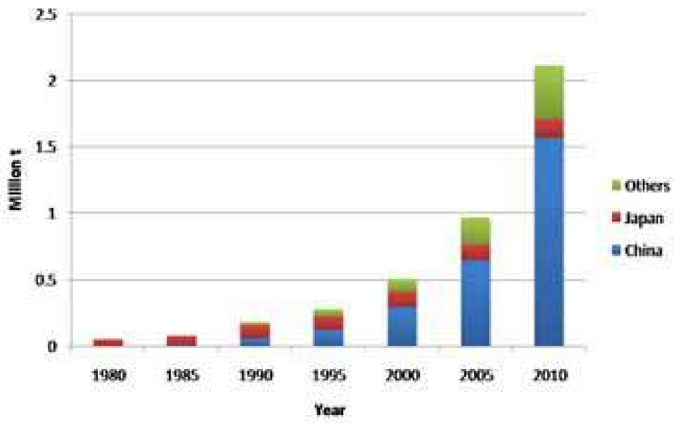


**Figure 8.** Growth in world production of *Flammulina velutipes*, 1980-2010 (Royse 1997, Chang 2005, Yamanaka 2011, Li 2012)

## *FLAMMULINA VELUTIPES*

From a production standpoint, volume increases of *Flammulina velutipes* (enoki) in Japan and China are similar to those for shiitake. Japan once dominated production of *F. velutipes* – until the mid 1990s when China equaled then surpassed Japan – a similar pattern to shiitake except for the magnitude (Figs. 5 & 8). Production of *F. velutipes* in China has increased from about 0.12 million t in 1995 to about 1.57 million t in 2010 (+1,208%). On the other hand, production in Japan has increased from about 0.11 million t in 1995 to about 0.14 million t (+27%) in 2010.

In the last five years, many new enoki farms have been constructed in China based on bottle technology first developed in Japan. In a description of one recent new farm in China, Dreve (2014) describes the first stage of a large climate controlled plant covering 6.7 hectares of land producing 60 t of enoki per day (21,900 t/year). Thus, this single farm produces an equivalent of about 6% of the total enoki production in Japan. About 80% of the farm's output is destined for the domestic market while the remainder is exported to countries in Southeast Asia and Europe.

## OUTLOOK

If history is a guide, the mushroom industry will continue to expand at a relatively rapid rate. Since mushroom production is a relatively labor-intensive industry, mushroom expansion is expected to increase at a faster rate in countries with lower

labor costs. In industrialized countries, greater use of mechanized systems and bulk handling of materials for preparation of substrate is expected.

The recent discovery of bioactive components in mushrooms with application to improving human health will provide an additional boost for the consumption of mushrooms. Health-conscious consumers will look to mushrooms to help satisfy their needs for a healthy diet.

Much more research is needed on the bioactive components in mushrooms to determine their biological responses in humans (Feeney *et al.,* 2014). Promising evidence suggests that ergothioneine, vitamin D, β-glucan, and selenium offer positive effects on immune function, intestine function, and weight management. It remains to be determined how often, how much and what species or mixture of species should be consumed to bring about a desired biological response in humans. In the meantime, we can enjoy the culinary characteristics and unique delicacies that mushrooms offer.

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# MUSHROOM BIODIVERSITY IN INDIA: PROSPECTS AND POTENTIAL

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## ABSTRACT

Mushrooms are an important and integral component of the ecosystem. Status of Indian Agaricales was reviewed first by Sathe and Rahalkar making 1825 as the base and then by Manjula in 1983, providing a very exhaustive list of Agricoid fungi from India and Nepal. The systematics of Agaricales can be divided in to three phases: Phase I (1825-1899), Phase –II (1900-1969) and Phase-III (1970-onwords). The following groups became active in phase III; Natarajan and his group in South India, Sathe and his Co Workers, in South West India, Kapoor and associates in and around Delhi, Rawala and his students, Saini and Atri and their students in North India, Lakhanpal and his co-worker, Kaul and his associates and Upadhyay *et. al* at DMR Solan in the Himalayan region. A family wise exploration which began with family Boletaceae in 1976 culminated in family wise monographic treatment of Boletaceae, Amanitaceae, Russulaceae and Morels.

**Keywords:** Agaricales, systematic, Boletaceae, Amanitaceae, Russulaceae, morels

Mushrooms are the macro or larger fungi which possess fleshy, subfleshy, or sometimes leathery, umbrella like fructifications, which bear their spore producing surface either on lamellae (gills) or lining the tubes, opening out by means of pores. Usually the lamellate members are called 'mushrooms' or toadstools' depending upon whether they are edible or poisonous and the tube bearing poroid members, as boletes. Mushrooms are seasonal fungi, which occupy diverse niches in nature in the forest ecosystem. They predominantly occur during the rainy season and also during spring when the snow melts.

Mushrooms have been by far most extensively studied in most of the countries of the world in the West. Though many papers compilations and treatments have been published periodically, "The Agaricales in Modern Taxonomy" by Singer [1] is still the most standard treatment. A new revised systematic treatment of all the fungi including mushrooms has been presented by Kirk *et al.* [2] in the "Dictionary of Fungi" based on molecular characters. Mutational rearrangements and placement least challenge the content given by Singer. Many other regional compendia and lists have been published periodically from different parts of the world.

Indian fungi has a chequered history, it was leisure past time for British amateurs, the pioneers in the investigations. Mushrooms, therefore, have a beleaguered history with gaps and punctuations. One of the reasons may be the lack of communication facilities and unapproachable destinations which are now comparatively easily accessible. The first list on Indian Fungi was published by Butler and Bisby [3], and then revised by Vasudeva [4]. Several additional lists appeared in between culminating with the fungi of India by Bilgrami *et al.* [5]. Status of Indian Agricales was reviewed first by Sathe and Rahalkar [6] making it as the base and then by Manjula [7], providing a very exhaustive list of Agaricoid and Boletoid fungi from India and Nepal. This is so far the best list which enumerates 538 valid genera and 20 families in the Agaricales. This list has been recently updated by Natrajan *et al.* [8]. The systematics of Agaricales can be divided into three phases; Phase I (1825-1899), Phase II (1900-1969) and Phase III (1970-onwards). The main key player in the first phase was Berkeley [9, 10, 11, 12, 13]. But Fries[14] appears to be the first to report and describe *Lentinus alopecinus* Fr. ex Fr. and *L. sajor-caju* Fr. from India. In 1855, he reported *L. molliceps*, Fr. and *Marasmius korthalsii* Fr. from Nicobar Islands [15]. Montagne [16] reported *Trogia belangeri* (Mont.) Fr. (as *Agaricus crepidotus*), T. *montagnei* Fr. (as *Cantherellus aploruitus* Mont.) *Xerotus pessotteitii* Lev. from Nilgiri hills (as *Agaricus catervarius* Lev.), *Lentinus javanicus* L. (as *L. decaisneanus* Lev.) from Bombay and *L. pergameneus* Lev., the type of which was deposited in Paris with the remarks "Herb de Canddle, Indes".

Berkeley made major notable contributions to the field of Agaricology in India [9, 10, 11, 12, 13]. He dealt with 159 species of mushrooms collected from Assam, Darjeeling, Sikkim, Calcutta, Masulipatnam and Madhya Pradesh. Cooke [17] recorded nine mushroom species from Bombay, Andaman, Island, Saharanpur, Madras and Nepal. Henning [18, 19]

described 72 species of mushrooms Bose and associates [20] added nearly 34 mushroom species to the Indian records. Similarly Ginai [21] recorded two species of *Coprinus* from Punjab, Benerji [22] listed 180 species of hymenomycates from Calcutta and suburbs which included 41 agarics belonging to 14 genera. Ghosh and Pathak [23] described three species of *Macrolepiota* from Lucknow (UP).

In the second phase Massee [24] recorded 32 species of mushrooms. The following groups became active in the III Phase: Natrajan and his group in South India, Sathe and co-workers, in South-West India, Kapoor and associates in and around Delhi, Rawla and his students, Saini and Atri and their students in North India, Lakhanpal and his co-workers, Kaul and his associates and Upadhyay *et al.* at DMR Solan in the Himalayan region.

The Himalayas represent the loftiest chain of mountain in the world. Himachal Pradesh is situated between 30$^{o}$22'-33$^{o}$ 12' N and 75$^{o}$47'-79$^{o}$04' E in the North-Western Himalayas along the northern border of India. It has a entirely mountainous terrain with altitude varying from 300 m to 7000 m. Physiographically it is characterized with hilly terrain with an intricate mosaic of hills, valleys, mountain range and snow clad peaks. It possesses a rugged topography with rich temperate flora. The forests vary from to tropical to alpine pastures. The vegetation predominantly consists of pine species, (*Pinus roxburghii, P. wallichiana and P. gerardiana*), high altitude conifers (*Picea smithiana, Abies pindrow, Abies specabilis), Taxus baccata, Cedrus deodara* and *Quercus incana, Q. leucotrichophora and Q. semecarpifolia*. These forests serve a congenial habitat for all sorts of fungi, especially mushrooms.

On the North-western Himalayan mycoflora, the prominent publications have been by Watlilng and Gregory [25], Hongo [26], Horak [27], Kaul and Kachroo [28], Abraham *et al.* [29], Saini and Atri [30-33], Saini *et al.* [34] Rawla and Sarwal [35], Sharma and Lakhanpal [36], Lakhanpal *et al.* [37-39], Sharma [40], Bhatt [41], Kumar [42], Shad [43-44], Kaisth [45], Sharma [46-48], Chaturvedi [49], Thakur[50], Lakhanpal and Shad [51-52], Lakhanpal *et al.* [53], Bhatt and Lakhanpal [54-56], Sagar and Lakhanpal 57], Kumar *et al.* [58], Lakhanpal [59], Lakhanpal *et al.* [60]. From a survey of mushroom in the N.W. Himalayas Lakhanpal and his associates recorded agarics belonging to 300 species, 59 genera and 15 families of Agaricales. This survey provides an inventory of the species occurring in the Himalaya, a list of species of mushrooms, which enter into mycorrhizal relationship with forest trees; and a list and description of non-conventional edible species discovered during the surveys.

Watlling and Gregory [25] presented a comprehensive list of 119 taxa from Jammu and Kashmir. Abraham [62] published a list of agarics with ecological notes from Kashmir Himalayas reporting 250 species. Lakhanpal reviewed exploratory work on the Himalayans agarics and concluded that the taxa recorded are least commensurate with the vastness and diversity in the mountainous range [62]. Atri and Saini [63] reviewed the work on Russulaceous fungi the world over and reported the Indian contribution, Atri and Saini [64] published the checklist of Indian Russulaceous wherein 48 species of *Lactarius* and 67 taxa of *Russula* have been listed. Saini and Atri [65] reviewed exploratory work on mushrooms from Punjab and listed 94 taxa spread over 24 genera. Gupta *et al.* [66] reviewed Indian work on Agaric systematics. While working on the taxonomy of the genus from North West India, Gupta [67] made 261 collections falling in 66 taxa, which include 4 new species. From South Indian region, excluding Kerala, Natarajan [68] reported 457 species of agarics spread over 76 genera. Work on mushrooms from Kerala has been reviewed by Bhawani Devi [69]. Patil *et al.* [70] listed 212 species of agarics spread over 63 genera from Maharashtra. Verma *et al.* [71] listed 95 additional species of mushrooms.

The wild mushroom seem to have been traditionally consumed by man since very early times, but these were then probably considered a food in wilderness, which now have come to occupy a very popular place in the modern dietic regimen because of its nutritive value.

## AGARICALES

A systematic survey of mushrooms and toadstools of N.W. Himalayas was started in 1976 and has been carried through all these years [36, 72, 73]. Since Agaricales is a large assemblage of mushrooms and toadstools, comprising more than 20 families [13], instead of initiating work on all families, it was decided to begin family wise survey, starting with the family Boletaceae [36, 40, 46, 72, 73].

In the family Boletaceae 7 genera and 57 species were recorded. The seven genera recorded are *Austroboletus* (1), *Boletus* (37), *Gyproporus* (2), *Leccinum* (6), *Strobilomyces* (3), *Suillus* (5)and *Tylopilus* (3).This work included 5 new species and an equal number of new varieties. The work on Boletaceae was compiled into a monograph: Mushrooms of India-Boletaceae [73]. The systematics was supplemented with data on 22 edible species in Boletaceae, *Boletus edulis* being the species of choice. Majority of the species were new records for India and *B. hoarkii* sp. nov. was designated as a new species. About 22 species of boletes were observed to form mycorrhiza with different predominant conifer tree species, especially with *C. deodara, P. wallichiana, A. pindrow and P. smithiana.*

In 1981, the mushroom exploration was extended to the families Amanitaceae, Russulaceae and Cantharellaaceae. The collections were primarily made from Shimla and adjoining areas. In these collections the genus *Amanita* in Amanitaceae was represented by 12 species; genus *Lactarius* and *Russula* in Russulaceae by 14 and 22 species, respectively and genus *Cantharellus* and *Craterellus* by 5 and 2 species respectively [41,74-76]. In 1983, work was extended to different parts of Himachal Pradesh and 6 more species in Amanitaceae were collected bringing the total number to 18 in the N.W. Himalayas and 25 in India [42]. This resulted in the publication of "Amanitaceae of India" [77]. This work included 4 taxa new to science, 13 species were mycorrhizal with different trees species. Out of this only *Amanita caesaria* and *A. vaginata* were reported to be edible and consumed locally by the people. Typical *A. muscaria* was not recorded however; only *A. muscaria var. flavivolvata w*as recorded. Similarly the exploration was also intensified on Russulaceae and Cantharellaceae [41].

The family *Cantharellaceae* was represented by 2 genera *Cantharellus* and *Craterellus* with 5 and 2 species, respectively and out of which one species each is new to science. *Cantharellus cibrarius* and *C. minor* and *Cratellus cornucopoides* are edible species and the former two are also mycorrhizal with *Cedrus deodara.* In the family Russulaceae, the genus *Lactatius* is represented by 14 species and *Russula* by 22 species and these include 4 species in the former and 5 new species in the latter. On the family Russulaceae very extensive work has been carried out at Punjabi University, Patiala by Saini and Atri [30-34, 63-65, 78-89]. They described 50 species of *Russula* from H.P. in addition to Watling and Gregory[25] and Rawla and Sarwal, [35]. Bhatt *et al.* [90] described 4 species of *Russula* from Uttrakhand. Eleven taxa are reported to be mycorrhizal with different trees species and 13 species are observed to be edible in literature out of which only *Lactarius delicious*, *L. sanguifluus* and *Russula brevipes* are the most favoured ones in the Himalayan region. A monograph on Russulaceae and *Cantharellus* has been prepared but not yet published (Bhatt and Lakhanpal, unpublished).

The systematic work was further extended to families Agaricaceae, Hygrophoraceae, Pluteaceae and Tricholomatatceae (Lakhanpal [91]; Kumar [42]). In the family Agaricaceae 6 genera have been recorded: *Agaricus, Cystoderma, Lepiota, Macrolepiota, Leucoagaricus* and *Leucocoprinus.* The last two were unrecorded from N.W. Himalaya earlier. In the genus *Agaricus* 8 species are reported from N.W. Himalayas [18, 25, 92-96]. Lakhanpal [91] and Kumar [42] described 4 species in this genus from Himachal Himalaya; *Agaricus angustus* Fr., *A. arvensis* Schaeff. *A. campestris* L.ex Fr. And *A. placomyces* Peck. Atri and his student have described around 25 species in the genus from different parts of Punjab and Dhancholia and Bahukhadi [97] described two species from Garhwal Himalayas in Utrakhand.

The genus *Cystoderma* was so far unrepresented from N.W. Himalayas. One species, *C. amianthinum* (Fr.) Fayod, has now been reported in the genus from N.W. Himalaya [91, 42]. The species is mycorrhizal with *Cedrus deodara.*

No. species in the genus *Lepiota* was so far known from N.W. Himalaya. The two species known from the Himalayan region are from Eastern Himalayas. Lakhanpal [91] and Kumar [42] recorded three species in this genus: *L. acutesquamosa* (Weinon) Kummer, *L. clypeolaria* (Bull ex Fr.) Kummer and *L. cristata* (Fr.) Kummer, The last two are mycorrhizal with *Cedrus deodara.*

The genus *Leucocoprinus* is represented by *L.cepaestipes* (Sow. ex Fr.) Pat and a new species *Leucocoprinus* sp. nov. and in the genus *Leucoagaricus,* only one species, *L. rubrotinctus* (Peck) Singer has been recorded. *M. procera* (Scop. ex Fr.) Singer a new species, *Macrolepiota sp.* Nov., *A. angustus and Leucoagaricus rubrotinctus* are new records

from India. *A. arvensis, C. amianthium, L. acutesquamosa, L. clypeolaria, L. cepaestipes* have been recorded for the first time from N.W. Himalaya and *A. campestris* and *L. cristata* have been recorded for the first time from Himachal Pradesh.

In the family Hygrophoraceae, only two genera *Hygrophorus* and *Hygrocybe* were represented in the N.W. Himalayas. Kumar [42] also recorded *Camerophyllus* for the first time. In *Hygrophorus* the following taxa have been described: *H. eburneus* (Bull. ex Fr.) Fr., *H. pudorinus* (Fr.) Fr., *H. pudorinus* var. *fragrans* (Murr.) Hesler and a new variety in *H. pustulatus*. Earlier only two species in the genus were known. In *Hygrocybe* only one species was known so far from N.W. Himalaya i.e. *H. psittacina* (Schaeff. ex Fr.) Kummer. Kumar [42] described 3 more species: *H. conica* (Scop ex Fr.) Kummer and H. *calopus* sp. nov. which forms mycorrhiza with *Q. incana.*

The genus *Camerophyllus* has been found to be represented by one species *C. pratensis* (Pers. ex Fr.) Kummer, and is recorded for the first time. Earlier it was known only from Maharashtra. Only one species *Gomphidius maculates* (Scop. ex Fr.) has been reported in the family Gomphidiaceae [91]. However, earlier Watling and Gregory [25] reported *Gomphus clavatus* (Pers. ex Fr.) S.F. Gray in the family Gomphidiaceae from Kashmir.

In the first family Pluteaceae, out of the three genera recorded from India, two are represented in the N.W. Himalaya. These are *Pluteus* and *Volvariella.* Out of the 6 species of *Pluteus* in India, 5 are represented in the Himalayan region but only one i.e. *Pluteus cervinus* (Schaeff. ex Fr.) Kummer in N.W. Himalaya [25, 42]. In the genus *Volvariella,* only *V. volvacea* (Bull. Ex Fr.) Singer was so far known from N.W. Himalayas [19]. Lakhanpal *et al.* [38] described *V. bombycina* (Schaeff, ex, Fr.) Singer from H.P. collected on the living decorticated trees of *Picea smithiana* (7000-9000 ft.). It was a low temperature loving species and could be cultured easily. Lakhanpal [91] and Kumar [42] respectively listed and described *V. pussila* (Pers. ex Fr.), Kumar [42] described a new species of *Volvariella,* apparently which was collected on soil, associated with *C. deodara.* Natrajan *et al.* [8] list two additional species in *Pluteus* and 12 in the genus *Volvariella.*

In the family Pleurotaceae,the genus *Pleurotus* is represented by 6 species, all collected from Jammu & Kashmir (Watling and Gregory)[25]as: *P. dryinus* (Pers. ex Fr.) Kummer, *P. ostreatus* (Kaul and Kachroo[28], *P. membranaceus* Massee and *P. fossulatus* (*Cooke*) Sacc. Lakhanpal [91] recorded *P. ostreatus* from Himachal Pradesh and Chaturvedi [49] conducted experimental trials on its cultivation.

In the genus *Lentinus* only two species are known from N.W. Himalayas, *L. strigosus* (Schwein) Fr. and *L. tigrinus* (Watling and Gregory [25], Lakhanpal [91], Natrajan *et al.* [8] treat these two genera in Polyporaceae listing two species in *Lentinus* and three in *Pleurotus.*

The family Tricholomataceae is one of the largest families of the Agricales with its members distributed far and wide and occupying a variety of ecological niches. Out of the 98 genera reported from all over the world, 41 genera are represented in India as well (Manjula, [7], However, Natrajan *et al.* [8] reported 34 genera in this family from 1984-2002. Twenty five genera have been reported from the Himalaya and only 5 from N.W. Himalaya. These genera are: *Armillaria, Tricholoma, Collybia, Leucopaxillus* and *Melanoleuca.* Kumar [42] recorded in addition to these, 8 more genera from N.W. Himalayas. These are: *Asterophora, Clitocybe, Lepista, Mycena, Oudmansiella, Tricholomopsis, Laccaria* and *Marasmius.* Lakhanpal [91] has also recorded these and two additional genra i.e. *Lyophyllum* and *Flammulina* from N.W. Himalaya.

So far only *A. mellea* (Vahl. Ex Fr.) Kummer, and *A. obscura* (Pers. ex Secr.) Romagn are known from Himalaya. We have recorded only *A. mellea* from Himachal Pradesh. Similar has been the case with the genus *Asterophora* in which only one species *A. lycoperdoides* (Bull ex Mirat) Ditmat ex Fr. was for the first time recorded from India by Kumar *et al.* [98], which was collected growing gregariously on the carpophores of *Russula.*

The genus *Clitocybe* hitherto unrecorded from N.W. Himalayas, is known to be represented by four species (Kumar, [42]; Lakhanpal, [91]). *C. clavipes* Fr.) Kummer, *C. dilatata* Pres. ex Karsten*, C. gibba* (Fr.) Kummer and *C. squamulosa* (Fr.) Kummer. All the four have been seen to form mycorrziza. Only three species in the genus *Collybia* are so far known from N.W. Himalaya. Out of these *C. peronata* (Boltr. exFr.) Kummer was recorded from Mussoorie (U.P.) by Hennings

[19]. The other two were recorded by Watling and Gregory [25] from Kashmir viz. *C. dryophila* (Bull ex Fr.) Kummer and *C. fuscopurpurea* (Pers ex Fr.) Kummer. In the genus *Laccaria, L. laccata* (Scop. ex Fr.) Cooke, and *L. amethysta* (Bull ex Gray) Murrill (earlier known from Nilgiri hills) has been recorded from N.W. Himalaya. The fungus has gained much prominence in the last few decades being a good mycorrhizal species, which forms mycorrhizal associations with many trees. In the genus *Lepista,* only one species, *L, nuda* (Bull. Ex Fr.) Cooke, has been recorded from N.W. Himalaya [42, 91] which is mycorrhizal with *Quercus semicarpifolia.*

In the genus *Leucopaxillus,* four species have been reported from N.W. Himalaya [25]; *L. albissimus* (peck) Singer var. *piceinus* Peck) Singer and Smith, *L. laterarius* (Peck) Singer and Smith, *L. amarus* (A&S ex Fr.) Kuhn, *L. rosebrulles* (Murr.) Singer and Smith and *L. giganteus* (Sow, ex Fr.).The genus *Marasmius* surprisingly has been reported to be represented by just one species in the N.W. Himalaya, *M. cohaerens* (A&S. ex Fr.) Cke & Quel. Kumar *et al.* [99] reported a new species, *M. ellipsoidosporus* and a new record *M. siccus* (Schw.) Fr. from H.P.

In the genus *Melanoleuca* two species *M. subpulverulenta* (Pers. ex Fr.) Singer and *M. melanoleuca* (Pers. ex Fr.) Murr. were so far known from Kashmir Himalaya [25, 42] recorded *M. alboflavida* (Pk.) Murrill from H.P. for the first time. The genus *Mycena* is so far represented by five species in the N.W. Himalayas: *M. aetites* (Fr.) Quel., *M. atrocyanea* (Fr.) Gillet, *M. golericulata, M. epiterygia* (Scop. ex Fr.) Kummer. The last two have been reported from Himachal Pradesh [42, 91]. The genus *Oudemansiella,* is observed to be represented only by one species i.e. *O. radicata* (Relh.) ex Fr.) Singer from N.W. Himalayas [42, 91].

*Tricholoma,* a widely distributed and represented genus the world over, has only *T. terreum* (Bull. ex Fr.) Kummar recorded from N.W. Himalaya [25]. Two more species have been now recorded from N.W. Himalayas. These are: a new species of *Tricholoma* and *T. virgatum* (Fr. ex Fr.) Kummer. The latter has been observed growing in mycorrhizal association with *C. deodara* [42, 91].

The genus *Tricholomopsis* is so far represented by two species from India, one from U.P. [92] and other from eastern Himalaya (*T. rutilana)* [92(a)]. Kumar [42] and Lakhanpal [91] recorded *T. rutilans* (Schaeff. ex Fr.) Singer and *T. sulphureoides* (Peck) Singer from N.W. Himalaya for the first time. In the genus *Flammulina* only *F. velutipes* (Curt ex Fr.) Karst. has been reported so far from Indian and Himalaya. The genus *Lyophyllum* isrepresented by four species in India. Lakhanpal [91] recorded *L. decastes* (Fr. Ex Fr.) Singer from HP. In the genus *Resupinatus* Watling and Gregory [25] recorded *R. applicatus* from Kashmir and in the genus *Xeramphalina,* out of the three species recorded from India and Himalaya, *X. aurara* Horak and *X. campanella* (Batsch. ex Fr.) Maire, have been recorded from N.W. Himalaya [25].

## SYSTEMATICS OF MORELS

The genus *Morchella* (Ascomycetes) commonly known as morels and 'Guchhi' in the Indian market has been investigated for different aspects of morel biology (Shad [44]; Lakhanpal and Shad [100], Shad and Lakhanpal [101], Thakur [102], Rana, [103], Lakhanpal *et al.* [60]. All the six classical species viz. *M. angusticeps, M. conica, M. crassipes, M. deliciosa, M. esculenta* and *M. semilibra* have been collected and described from the Himalayan region. In addition *M. tibelica* and *M. simlensis* sp. nov have been recorded from HP. The polymorphism and genetic analysis in the species *Morchella* has been assessed at molecular level [60].

Similar studies have been conducted on the taxonomy, ecology, physiology and nutritional requirements, ethnic uses and analysis of nutritive components [104]. On *Helvella crispa, Gyromitra esculenta* among members of *Ascomycotina, Lactarius delicious, L. sanguifluus, Macrolepiota procera* and *Russula brevipes* among gilled members and *Boletus edulis, B. erythropus, B. horakii, Cantharellus cibarius, Hydnum repandum, Ramaria botrytoides* and *Sparassis crispa* among the non-gilled members of Basdiomycotina. These mushrooms can be exploited for cultivation as they are already accepted for consumption by local people.

Nutraceutical potential of morels has been investigated [60]. Nutraceutical attributes of morels compare favourably with the mushroom species listed above. They are even a better source of polysaccharides, crude fibre, nucleic acids, minerals especially Se, Zn, K, Cu, Na, and Ca, Vitamin ($B_1$, $B_2$, C, A, D and K; proteins and all the essential amino acids. They are free from cholesterol. Hence they are of good nutraceutical use.

Ethnomycological studies were initially conducted on morels and some other mushrooms: *Lactarius delicious, L. sanguifluus, Amanita veginata, Russula brevipes, Sparassis crispa, Hydnum repandum,* specie of *Clavaria, Macrolepiota procera* etc. [45, 104-106]. The general myths are almost the same for all these mushrooms. Even the recipes are similar but the varieties are more. Previously most of these mushrooms were dried for use in winter months by the local inhabitants when most of the areas in HP are snow bound. Now the use is declining with the ready availability of vegetables and other crops.

The ecological data was collected from forest lying in the outer, middle and inner ranges (Tara Devi, Glan and Jakhu Forests) and also along a gradient with altitude varying from 6000-10,000 ft. msl. (Narkanda forest).The studies were aimed at (i) to determine the distribution/occurrence of particular species of fungi in different elevational/vegetational zones (ii) to identify the species of fungi entering into mycorrhizal association with particular species of trees, and (iii) to quantify fruiting phenlogy of different species of fungi.

Cultivation technology was developed for two mushrooms. Cultivation of *Lentinula edodes* (Shitake) was achieved for the first time in India on local sawdust substitutes [107, 108]. Similarly package of practices was developed for the first time for *Calocybe indica* [109, 110].

The research on ectomycorrhiza at HP University during the last three decades has been carried out on almost all the dominant conifer species: *P. roxburghii, P. wallichiana, P. gerardiana, Cedrus deodara, Abis pindrow, Picea smithiana* and *Taxus baccata.* In addition studies on the mycorrhiza of Oak*, Rhododendron arboreum, Monotropa,* Orchids*, Aesculus indica* and Apple has also been carried out [72, 102, 106, 111, 113]. The survey of mushroom that enters into mycorrhizal synthesis with different species of conifers provided the specific mushroom species for *in-vitro* myconhizal synthesis which has been achieved successfully.

## III. HYPOGEOUS FUNGI

Truffle and truffle like Fungi are virtually unknown in India except *Tuber indicum* Cooke & Massee. There have been no systematic explorations. The author has recorded the following genera from N.W. Himalaya: *Astreus hygrometricus,* (Pers.) Margan*, Gauteria trabuti* (Chatin) Pat.*, Histerangium membranaceum* Vitt., *Melanogaster broomeianus* Berk., *Rhizopogon rubescens* Var. *Ochra ceous* A.H. Smith, *Tuber mesentricum* and *Trappeinda himalayasis* [112, 114].

These are least representative of the vast Himalayan ranges with so great diversity of vegetation and climate. These need to be explored more intensively and extensively. Nevertheless, a small and humble beginning has been made for enthusiasts to pursue further.

It is clear that mushroom diversity so presented, holds great promise and potential for exploration, experimentation and amelioration of the environment. It demands great interest, commitment and of course, encouragement from living fossils-the systematics.

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# COLLECTION, IDENTIFICATION AND MORPHOLOGICAL CHARACTERIZATION OF INDIGENOUS MUSHROOMS IN COASTAL KENYA

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## ABSTRACT

Mushrooms are consumed all over the world as part of the regular diet. They are consumed for their ample nutrition, medicinal value and enticing flavour. In Kenya, both cultivated and wild mushrooms are consumed, the latter forming an integral part of a long standing cultural practice. However, many people shy off from such wild sources due to fear of poisoning. That notwithstanding, these wild resources risk extinction due to climate change, over exploitation and wanton destruction of their natural habitat. To improve on their utilization, a survey was conducted in coastal Kenya to document the edible species which are known by communities but completely unknown to science. Species collected were identified and characterized using habitat, morphological features and any phenotypic features easily identifiable and categorized as edible hence a food source, poisonous or ornamental. The edible included *Ganoderma* spp, *Cantharellus* spp, *Agaricus* spp, *Pleurotus* spp, *Russula* spp, *Auricularia* spp and *Termitomyces* spp; poisonous species included the deadly *Amanita* spp, *Lactarius* spp and stinkhorn spp while ornamental included the beautiful ringed *Microporous* spp. The survey revealed a rich diversity of economic importance especially for food security if well exploited. Information obtained can be used as a baseline for future studies on genetic diversity, trends associated with climate change and on species for domestication. This information can also be used to improve the management strategy on sustainable utilization of edible species from the forests. However, further studies using modern methods of characterization involving molecular tools are required to improve on such strategies.

**Keywords:** indigenous mushrooms, edible species, poisonous species, ornamental species, sustainable utilization.

## INTRODUCTION

Mushrooms are consumed all over the world as part of the regular diet. While some are cultivated, some are just collected from the wild and consumed. In Kenya, majority of the locals depend on wild mushrooms to spice their diets as cultivated species are exorbitantly expensive and unaffordable to many. Of the 42 tribes living in the country, 38 are known to consume them [1]. They form an integral part of a long standing cultural practice which is passed on from generations to generations[2]. Extensive consumption is however hampered by cultural bias in some communities and lack of adequate knowledge on edible and poisonous species.

According to Hawksworth [3] and Bates [4] the estimated number of fungal species worldwide is 1.5 million species and less than 5% have been described. Fungi producing conspicuous sporocaps are collectively called macrofungi and include gilled fungi, bracket fungi, coral fungi, jelly fungi, stinkhorns, birds nest fungi and puffballs [5]. A mushroom is defined by Chang [6] as a macrofungi with a distinctive fruiting body large enough to be seen with the naked eye and to be picked by hand. Each mushroom has its own special ecological niche and occurs only in certain habitats. They are found growing prolifically all over the world [7] and have been used since time immemorial as part of human diet. To date, they are collected and sold in over 80 countries and collections amount to several million tones with a minimum value of USD 2 billion [8].

Mushrooms can solve world's food shortage problem because of the fact that they occupy a place above vegetables and legumes but below the first class proteins in meat, fish and poultry [9]. They can also solve most of the world's health problems because they are endowed with bioactive compounds that are of medicinal value [10]. Due to their good nutrition and medicinal values, mushrooms are considered ideal for vulnerable groups in the society such as children, breast feeding mothers, the old and the sick especially those suffering from diabetes, heart diseases, cancer and HIV/aids.

In Kenya, several exotic varieties are cultivated which include *Agaricus bisporus, Pleurotus* spp, *Lentinula edodes* and *Ganoderma lucidum* [11,12]. However, there is preference for wild species as expressed during a KARI's stake holders work shop held in February 2007 [13]. But they risk extinction due to climate change and wanton destruction of their natural habitat which also poses a threat to biodiversity conservation. The wide spread practice of collecting wild mushrooms

for consumption among local communities also poses the danger of illnesses and possible deaths from poisonous species. Cultivated mushrooms are therefore the only safe and sustainable source of this delicacy, necessitating domestication of these wild types.

However, there exists scarce knowledge for these types of mushrooms in Kenya. Many different kinds are consumed from the wild and though well known by the local communities, they are completely unknown to science. Studies on the taxonomy and diversity of the same are gaining importance as many are facing extinction due to global warming, habitat destruction and or overexploitation. The present study was undertaken to collect, identify and characterize important indigenous mushrooms (both edible and poisonous) of Kenya, conserve specimens in a gene bank and document indigenous technical knowledge (ITK) about them from the locals. This paper highlights the identification and morphological characterization of the mushroom species collected during the study.

## MATERIALS AND METHODS

Field surveys were conducted among the key indigenous mushroom consuming communities in coastal Kenya. Arabuko Sokoke forest in North coast and Kaya Teleza forest shrines at the South coast were surveyed. The forests were the main target but farms were also visited for collections as mushrooms grow everywhere during the rainy season.

### A brief description of the sites

Arabuko Sokoke forest is the largest stretch of coastal dry forest extending from south of Somalia to Northern Mozambique. The forest comprises Brachystegia woodlands which are a perfect habitat for Mycorrhizal fungi. It extends 3° 19' 60'' and 39° 52' 0'' in DMS. Average annual precipitation ranges from 900mm in the Northwest to 1100 mm in the East with mean temperature of 31 °C. The elevation is 138 m above sea level. The Kaya Teleza forest shrines are part of Simba Hills which are a naturally rich biodiversity, barely disturbed for many years. The area is situated between 4°10' N and 39°10' S. Elevation is 251 meters above sea level. The annual precipitation ranges between 400 and 1680 mm with about 90% occurring between October-December (short rains) and March-June (long rains). The annual mean air temperature is 25 °C.

### Mushrooms survey and sampling

The museums of Kenya and key informants were used in the selection of suitable sites for mushroom collection. The major criteria used for site selection was knowledge and utilization of mushrooms by the local communities. Fruit body surveys were considered for the primary basis of documenting mushroom diversity and were limited to epigeous macromycetes of soil and wood inhibiting fungi that were of appreciable size. Sampling in the forest was done across along transect to a distance of 1000 m x50 m along the forest line. Communities are not able to go deep into the forest for fear of dangerous wild animals. Sampling was done from three communities, two in north coast (from Malindi and Kilifi) and one in south coast (Matuga). Collection of various macro fungi was made and occupational habitat, morphological features and any other phenotypic parameters noted while the mushrooms were still fresh. Where possible, spores prints were also taken for colour. Specimens of fruit bodies were later dried using a field drier at a temperature not exceeding 40 °C. Dried specimens were carefully tagged, packed and tightly sealed in polythene bags for transfer to the museums of Kenya for further identification and characterization.

According to natural habitats, mushrooms were placed in any of the four habitation groups which include

i) Humicolous or Folicolous: humus inhabiting mushrooms which may be either purely saprophytic or growing in symbiotic with green plants without parasitic tendencies.
ii) Lignicolous: wood inhabiting fungi some of which are purely saprophytic but others facultative or obligatory parasitic
iii) Coprophilous: dung inhabiting mushrooms and saprophytic in nature
iv) Fungicolous: fungus inhabiting and saprophytic in nature

### Fruit body identification and description

Specimens were initially marched with descriptions in books to facilitate classification. Identification was based on their reproductive structures visible to the naked eye above the ground. Specimens were later described for morphological characters such as size, shape, odour, texture and colour. Other morphological characters such as the ornamentation on the surfaces of the pileus and stipe, the presence of a ring on the stipe and volva at the base of the stipe were also used to describe and identify the mushrooms. As there is very limited reference collection, each specimen was fully described and

where possible, spores print taken for colour to confirm the specific taxon. The listed taxons were according to [14]. The specimens were also preserved in a herbarium at the Museums of Kenya to be used later for spawn production and further studies.

## RESULTS

During the survey, several species of mushrooms were collected and described. The photos below provide a sample of the specimens.

**Figure 1.** *Trametes* spp **Figure 2.** *Ganoderma* spp **Figure 3.** Stinkhorn spp

**Figure 4.** *Catherellus* spp **Figure 5.** *Auricularia* spp **Figure 6.** *Ganoderna* spp

**Figure 7.** *Termitomyces* spp **Figure 8.** *Lactarius* spp **Figure 9.** *Microporous* spp

**Figure 10.** *Amanita* spp **Figure 11.** *Russula* spp **Figure 12.** *Pleurotus* spp

The survey collected mushrooms from the first three inhabitations (see Table. 1)

**Table 1.** Shows species collected, their habitation, morphological features noted, local name where applicable and possible usage

| Species of mushroom | Habitation | Morphological features | Local name | Possible usage |
|---|---|---|---|---|
| *Ganoderma* spp (Bracket mushroom) Fig. 2, 6 | Lignicolous-Found growing on decaying logs and stumps of hardwood | Cap thick, hard, flat and shinny, kidney-bean shaped. creamy white margin, yellowish in the middle orange to red at the center. Pileus large (6-10cm). Pores on the underside. Spore print brown. | | Medicinal |
| *Cantharellus* spp (Gilled mushroom) Fig. 4 | Foliculous- Found growing on forest floor on roots of Brachystagia trees | Cap (3-5cm), smooth, yellow or orange, funnel shaped and depressed at the centre. Gills forked and run all the way down to the stipe (decurrent). Stipe fleshy and centrally placed. Same colour on entire fruit body. Smells nice and has a mildly peppery taste. Spore print white | Choga Kapilipili | Food |
| *Agaricus* spp (Gilled mushroom) | Carpophilous- Found growing on the forest debri. | Cap, thick, conic, white in colour and button shaped. Stalk hollow and short with a ring. Dark brown hymenium with gills. Spore print brown | | Food |
| *Pleurotus* spp (Gilled mushroom) Fig. 12 | Lignicolous- Found growing ondecidous trees | Carps are spongy, funnel shaped and white growing in clusters. Stipe reduced and off centre. Gills white in colour and decurrent. Spore print white. Have fishy smell | | Food |
| *Russula* spp (Gilled mushroom) Fig. 11 | Hemicolous- Found growing on the forest floor on roots of hardwoods | Cap pinkish to purplish, convex to flat (5cm). Gills white to cream, forked near the margin. Fresh white and skin easily peels. Stipe long and white in colour. Spore print white | Hakoranyani | Food |
| *Amanita* spp (Gilled mushroom) Fig. 10 | Humicolous- grown on forest floor on roots of trees | Cap greyish covered with pyramid like warts, thick and conical. Stalk well visible with a ring and volva at the base. | Choga Nyere | Poisonous-Industrial |
| *Microporous* spp Fig. 9 | Lignicolous- found growing on fallen woody branches of trees either one or two close together | Cap hard and corky with beautiful concentric rings which assume various colours. Margin sharp Pileus round 3-5cm with stipe centrally placed. Very beautiful | | Ornamental |

| Species of mushroom | Habitation | Morphological features | Local name | Possible usage |
|---|---|---|---|---|
| *Lactarius* spp (Gilled mushroom) Fig.8 | Humicolous grown on forest floor on roots of deciduous trees | Caps (5 - 6 cm), white, convex to flat. Gills white, decurrent and exude a milky substance with a peppery taste. Stipe long and white without a ring. Spore print white. | Choga Mazia | Poisonous-Industrial |
| *Auricularia* spp (Jelly mushroom) Fig. 5 | Lignicolous- found growing on dead decaying wood, many clustered together | Caps ear shaped and smooth. Flesh jelly-like, elastic texture. Caps dark brown with a purplish tint. No gills. Stalk very short or absent. | | Food |
| *Termitomyces* spp – (Gilled mushroom) Fig. 7 | Coprophilous- found growing on soil with termites | Caps very large and white in colour. Stipe is long with a subterranean elongation | Choga Nyama | Food |
| *Trametes* spp Fig. 1 | Lignicolous-found growing on fallen woody logs | Caps thin but tough. Flat and round. Show concentric zones of different colours with a conspicuous brown zone. Pores on the underside. Mushrooms grow in tiled layers lacking a stipe. | | Ornamental |
| Stinkhorn spp *Phallaceae* Fig.3 | Humicolous – found growing on wooden decomposing debri | Cap pink and spherical. White coloured stalk with a volva at the base. Mushroom covered in a foul smelling slime with insects | | Poisonous |

## DISCUSSION

Species found utilized by the communities included *Cantharellus* spp (Fig. 4), *Pleurotus* spp (Fig.12), *Russulla* spp (Fig. 11), *Auricularia* spp (Fig. 5) and *Termitomyces* spp (Fig. 7). Among them the *Cantharellus* is the most prominent especially in North Coast, having been harvested and traded locally and internationally [15]. The mushroom has also been reported by [16 ] to be popular in Tanzania where it is harvested from the Miombo woodlands. Despite its good prospects, the mushroom cannot be grown artificially because of its dependency on a living plant host. The same goes for the *Rusulla* spp which is also mycorrhizal. The *Pleurotus* spp and *Auricularia* spp are promising as they can be artificially grown. These two mushrooms were only found in South Coast and are not popular with the locals because they are not abundant. Their occurrence depends largely on the presence of decaying logs in the forests. The description of the *Auricularia* spp from south coast match the description of that collected from Kakamega forest, which has been fully described by [17]

The *Termitomyces* spp is another popular mushroom among the locals growing near homesteads on areas with termites. It is typified by symbiotic life with termites and therefore quite difficult to grow artificially. The mushroom is a tropical species reported in many parts of Africa. The genus comprises of the largest mushrooms in the world such as *T. titanicus* of West Africa and Zambia whose cap reaches 1m in diameter [18]. The termite mushrooms are a great contributor to the livelihoods of rural communities in Africa through income generation and food security [8]

The precious *Ganoderma* spp (Fig. 2) is utilized by very few in the community perhaps because it is also arare occurrence and the locals have little knowledge of its medicinal benefits. The mushroom is however reported to have a worldwide distribution in both tropical and temperate geographical regions including North and South America, Africa, Europe, Asia and Australia [18]. The *Agaricus* spp whose description matches that of *Agaricus bisporus* is hardly utilized by the locals

for fear of poisoning. The species is said to resemble the *Amanita* species which the locals say is deadly. *Agaricus bisporus* is the world premier mushroom and 95% of Kenyan production comprises this mushroom [11, 12]

*Amanita* spp (Fig. 10), *Lactarius* spp (Fig. 8) and Stinkhorn spp (Fig. 3) were all labeled poisonous by the local communities. Although *Amanitas* are always feared to be poisonous, Smith [7] indicates that some 81 edible species are reported from 31 countries. For example *A. ceasarea* is reported to be highly valued in Mexico, Turkey and Napal. The *Lactarius* spp also has several species reported as edible and traded in Europe. The *Lactarius* described above resembles *L. piperatus* (no other *Lactarius* spp exudes a peppery milk substance) which is also reported by Metzler and Metzler [19] as toxic. The Stinkhorn (Fig. 3)which has such an awful smell is difficult to consume and passes out easily as poisonous though it may not be. The locals use smell as one of the ways to identify poisonous species.

The *Trametes* spp (Fig. 1) and *Microporous* spp (Fig. 9) are unpalatable but display unmistaken Beauty. With little value addition, these species can be of economic importance as ornamentals. However, the *Trametes* spp described above which resembles *T. versicolor* could be of medicinal value. There is scientific evidence from the [20] that substances referred to as polysaccharides derived from parts of the mushroom may be useful against cancer. Polysaccharide-K displays anticancer activity in preliminary human research [21]. Careful studies on such species may be very beneficial

It was noted that the communities highly utilize mushrooms either collected from the forest or within the homesteads. This was established to be because of knowledge of edible species as well as methods of preparation of collected mushrooms. Forests closets to homesteads had lowest species richness due to wood collected for fuel and perhaps over exploitation.

## CONCLUSION

The survey has demonstrated that Arabuko Sokoke and Kaya Teleza forests provide a habitat for diverse macro fungal species some of which are used by the locals as food. Some of the edible species collected are mycorrhizal and cannot be cultivated necessitating employment of good management strategies for sustainable utilization. The species collected can be recognized for industrial usage, ornamental display, pharmaceutical application or food security. The information obtained can be used as a baseline for future studies including fungal genetic diversity and populations, future trends associated with climate change, indigenous knowledge application to current usage of mushrooms and species likely to go for domestication. The information can also be used to improve the management strategy on sustainable utilization of all the edible mushroom resources from the forests.

## RECOMMENDATIONS

Further studies using modern technology of characterization involving molecular tools should be undertaken on economically important indigenous mushrooms among the communities. This is because effective strategies for conservation and utilization of fungal resources require a clear understanding of the populations of the target mushroom species. Population genetic studies of selected edible mushroom species will provide more information about genetic diversity within single species and contribute to improving management strategy on conservation and utilization of indigenous edible fungal resources in Kenya. Genetic studies will also give a mushroom a scientific name which can clearly tell whether mushroom is edible or not and also provide a clue to other important properties. Further research is also required to map out mycorrhizal partners of edible species. A data base linking edible species to tree hosts would also help to develop management strategies for sustainable use. Investigation on culturing, nutrition and pharmaceutical studies of these species should also be undertaken.

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# DIVERSITY OF RESUPINATE, NON-POROID AGARICOMYCETOUS FUNGI IN THE HIMALAYA AND ADJOINING AREAS

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## ABSTRACT

An account of more than 400 taxa belonging to more than 105 genera of the different families and orders of class *Agaricomycetes* (phylum *Basidiomycota*, subphylum *Agaricomycotina*) has been given. These include one genus (*Confertextum*), 12 species (*Byssocorticium microsporum*, *Confertextum macrosporum, C. microsporum*, *Corticium mussooriensis*, *Cylindrobasidium indicus*, *Leptosporomyces singularis*, *Tomentella garhwaliana*, *Vararia himalayana*, *V. indica*, *Xylodon mussooriensis, X. subglobosus* and one variety (*Scytinostroma phaeosarcum* var. *angustispora*) new to science;six genera (*Cordochaete, Dendrophlebia, Hallenbergia, Radulomycetopsis, Repetobasidiopsis*, *Trimitiella*), two sub genera (*Stereum* subgen. *Acanthostereum, Stereum* subgen. *Aculeatostereum*), as many as 48 species (*Aleurodiscus himalaicus, A. indicus, Athelopsis parvispora, Candelabrochaete himalayana, Ceraceomyces bizonatus, Clavulicium hallenbergii*, *Conohypha grandispora*, *Cristinia tubulicystidiata*, *Dendrophlebia crassispora, Flavophlebia sphaerospora, Fibulomyces cystoideus, Hallenbergia singularisa, Hyphoderma bicystidiata, H. claruspróprietas, H. densustextum, H. hallenbergii, H. parvisporum, H. sikkimia, H. sporulosum, H. subglobosum, Hyphodontia caulicystidiata, H. dhingrae, Leptocorticium indicum, Leucogyrophana thimphina, Paullicorticium indicum, Peniophora hallenbergii, Phlebia crassisubiculata, P. interjacenoides, P. microspora, P. kamengii, P. singularisa, P. thindii, Phlebiopsis darjeelingensis, P. himalayensis, P. mussooriensis, Radulodon indicus, R. acaciae, Radulomycetopsis cystidiata, Repetobasidiopsis grandisporus, Scytinostroma pulverulentum, S. renisporum, Sistotrema angustispora, Sistotremastrum roseum*, *Stereum peculiare, T. kalatopii, T. unicusa, Trimitiella indica, V. longicystidiata*) and 8 varieties (*Amphinema byssoides* var. *macrospores*, *Botryobasidium subcoronatum* var. *crassispora*, *Ceraceomyces sublaevis* var. *grandisporus*, *Conohypha albocremea* var. *angustisporum*, *Hyphoderma roseocremeum* var. *minutisporum*, *H. setigerum* var. *bicystidium*, *Tomentella cladii* var. *grandii, Tubulicium vermifer* var. *hexasterigmatum*) published new taxa and 225 new reports for the Himalaya.

**Keywords:** *Agaricomycetes,* non-poroid fungi, *Auriculariales*, *Cantharellales*, *Corticiales*, *Gloeophyllales, Hymenochaetales*, *Polyporales*, *Russulales*, *Sebacinales*, *Thelephorales, Trechisporales*

## INTRODUCTION

Resupinate, non-poroid agaricomycetous fungi are generally lignicolous with unilateral and gymnocarpic hymenium and belong to class *Agaricomycetes* (phylum *Basidiomycota*, subphylum *Agaricomycotina*). These fungi have been assigned to two subclasses *Agaricomycetidae* (orders *Agaricales, Atheliales, Boletales*) and *Agaricomycetes* incertae sedis (orders *Auriculariales*, *Cantharellales*, *Corticiales*, *Gloeophyllales, Hymenochaetales*, *Polyporales*, *Russulales*, *Sebacinales*, *Thelephorales* and *Trechisporales*). The diversity study of these fungi is an outcome of extensive work of almost three and a half decades in the Eastern and the North Western Himalaya and adjoining areas covering a distance of about 2500 km from West to East, with 100–400 km average width along the entire longitudinal extension and lot of variation in altitude gradients. Based on macroscopic and microscopic observations of more than 4150 collections, using standard techniques, more than 400 taxa have been identified, which belong to more than 105 genera of the different families and orders. Detailed decriptions supported by photographs and line diagrams have been given for the new taxa, distribution in the Himalaya with herbarium numbers within bracts for the new taxa already published and tabulated account for new records for the Himalaya.

## MATERIALS AND METHODS

Fungi have been collected from the various localities of the Himalaya and adjoining areas from 1978 to 2014. Microscopic details related to hyphae, cystidia, basidia and basidiospores of the specimens and their arrangement were studied by

making crush mounts and hand cut sections in water, 3–5% KOH solutions and staining in Congo red, Phloxine, Cotton Blue, Melzer's Reagent and Sulphovanillin and line diagrams were made by using compound microscope and camera lucida. Color standards are as per Methuen's Handbook of colors by Cornerup and Wanscher [1]. Specimens are kept in herbarium of Department of Botany, Punjabi University, Patiala, India (PUN), Herbarium of Department of Botany, Panjab University, Chandigarh, India (PAN) and some duplicates in herbaria of University of Gothenburg, Gothenburg, Sweden (GH) and Biology Institute, University of Oslo, Blindern, Oslo, Norway (O). Nomenclature follows Blackwell *et al.* [2], James *et al.* [3], Hibbett *et al*. [4], Kirk *et al*. [5], Bernicchia and Gorjün [6] and www.mycobank.org (2014) as far as possible.

## NEW TAXA

### Genus new to science

***Confertextum*** Priyanka & Dhingra gen. nov.

Mycobank MB810722

The new genus differs from *Phlebia* on the basis of dense texture due to the presence of microbinding hyphae along with generative hyphae and having different shape of basidia

Basidiocarps resupinate, effused, closely adnate; hymenial surface smooth to tuberculate, grayish white to orange white to pale yellow; margins thinning, paler concolorous to abrupt. Hyphal system dimitic. Generative hyphae branched, septate, clamped; basal hyphae parallel to the substrate, loosely arranged; sub hymenial hyphae vertical, densely packed. Microbinding hyphae thin, laxly branched, without septa and clamps. Cystidia fusiform to cylindrical, thin-walled, with or without oily contents, with basal clamp.Basidia clavate to subclavate, 4–sterigmate, with basal clamp. Basidiospores ellipsoid to subcylindrical, apiculate, thin-walled, smooth, inamyloid, acyanophilous.

**Type species:** *Confertextum microsporum*

**Distribution in India:** Himachal Pradesh

**Remarks:** This genus is close to *Phlebia* in having branched, septate, clamped generative hyphae, some kind of cystidia and ellipsoid to subcylindrical, acyanophilous, inamyloid basidiospores, but differs on the basis of dense texture due to the presence of microbinding hyphae along with generative hyphae and having different shape of basidia. The new genus is being described on the basis of two new species.

### Species new to science

***Byssocorticium microsporum*** Samita, Sanyal and Dhingra sp. nov.

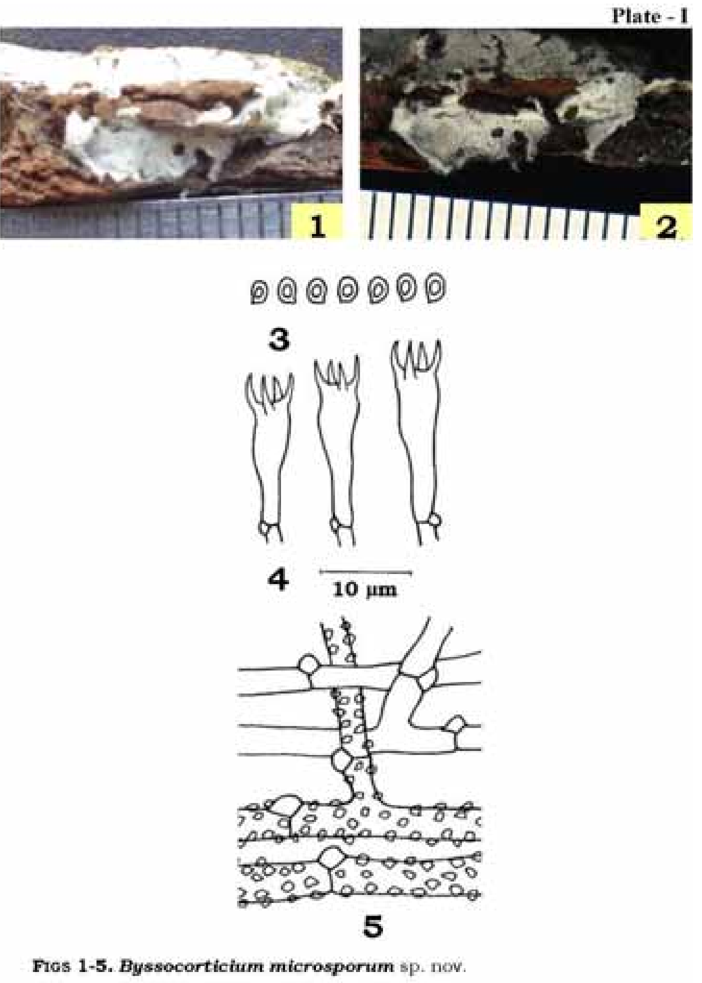
Mycobank 808608

The new species differs from *B. lutescens* in having clamped hyphae and small ellipsoid to subglobose basidiospores.

**Type:** India, Uttarakhand: Tehri Garhwal, Surkanda Devi, on log of *P. roxburghii*, Samita 6019 (PUN, holotype), September 02, 2012.

**Etymology:** The epithet refers to the smaller size of basidiospores.

**Basidiocarp** resupinate, effused, loosely adnate, up to 130 µm thick in section; hymenial surface hypochnoid to smooth, grayish yellow to pale yellow when fresh, not changing much on drying; margins thinning, fibrillose, concolorous, to indeterminate. **Hyphal system** monomitic. Generative hyphae up to 4.5 µm wide, septate, clamped, thin-walled; basal hyphae parallel to the substrate, less branched, with crystalline encrustation which dissolves in 3% of KOH solution; subhymenial hyphae, vertical, more branched. **Cystidia** none. **Basidia** 12.0–18.0 × 3.0–4.5 µm, clavate, somewhat




**FIGS 1-5.** ***Byssocorticium microsporum*** **sp. nov.**
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry); 3. basidiospores; 4. basidia; 5. generative hyphae

sinuous, 4–sterigmate, with basal clamp; sterigmata up to 3.0 µm long. **Basidiospores** 2.0–2.5 × 1.5–1.8 µm, ellipsoid to subglobose, thick-walled, smooth, generally uniguttulate, inamyloid, acyanophilous.

**Remarks** – This species differs from *B. lutescens* in having smaller basidiospores (2.0–2.5 × 1.5–1.8 µm as compared to 3.5–4.5 µm) and hyphae with clamps at all septa as compared to hyphae with scattered clamps **(Plate 1).**

***Confertextum macrosporum*** Priyanka & Dhingra **sp. nov.**

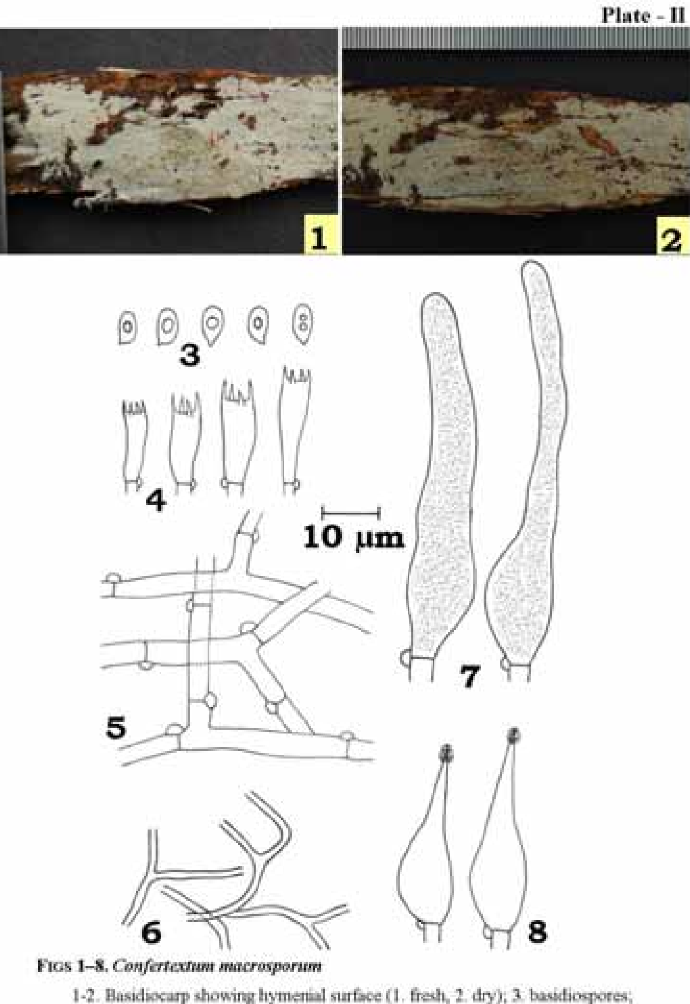
Mycobank MB810723

The new species is different from *C. microsporum* in having two types of cystidia and bigger basidiospores.

**Type:** India, Himachal Pradesh: Shimla, Kandiyali, on decaying wood of *C. deodara*, Priyanka 4620 (PUN, holotype), August 06, 2011.

**Etymology:** The epithet refers to the bigger size of basidiospores **(Plate 2).**

**Basidiocarp** resupinate, effused, closely adnate, up to 350 µm thick in section; hymenial surface smooth to tuberculate, creamish white to pale yellow when fresh, becoming pale orange to grayish orange on drying; margins thinning, paler concolorous to abrupt. **Hyphal system** dimitic. Generative hyphae up to 4.5 µm wide, thin-walled, branched, septate, clamped; basal hyphae parallel to the substrate; subhymenial hyphae vertical. Microbinding hyphae up to 1.9 µm wide, thin-walled, laxly branched. Texture dense due to the presence of microbinding hyphae along with generative hyphae. **Cystidial elements** of 2 kinds: **i) Leptocystidia** 66.0–74.0 ×5.0–8.8 µm, subcylindrical, with broad base, thin-walled, smooth, with basal clamp and oily contents. **ii)** 31.0–37.0 ×9.3–10.6 µm, bladder shaped with pointed apical region, thin-walled, resinous encrustation present on tip, with basal clamp. **Basidia** 12.5–18.5 × 3.7–6.3 µm, subclavate to clavate, 4–sterigmate, with basal clamp; sterigmata up to 3.8 µm long. **Basidiospores** 6.2–7.5 × 3.1–3.8 µm, ellipsoid to subcylinrical, apiculate, thin-walled, smooth, inamyloid, acyanophilous, with oily contents.




**FIGS 1–8.** ***Confertextum macrosporum***
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry); 3. basidiospores; 4. basidia; 5. generative hyphae; 6. microbinding hyphae; 7. leptocystidia; 8. cystidia

**Remarks**–This species is similar to *C. microsporum* in having dense texture, dimitic hyphal system, presence of microbinding hyphae, but is different in having two types of cystidia and bigger basidiospores.

***C. microsporum*** Priyanka & Dhingra **sp. nov.**

Mycobank MB810724

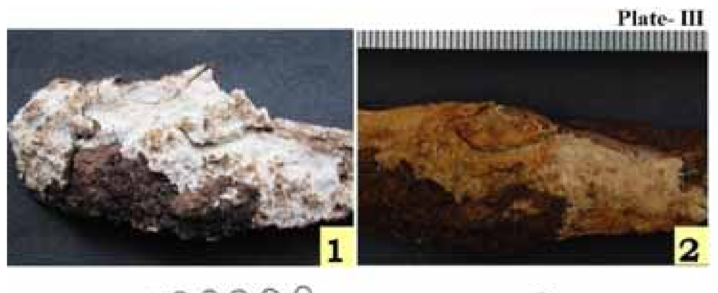
It is different from *C. macrosporum* in having smaller basidiospores

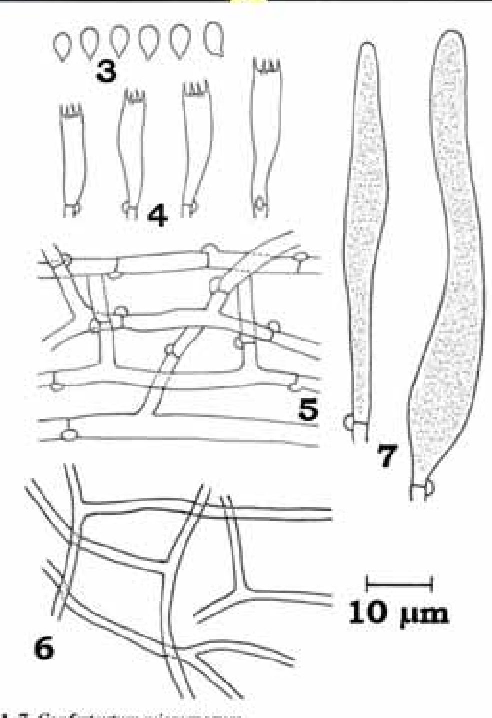
**Type:** India, Himachal Pradesh: Kangra, Nargala, on wood of *Ficus religiosa*, Priyanka 4618 (PUN), August 24, 2009.

**Etymology:** The epithet refers to the smaller size of spores

**Basidiocarp** resupinate, effused, closely adnate, up to 950 µm thick in section; hymenial surface smooth, orange white to yellowish white when fresh, brown on bruising, becoming pale orange to yellowish orange to golden yellow on drying; subiculum paler colored and visible through the cracks; margins thinning, paler concolorous to abrupt. **Hyphal system** dimitic. Generative hyphae up to 3.8 µm wide, thin-walled, branched, septate, clamped; basal hyphae parallel to the substrate; subhymenial hyphae vertical. Microbinding hyphae up to 2.0 µm wide, thin-walled, laxly branched. Texture dense due to the presence of microbinding hyphae along with generative hyphae. **Cystidia** 57.0–70.0 ×5.0–6.3 µm, subfusiform, thin-walled, with basal clamp and oily contents. **Basidia** 13.4–20.0 × 3.1–4.7 µm, clavate to subclavate, 4–sterigmate, with basal clamp; sterigmata up to 2.2 µm long. **Basidiospores** 4.3–5.3 × 2.8–3.1 µm, ellipsoid, apiculate, thin-walled, smooth, inamyloid, acyanophilous **(Plate 3).**

Plate- III






FIGS 1–7. *Confertextum microsporum*

1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry); 3. basidiospores; 4. basidia; 5. generative hyphae; 6. microbinding hyphae; 7. cystidia

**Remarks:** The newly described species is marked by the presence of dense texture, cystidia with oily contents and smaller, ellipsoid basidiospores.

***Corticium mussooriensis*** Samita, Sanyal & Dhingra sp. nov.

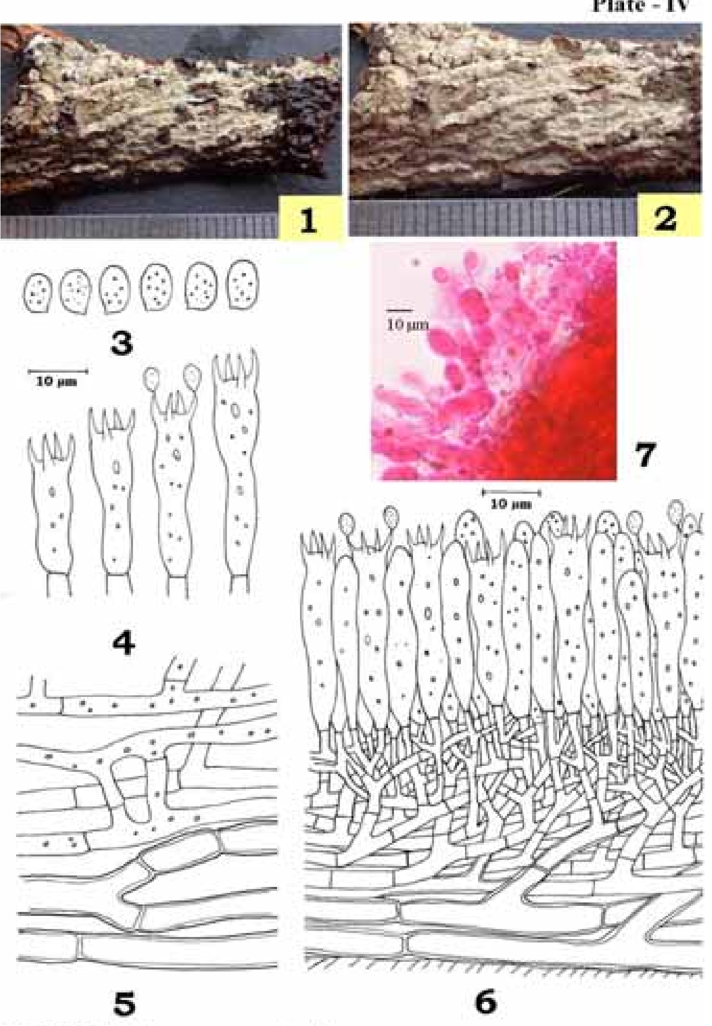
Mycobank 808613

This species differs from *Corticium albidocremeum* in having larger basidia and broadly ellipsoid basidiospores.

**Type:** India, Uttarakhand: Dehradun, Mussoorie, Mall road, on angiospermous log, Samita 6069 (PUN, holotype), August 20, 2010.

**Etymology:** The epithet refers to the locality of collection.

**Basidiocarp** resupinate, effused, adnate, up to 80 µm thick in section; hymenial surface porulose to smooth, grayish yellow when fresh, not changing much on drying; margins thinning, paler concolorous, to indeterminate. **Hyphal system** monomitic. Generative hyphae up to 5.5 µm wide, septate, without clamps; basal hyphae parallel to substrate, thick–walled, less branched; subhymenial hyphae vertical, short celled, thin–walled, more branched. **Cystidia** absent. **Basidia** 20.0–34.0 × 5.8–7.5µm, clavate to subclavate, somewhat sinuous, 4–sterigmate, without basal clamp, with oily contents; sterigmata up to 7.5 µm long. **Basidiospores** 6.0–8.3 × 4.0–5.5 µm, broadly ellipsoid, thin–walled, with oily contents, inamyloid, acyanophilous **(Plate 4).**




IGS 1-7. *Corticium mussooriensis* sp. nov.
-2. Basidiocarp showing h9ymenial surface (1. fresh, 2. dry);
. basidiospores; 4. basidia; 5. generative hyphae; 6. V.S of basidiocarp;
. Microphotograph showing basidia and basidiospores

**Remarks:** This species differs from *Corticium albidocremeum* Rehill and Bakshiin having larger basidia (20.0–34.0 × 5.8–7.5µm as compared to 11.2–14.4 × 4.8–6.4µm) and broadly ellipsoid basidiospores in comparison to oval or pip-shaped.

***Cylindrobasidium indicus*** Samita, Sanyal & Dhingra sp. nov.

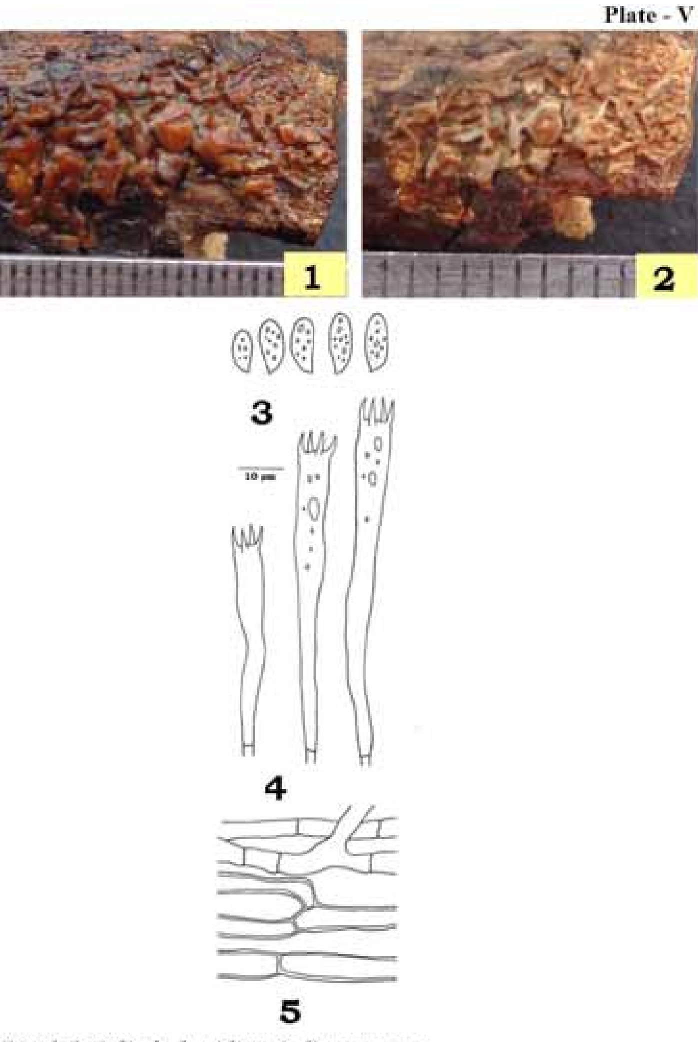
Mycobank 808607

This species differs from *C. evolvens* in having simple septate hyphae, absence of cystidia and larger basidiospores.

**Type:** India, Uttarakhand: Tehri Garhwal, Dhanaulti, on angiospermous stick, Samita 6006 (PUN, holotype), August 21, 2010.

**Etymology:** The epithet refers to the country of collection.

**Basidiocarp** resupinate, loosely adnate, effused, gelatinous, up to 450 µm thick in section; hymenial surface smooth to cracked, brownish red to reddish brown when fresh, not changing much on drying; shrinking with cracks appearing prominent; margins thinning, paler concolorous, to indeterminate. **Hyphal system** monomitic. Generative hyphae up to 5.0 µm wide, septate, without clamps; basal hyphae parallel to the substrate, thin– to somewhat thick–walled compactly arranged, less branched; subhymenial hyphae vertical, loosely arranged, thin–walled, more branched. **Cystidia** absent. **Basidia** 48.0–80.0 × 5.5–6.3 µm, narrowly clavate, somewhat sinuous, 4–sterigmate, with or without oily contents, without basal clamp; sterigmata up to 5.0 µm long. **Basidiospores** 9.3–14.4 × 4.5–5.5 µm, fusoid to lacrymoid, thin–walled, smooth, with oily contents, acyanophilous, inamyloid **(Plate 5).**




FIGS 1-5. *Cylindrobasidium indicus* sp. nov.
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry);
3. basidiospores; 4. basidia; 5. generative hyphae

**Remarks**– This species differs from *C. evolvens* in having simple septate hyphae, absence of cystidia and larger basidiospores (9.3–14.4 × 4.5–5.5 µm as compared to 7.5–9.5 × 4.3–5.0 µm).

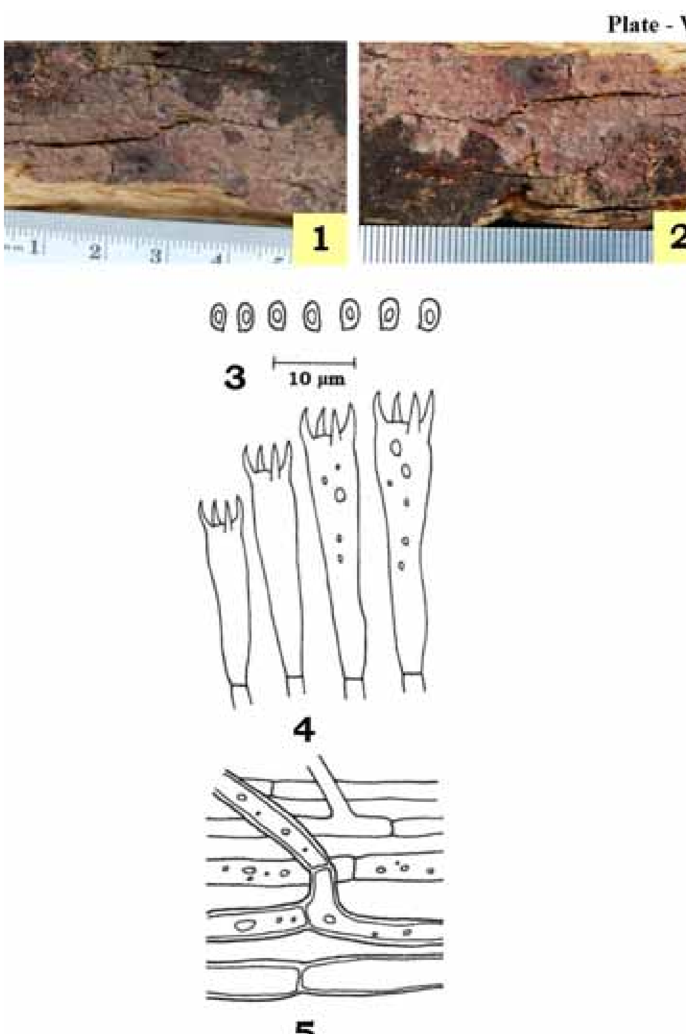
***Leptosporomyces singularis*** Samita, Sanyal & Dhingra

Mycobank 808610

This species is peculiar in having membranaceous basidiocarps, grayish red to brownish red hymenial surface and simple septate hyphae.

**Type:** India, Uttarakhand: Dehradun, Mussoorie, Lal Tibba, on angiospermous stump, Samita 6022 (PUN, holotype), August 19, 2009.

**Etymology:** The epithet refers to the unique combination of characters.




**FIGS 1-5. *Leptosporomyces singularis*** sp. nov.
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry);
3. basidiospores; 4. basidia; 5. generative hyphae

**Basidiocarps** resupinate, adnate, effused, membranaceous, up to 250 µm thick in section; hymenial surface smooth, grayish red to brownish red when fresh, not changing much on drying; margins thinning, somewhat fibrillose, whitish to paler concolorous, to indeterminate. **Hyphal system** monomitic. Generative hyphae up to 5.2 µm wide, septate, without clamps, with oily contents; basal hyphae parallel to the substrate, thin– to somewhat thick–walled, less branched, with or without oily contents; subhymenial hyphae up to 2.0 µm wide, vertical, thin–walled, more branched. **Cystidia** none. **Basidia** 19.4–30.0 × 4.7–7.5 µm, narrowly clavate, somewhat sinuous, 4–sterigmate, without basal clamp, with or without oily contents; sterigmata up to 4.3 µm long. **Basidiospores** 2.9–3.5 × 1.7–2.3 µm, ellipsoid, thin–walled, smooth, uniguttulate, inamyloid, acyanophilous **(Plate 6).**

**Additional collection examined–** India, Uttarakhand: Dehradun, Mussoorie, Lal Tibba, on angiospermous log, Dhingra 6023 (PUN), August 19, 2009.

**Remarks:** This species is peculiar in having membranaceous basidiocarps, grayish red to brownish red hymenial surface and simple septate hyphae. It does not match with any of the known species of the genus, hence described as a new taxon.

***Tomentella garhwaliana*** Samita, Sanyal & Dhingra

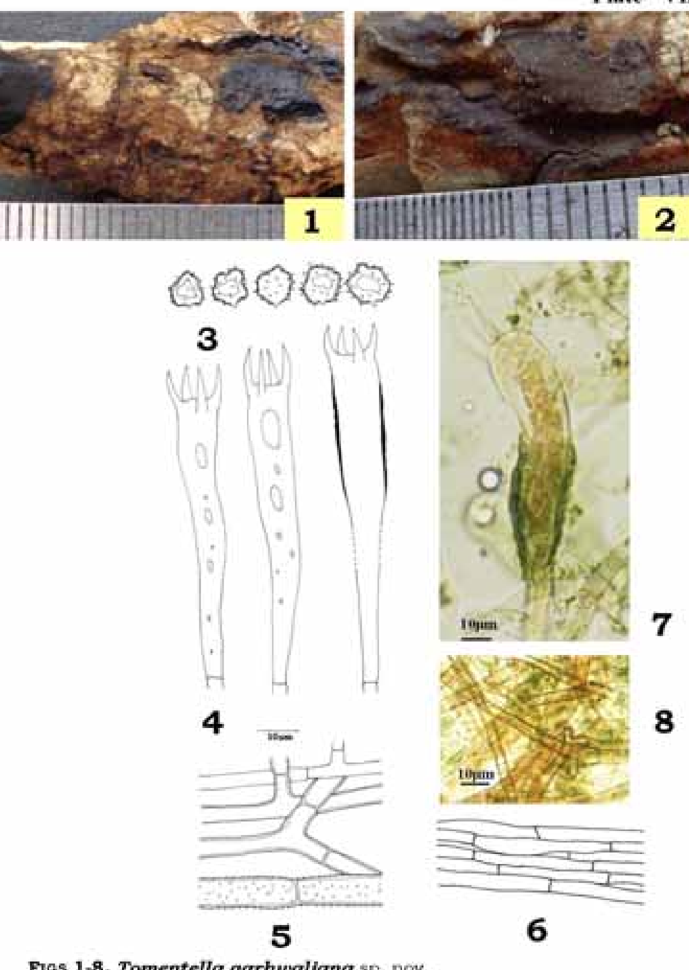
Mycobank 808625

Differs from rest of species of *Tomentella* in having deep violet to blackish basidiocarps and basidia with covering of greenish material.

**Type:** India, Uttarakhand: Tehri Garhwal, Dhanaulti, on angiospermous stump, Samita 6329 (PUN, holotype), September 02, 2012.

**Etymology:** The epithet refers to Garhwal region of the state of Uttarakhand.

**Basidiocarp** resupinate, effused, adnate; up to 300 µm thick in section; hymenial surface smooth, deep violet to blackish when fresh, not changing much on drying; margins thinning, paler concolorous, to indeterminate. **Hyphal system** monomitic. Generative hyphae septate, without clamps; basal hyphae up to 7.7 µm wide, parallel to the substrate, thick-walled, distantly septate, less branched, dark brown, with rough to spinulose outer wall; subhymenial hyphae up to 5.6 µm wide, vertical, closely septate, thin- to somewhat thick-walled, more branched. **Hyphal cordons** up to 20.0 µm




**Figs 1-8. *Tomentella garhwaliana*** sp. nov.
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry);
3. basidiospores; 4. basidia; 5. generative hyphae; 6. portion of hyphal cordon; 7-8. Microphotographs (7. basidium; 8. hyphae)

wide, dull brown, individual hyphae up to 3.3 μm wide, septate, without clamps. **Cystidia** none. **Basidia** 70.0–88.0 × 10.0–12.5 μm, narrowly clavate, somewhat sinuous, 4–sterigmate, thin- to thick-walled, covered by a thick, greenish material, without basal clamp, with or without oily contents; sterigmata up to 12.5 μm long. **Basidiospores** 8.0–10.6 μm across, irregular in outline to lobed, thick-walled, with oily contents, echinulate, yellowish brown **(Plate 7).**

**Remarks–** This species is peculiar in having smooth, deep violet to blackish basidiocarps, simple-septate hyphae, narrowly clavate basidia having a covering of greenish material and irregular to lobed basidiospores and does not match with any of the known species Larsen, Kõljalg, Rattan, Dhingra, Dhingra and Rani, Harpreet *et al.* [7-10].

***Vararia himalayana*** Samita, Sanyal & Dhingra

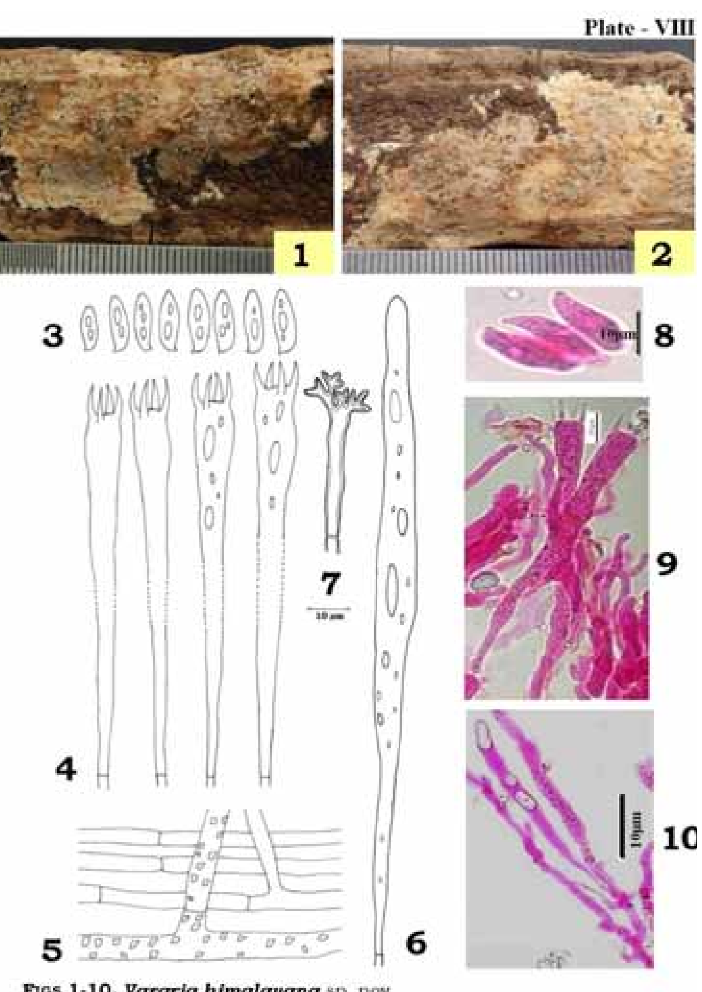
Mycobank 808623

This species differsfrom *V. gomezii* in having dendrohyphidia only in the subhymenium, very long gloeocystidia and longer basidia.

**Type:** India, Uttarakhand: Tehri Garhwal,Surkanda Devi,on stump of *Q. leucotrichophora*, Dhingra 6263 (PUN, holotype), September 28, 2012.

**Etymology:** The epithet refers to the Himalayan region of collection.

**Basidiocarps** resupinate, adnate, effused, up to 410 μm thick in section; hymenial surface smooth to tuberculate, pale orange to grayish orange to brownish orange when fresh, not changing much on drying; margins thinning, fibrillose, paler concolorous, to indeterminate. **Hyphal system** monomitic. Generative hyphae septate, without clamps, thin–walled; basal hyphae up to 6.3 μm wide, parallel to the substrate, less branched, heavily encrusted; subhymenial hyphae up to




**Figs 1-10. *Vararia himalayana*** sp. nov.
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry);
3. basidiospores; 4. basidia; 5. generative hyphae; 6. gloeocystidium;
7. dendrohyphidium; 8-10. Microphotographs (8. basidiospores;
9. basidia; 10. gloeocystidia)

3.5 µm wide, vertical, more branched. **Dendrohyphidia** few, present only in the subhymenium, dichotomously to irregularly branched, branches thick–walled, with blunt to somewhat pointed endings, dextrinoid. **Gloeocystidia** 87.0–166.0 × 6.3–10.0 µm, tubular, smooth, thin–walled, without basal clamp, with oily contents negative to sulphovanillin, projecting up to 30 µm out of hymenium. **Basidia** 80.0–108.0 × 8.8–10.0 µm, narrowly clavate, sinuous, 4–sterigmate, with or without oily contents, without basal clamp; sterigmata up to 6.5 µm long. **Basidiospores** 11.1–15.5 × 4.4–5.5 µm, subfusiform to navicular, thin–walled, smooth, with oily contents, acyanophilous, inamyloid, sometimes in groups of two or three **(Plate 8).**

**Remarks** – This species differs from *V. gomezii* in having dendrohyphidia only in the subhymenium, very long, thin–walled gloeocystidia and longer basidia (80.0 – 108.0 × 8.8 – 10.0 µm in comparison to 34.0–45.0 ×7.0–8.0 µm).

***Vararia indica*** Samita, Sanyal & Dhingra

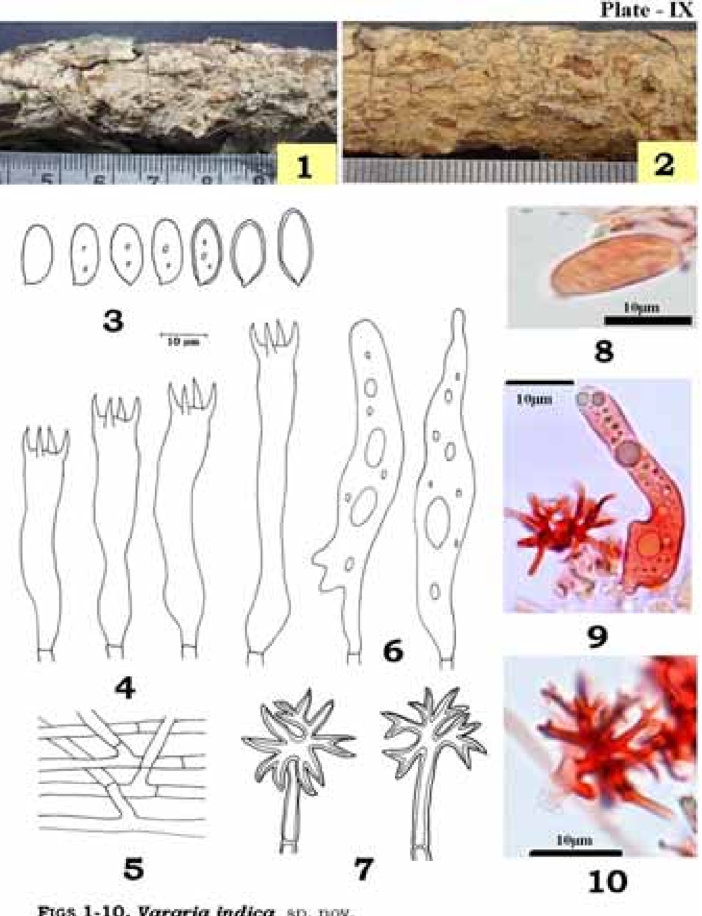
Mycobank 808624

Differs from *V. ellipsospora* in having bigger, ellipsoid to ovoid basidiospores.

**Type:** India, Uttarakhand: Pauri Garhwal, Adwani, on stump of *Q. leucotrichophora*, Dhingra 6265 (PUN, holotype), July 23, 2011.

**Etymology:** The epithet refers to the country of collection.

**Basidiocarp** resupinate, adnate, effused, up to 160 µm thick in section; hymenial surface smooth to somewhat tuberculate, grayish white to grayish yellow when fresh, pale yellow to grayish orange on drying, margins thinning, paler concolorous, to indeterminate. **Hyphal system** monomitic. Generative hyphae up to 3.0 µm wide, septate, without clamps; basal hyphae thin– to thick–walled, parallel to the substrate, less branched; subhymenial hyphae vertical, thin–walled, more branched. **Dendrohyphidia** abundant, present both in the hymenium and subhymenium, dichotomously to irregularly branched, branches thick–walled, with blunt to somewhat pointed endings. **Gloeocystidia** 70.0–75.0 × 10.0–11.8 µm, variable in shape, generally fusiform, tip pointed, moniliform to round, base narrow to swollen, smooth, thin–walled, with oily contents negative to sulphovanillin, without basal clamp, projecting up to 30 µm out of hymenium. **Basidia** 41.0–65.0 × 6.5–7.2 µm, clavate to subutriform, somewhat constricted, 4–sterigmate, without basal clamp; sterigmata up to 5.0 µm long. **Basidiospores** 12.7–17.3 × 5.4–7.2 µm, ellipsoid to ovoid, thin– to somewhat thick–walled, smooth, with or without oily contents, acyanophilous, amyloid **(Plate 9).**



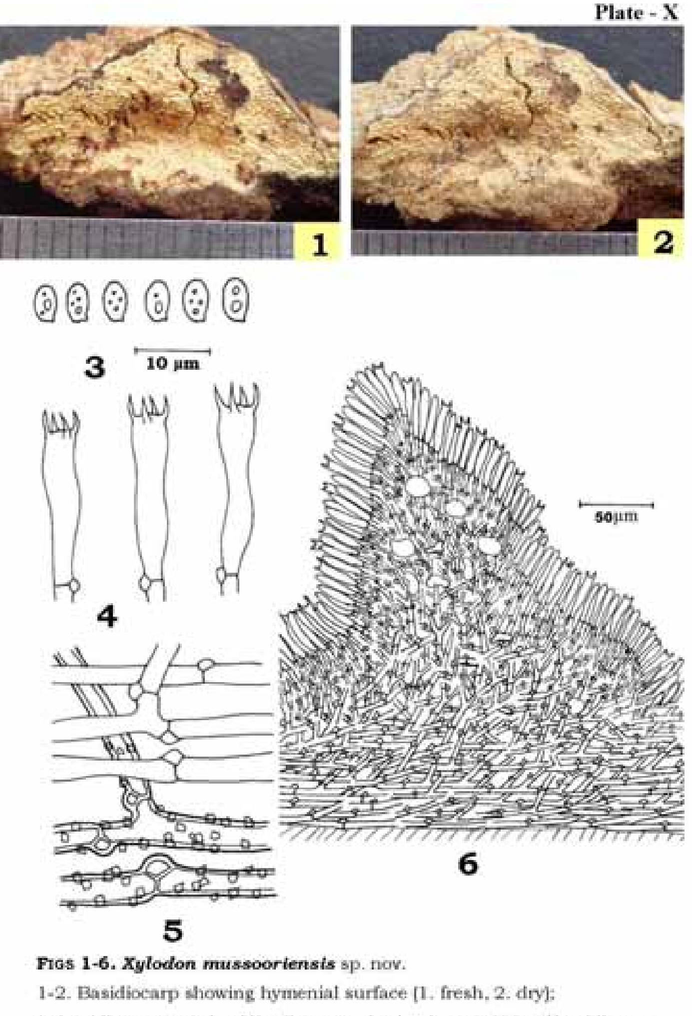

FIGS 1-10. ***Vararia indica*** sp. nov.
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry); 3. basidiospores; 4. basidia; 5. generative hyphae; 6. gloeocystidia; 7. dendrohyphidia; 8-10. Microphotographs (8. basidiospore; 9. cystidium; 10. dendrohyphidia)

**Remarks** – This species differs from *V. ellipsospora* in having bigger (12.7–17.3 × 5.4–7.2 µm in comparison 8.0–12.0 × 5.5–6.5 µm), ellipsoid to ovoid basidiospores.

***Xylodon mussoriensis*** Samita, Sanyal & Dhingra

Mycobank 808620

The new species differs from *X. asperus* in lacking capitate hyphal ends in the aculei.




FIGS 1-6. *Xylodon mussooriensis* sp. nov.
1-2. Basidiocarp showing hymenial surface [1. fresh, 2. dry];
3. basidiospores; 4. basidia; 5. generative hyphae; 6. V.S. of basidiocarp

**Type:** India, Uttarakhand: Dehradun, Mussoorie, Mall Road, on angiospermouslog, Samita 6207 (PUN, holotype), August 20, 2010.

**Etymology:** The epithet refers to the locality of collection.

**Basidiocarp** resupinate, effused, adnate, up to 320 µm thick in section; hymenial surface odontioid, aculei dense, conical, pale orange to grayish orange when fresh, not changing much on drying; margins thinning; paler concolorous, to indeterminate. **Hyphal system** monomitic.Generative hyphae, branched, septate, clamped; basal hyphae up to 4.5 µm wide, parallel to the substrate, encrusted, thick–walled, loosely arranged; subhymenial hyphae up to 3.5 µm wide, vertical, thin–walled, compactly arranged. **Cystidia like hyphal ends** none. **Basidia** 21.0–24.0 × 4.1–5.2 µm, narrowly clavate, somewhat sinuous, 4–sterigmate, with basal clamp; sterigmata up to 4.0 µm long. **Basidiospores** 5.2–5.8 × 3.1–3.5 µm, ellipsoid to broadly ellipsoid, smooth, thin–walled, with oily contents, inamyloid, acyanophilous **(Plate 10).**

**Remarks**– This speciesis different from the closely related *X. asperus* in lacking capitate hyphal ends in the aculei and smaller basidiospores with oily contents.

***Xylodon subglobosus*** Samita, Sanyal & Dhingra

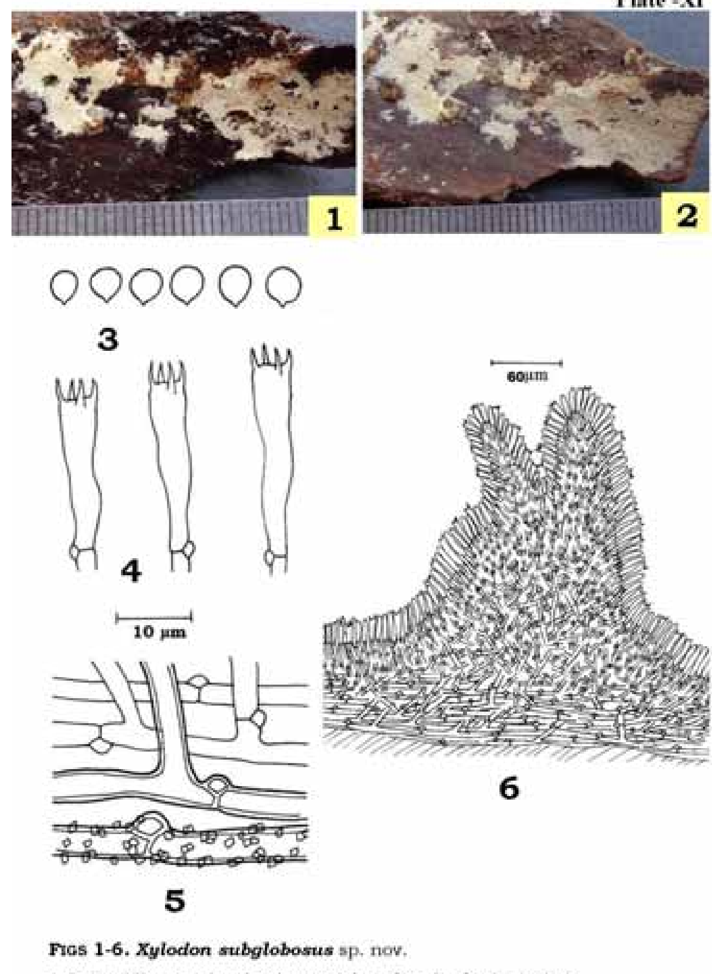
Mycobank 808621

This species is peculiar in having odontioid hymenial surface and small, subglobose basidiospores.

**Type:** India, Uttarakhand: Tehri Garhwal, Dhanaulti, on angiospermous stump, Samita 6208 (PUN, holotype), August 21, 2010.

**Etymology:** The epithet refers to the shape of basidiospores.

**Basidiocarp** resupinate, effused, adnate, up to 300 µm thick in section; hymenial surface odontioid, aculei dense, conical, pale orange when fresh, orange gray to grayish orange on drying; margins thinning, fibrillose, paler concolorous, to indeterminate. **Hyphal system** monomitic. Generative




FIGS 1-6. *Xylodon subglobosus* sp. nov.
1-2. Basidiocarp showing hymenial surface [1. fresh, 2. dry];
3. basidiospores; 4. basidia; 5. generative hyphae; 6. V.S of basidiocarp

hyphae, branched, septate, clamped; basal hyphae up to 4.7 µm wide, parallel to the substrate, thick–walled, loosely arranged, encrusted; subhymenial hyphae up to 3.5 µm wide, vertical, thin–walled, compactly arranged. Prominent patches of encrustation in the aculei. **Cystidia like hyphal ends** none. **Basidia** 20.0–26.0 × 4.7–5.3 µm, clavate, somewhat sinuous, 4–sterigmate, with basal clamp; sterigmata up to 4.0 µm. **Basidiospores** 4.2–5.2 × 3.0–5.0 µm, subglobose, smooth, thin–walled, inamyloid, acyanophilous **(Plate 11).**

**Remarks**– This species differs from the closely related *X. pruni* in having smaller (4.2–5.2 × 3.0–5.0 µm), subglobose basidiospores as compared to bigger (5.5–7.0 × 3.5–4.5 µm), ellipsoid ones.

***Scytinostroma phaeosarcum*** var. ***angustispora*** Samita, Sanyal & Dhingra var. nov.

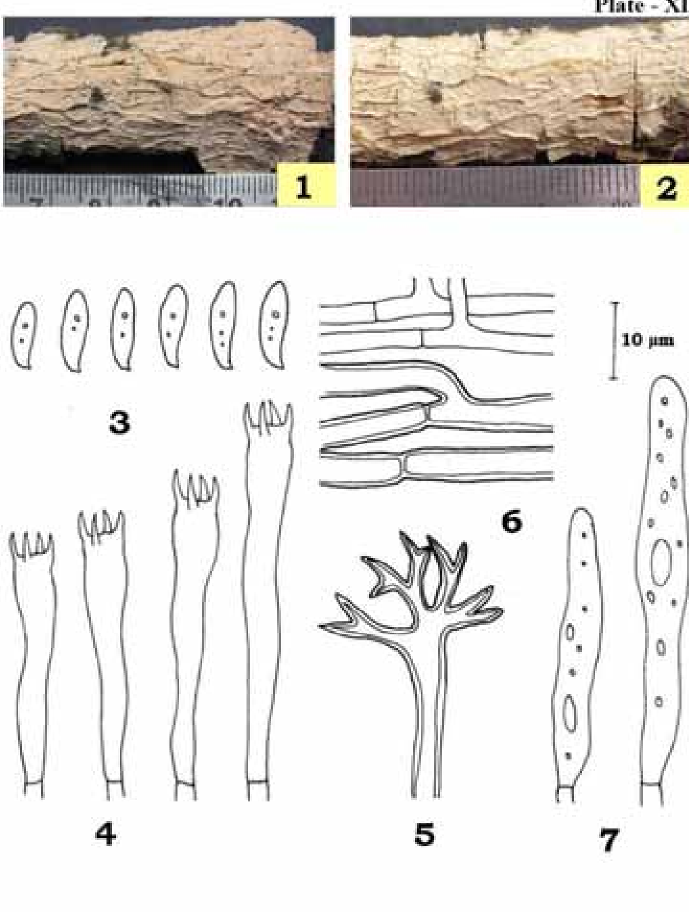
Mycobank 808622

The new variety differs from *S. phaeosarcum* in having narrower, suballantoid to subfusiform basidiospores**.**

**Type:** India, Uttarakhand: Pauri Garhwal, Adwani, on burnt stump of *R. arboreum*, Samita 6256 (PUN, holotype), August 23, 2011.

**Etymology:** The epithet refers to the shape of basidiospores.

**Basidiocarp** resupinate, adnate, effused, up to 210 µm thick in section; hymenial surface smooth to tuberculate, pale orange to grayish orange when fresh, not changing much on drying; margins thinning, paler concolorous, to indeterminate. **Hyphal system** dimitic. Generative hyphae up to 4.5 µm wide, branched, septate, thin**-**to thick**-**walled, without clamps. Skeletal**-**binding hyphae up to 5.3 µm wide, dichotomously to irregularly branched, restricted to basal zone, aseptate, thick**-**walled, cyanophilous. **Gloeocystidia** 36.0–54.0 × 5.2–7.0 µm, subcylindrical, smooth, thin**-**walled, with oily contents positive to sulphovanillin, without basal clamp. **Basidia** 29.0–48.0 × 4.7–6.0 µm, clavate, somewhat sinuous, 4–sterigmate, without basal clamp; sterigmata up to 5.5 µm long. **Basidiospores** 8.0–11.2 × 2.9–3.5 µm, suballantoid to subfusiform, thin**-**walled, smooth, with oily contents, acyanophilous, inamyloid **(Plate 12).**




**FIGS 1-7.** ***Scytinostroma phaeosarcum*** **var.** ***angustispora*** var. nov.
1-2. Basidiocarp showing hymenial surface (1. fresh, 2. dry);
3. basidiospores; 4. basidia; 5. skeletal-binding hyphae; 6. generative hyphae; 7. gloeocystidia

**Remarks:** The new variety differs from *Scytinostroma phaeosarcum* in having narrower (8.0–11.2 × 2.9–3.5 µm) in comparison to (7.5)-8.0–10.0-(11) × 4.5–5.0-(6) µm], suballantoid to subfusiform basidiospores in contrast to broadly ellipsoid ones.

**New taxa already published**

**New genera**

***Cordochaetec*** Sanyal, Samita, Dhingra & Avneet P Singh, Mycotaxon 123: 103, 2013.
Distribution in the Himalaya – Uttarakhand
***Dendrophlebia*** Dhingra & Priyanka, Mycotaxon 116: 157, 2011.
Distribution in the Himalaya – Arunachal Pradesh
***Hallenbergia*** Dhingra & Priyanka, Mycotaxon 118: 289, 2011.

Distribution in the Himalaya – Bhutan
***Radulomycetopsis*** Dhingra, Priyanka & J. Kaur., Mycotaxon 119: 133, 2012.
Distribution in the Himalaya – Bhutan
***Repetobasidiopsis*** Dhingra & Avneet P. Singh, Mycotaxon 105: 421, 2008.
Distribution in the Himalaya - Arunachal Pradesh
***Trimitiella*** Dhingra, Mycotaxon 97: 125, 2006.
Distribution in the Himalaya - Arunachal Pradesh
Distribution in the Himalaya - Manipur

**New subgenera**

***Stereum*** subgen**. *Acanthostereum*** Boidin, Parmasto, Dhingra & Lanquetin, Persoonia 10: 320, 1979.
Distribution in the Himalaya - Manipur
***Stereum*** subgen***.Aculeatostereum*** Boidin, Parmasto, Dhingra & Lanquetin, Persoonia 10: 320, 1979

**New species**

***Aleurodiscus himalaicus*** Maninder K, Avneet P. Singh, Dhingra & Ryvarden, Synopsis Fungorum 32: 5, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (5200, 5985, 5986)]
***A. indicus*** Ryvarden, S.K. Sanyal& Dhingra, Synopsis Fungorum (Oslo) 30: 14, 2012**.**
Distribution in the Himalaya - Uttarakhand [PUN (4413, 6292)]
***Athelopsis parvisporus*** Avneet P. Singh, Dhingra & J. Kaur, Mycotaxon 113: 327, 2010.
Distribution in the Himalaya - Himachal Pradesh [PUN (4860)]
***Candelabrochaete himalayana*** Dhingra, Synopsis Fungorum 29: 26, 2011.
Distribution in the Himalaya - Bhutan [PAN (19430)]
***Ceraceomyces bizonatus*** Dhingra & Avneet P. Singh, Mycotaxon 106: 399, 2009.
Distribution in the Himalaya - Himachal Pradesh [PUN (3612, 3613)]
***Clavulicium hallenbergii*** Avneet P. Singh, J. Kaur & Dhingra, Mycotaxon 120: 353, 2012.
Distribution in the Himalaya - Himachal Pradesh [PUN (3683, 4898)]
***Conohypha grandispora*** Dhingra, Synopsis Fungorum 29: 28, 2011.
Distribution in the Himalaya - West Bengal [PAN (19286,19323)]
***Cristinia tubulicystidiata*** J. Kaur, Dhingra & Hallenberg,Mycotaxon 127: 89, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (4763)]
***Dendrophlebia crassispora*** Dhingra &Priyanka, Mycotaxon 116: 159, 2011.
Distribution in the Himalaya - Arunachal Pradesh [PAN (19726)]
***Flavophlebia sphaerospora*** Man. Kaur, Avneet P. Singh & Dhingra, Mycotaxon 126: 231, 2013.
Distribution in the Himalaya - Himachal Pradesh [PUN (5166)]
***Fibulomyces cystoideus*** Dhingra, Synopsis Fungorum 29: 30, 2011.
Distribution in the Himalaya - Bhutan [PAN (19365)]
***Hallenbergia singularisa*** Dhingra & Priyanka, Mycotaxon 118: 289, 2011.
Distribution in the Himalaya - Bhutan [PAN (19548)]
***Hyphoderma bicystidiatum*** Priyanka & Dhingra, Mycotaxon 119: 255, 2012.
Distribution in the Himalaya - Himachal Pradesh [PUN (4928)]
***H. claruspropr*i*etas*** Dhingra, Synopsis Fungorum 29: 32, 2011.
Distribution in the Himalaya - West Bengal [PAN (19239)]
***H. densustextum*** Dhingra, Synopsis Fungorum 29: 33, 2011.
Distribution in the Himalaya - West Bengal [PAN (19229)]
***H. hallenbergii*** Man. Kaur, Avneet P. Singh & Dhingra, Mycotaxon (in press).
Distribution in the Himalaya - Himachal Pradesh [PUN(6962)]
***H. parvisporum*** Avneet P. Singh, Priyanka, Dhingra & Singla, Mycotaxon 111: 71, 2010.

Distribution in the Himalaya - Himachal Pradesh [PUN (1623)]
***H. sikkimia*** Dhingra, Synopsis Fungorum 29: 37, 2011.
Distribution in the Himalaya - Sikkim [PAN (19349)]
***H. sporulosum*** Dhingra, Synopsis Fungorum 29: 39, 2011.
Distribution in the Himalaya - West Bengal [PAN (19526)]
***H. subglobosum*** Priyanka & Dhingra, Mycotaxon 119: 257, 2012.
Distribution in the Himalaya - Himachal Pradesh [PUN (4299)]
***Hyphodontia caulicystidiata*** Dhingra, Synopsis Fungorum 29: 41, 2011.
Distribution in the Himalaya - West Bengal [PAN (19262)]
***H. dhingrae*** Samita & Sanyal,Mycotaxon (in press).
Distribution in the Himalaya - Uttarakhand [PUN (5199)]
***Leptocorticium indicum*** Samita, Sanyal & Dhingra**,** Mycotaxon (in press).
Distribution in the Himalaya - Uttarakhand [PUN (6092)]
***Leucogyrophana thimphina*** Dhingra, Synopsis Fungorum 29: 44, 2011.
Distribution in the Himalaya - Bhutan [PAN (19566)]
***Paullicorticium indicum*** Dhingra, Synopsis Fungorum 29: 46, 2011.
Distribution in the Himalaya - West Bengal [PAN (19255)]
***Peniophora hallenbergii*** Samita & Dhingra, Mycotaxon 126: 235, 2013.
Distribution in the Himalaya - Uttarakhand [PUN (5167, 6285)]
***Phlebia crassisubiculata*** Avneet. P. Singh, Priyanka, Dhingra & Singla, Mycotaxon 112: 21, 2010.
Distribution in the Himalaya - Himachal Pradesh [PUN (1405)]
***P. interjacenoides*** Dhingra, Synopsis Fungorum 29: 49, 2011.
Distribution in the Himalaya - Bhutan [PAN (19628)]
***P. microspora*** Dhingra, Synopsis Fungorum 29: 51, 2011.
Distribution in the Himalaya - West Bengal [PAN (19203)]
***P. kamengii*** Dhingra, Synopsis Fungorum 29: 51, 2011.
Distribution in the Himalaya - Arunachal Pradesh [PAN (19690, 19693)]
***P. singularisa*** Dhingra, Synopsis Fungorum 29: 54, 2011.
Distribution in the Himalaya - Bhutan [PAN (19612)]
***P. thindii*** Dhingra, Synopsis Fungorum 29: 57, 2011.
Distribution in the Himalaya - West Bengal [PAN (19249, 19305)]
***Phlebiopsis darjeelingensis*** Dhingra, Nova Hedwigia, 44: 222, 1987.
Distribution in the Himalaya - West Bengal [PAN (19199)]
***P. himalayensis*** Dhingra, Nova Hedwigia, 44: 222, 1987.
Distribution in the Himalaya - Arunachal Pradesh [PAN (19862, 19888)], West Bengal[PAN (19202)]
***P. mussooriensis*** Priyanka, Dhingra & N. Kaur, Mycotaxon 115: 255, 2011.
Distribution in the Himalaya - Uttarakhand [PUN (3405)]
***Radulodon indicus*** Jyoti & Dhingra, Synopsis Fungorum 32: 38, 2014.
Distribution in the Himalaya - Jammu and Kashmir [PUN (5987)]
***R. acaciae*** G. Kaur, Avneet P. Singh & Dhingra 127: 111, 2014.
Distribution in the Himalaya - Chandigarh [PUN (5982, 5983, 5984)]
***Redulomycetopsis cystidiata*** Dhingra, Mycotaxon 119: 135, 2012.
Distribution in the Himalaya - Arunachal Pradesh [PAN (19767)]
***Repetobasidiopsis grandisporus*** Dhingra & Avneet P. Singh, Mycotaxon 97: 116, 2006.
Distribution in the Himalaya - Arunachal Pradesh [PAN (19706)]
***Scytinostroma pulverulentum*** Boidin & Dhingra, In Boidin & Lanquetin, Le Genre *Scytinostroma* Donk.(*Basidiomycètes*, *Lachnocladiaceae*), Bibliotheca Mycologica 114: 94, 1987.
Distribution in the Himalaya - Bhutan [PAN (19598)]

***S. renisporum*** Boidin, Lanquetin & Gilles, In Boidin & Lanquetin, Le Genre *Scytinostroma* Donk.(*Basidiomycètes*, *Lachnocladiaceae*), Bibliotheca Mycologica 114: 97, 1987.
Distribution in the Himalaya - Meghalaya [PAN (19029)]
***Sistotrema angustispora*** Dhingra, Synopsis Fungorum 29: 63, 2011.
Distribution in the Himalaya - West Bengal [PAN (19233)]
***Sistotremastrum roseum*** Jaspreet and Dhingra, Synopsis Fungorum32: 26, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (5057)]
***Stereum peculiare*** Parm., Boidin & Dhingra, Persoonia 10: 311, 1979.
Distribution in the Himalaya - Manipur [PAN (19093, 19787,19789, 19798, 19803, 19820, 19833, 19847, 19864, 19876)]
***T. kalatopii*** Dhingra & Malka, Synopsis Fungorum 32: 30, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (1748)]
***T. unicusa*** Dhingra & Malka, Synopsis Fungorum 29: 32, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (1756)]
***Trimitiella indica*** Dhingra, Mycotaxon 97: 126, 2006.
Distribution in the Himalaya - Arunachal Pradesh [PAN (19722)]
***V. longicystidiata*** Samita, Sanyal, Dhingra & Singh, Mycotaxon 120: 357, 2012.
Distribution in the Himalaya - Uttarakhand [PUN (4413)]

## New varieties

***Amphinema byssoides*** var. ***macrospores*** Dhingra & Avneet P. Singh, Synopsis Fungorum 32:10, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (3528)]
***Botryobasidium subcoronatum*** var***. crassispora***Dhingra, Synopsis Fungorum 29: 25, 2011.
Distribution in the Himalaya - Himachal Pradesh [PUN (4891)]
***Ceraceomyces sublaevis*** var***. grandisporus*** Dhingra & Avneet P. Singh, Synopsis Fungorum 32:13, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (3560)]
***Conohypha albocremea*** var***. angustisporum*** Priyanka &Dhingra,Synopsis Fungorum 32:14, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (4453)]
***Hyphoderma roseocremeum*** var***. minutisporum***Priyanka & Dhingra, Synopsis Fungorum 32:18, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (4470)]
***H. setigerum*** var***. bicystidium*** Dhingra & Singla, Synopsis Fungorum 32:18, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (1469)]
***Tomentella cladii*** var***. grandii*** Dhingra and Malka, Synopsis Fungorum 32:28, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (1749)]
***Tubulicium vermiferum*** var***. hexasterigmatum*** Jaspreet & Dhingra, Synopsis Fungorum 32: 33, 2014.
Distribution in the Himalaya - Himachal Pradesh [PUN (5069)]

## New Records for the Himalaya

| S. No. | Name of the taxon | Herbarium No. |
|---|---|---|
| 1. | *Acanthophysallum lividocoeruleum* (Karst.) Parmasto | PUN (5007 , 5008) |
| 2. | *Aleurodiscus amorphous* (Pers.) Schröt. | PUN (5003) |
| 3. | *Aleurodiscus cerussatus* (Bres.) Höhn. & Litsch. | PUN (6290, 6291) |
| 4. | *Aleurodiscus lapponicus* Litsch. | PUN (5005, 5006) |
| 5. | *Aleurodiscus oakesii* (Berk. & Curt.) Höhn. & Litsch. | PAN (19450) |
| 6. | *Alutaceodontia alutacea* (Fr.) Hjortstam & Ryvarden | PUN (4924) |
| 7. | *Amethicium luteoincrustatum* Hjortstam & Ryvarden | PUN (6951) |
| 8. | *Amphinema byssoides* (Fr.) John Erikss. | PAN (19620) |

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| 9. | *Amyloathelia crassiuscula* Hjortstam & Ryvarden | PUN (4841) |
| 10. | *Asterostroma boninense* Suhara & N. Maekawa | PUN (6214, 6215) |
| 11. | *Asterostroma cervicolor* (Berk. & Curt.) Mass. | PAN (19253) |
| 12. | *Athelia bombacina* Pers. | PUN (1851, 3415) |
| 13. | *Athelia pyriformis* (Christ.) Jülich | PUN (6017) |
| 14. | *Athelia salicum* Pers. | PUN (6016) |
| 15. | *Athelia tenuispora* Jülich | PUN (6015) |
| 16. | *Athelopsis lunata* (Romell ex Bourdot & Galzin) Parmasto | PUN (4859) |
| 17. | *Athelopsis subinconspicua* (Litsch.) Jülich | PUN (4863) |
| 18. | *Boidinia furfuracea* (Bres.) Stalpers & Hjortstam | PUN (4199, 5001) |
| 19. | *Boidinia lacticolor* (Bres.) Hjortstam & Ryvarden | PUN (5000) |
| 20. | *Botryobasidium botryosum* (Bres.) John Erikss. | PAN (19289) |
| 21. | *Botryobasidium laeve* (J. Erikss.) Parmasto | PUN (6502) |
| 22. | *Botryobasidium obtusisporum* J. Erikss. | PUN (3429, 4890) |
| 23. | *Botryobasidium pruinatum* (Bres.) J. Erikss. | PUN (3603, 3604) |
| 24. | *Botryobasidium subcoronatum* (Höhn. & Litsch.) Donk | PAN (19235) |
| 25. | *Botryohypochnus isabellinus* (Fr.) J. Erikss. | PAN (19622) |
| 26. | *Byssomerulius corium* (Fr.) Parm. | PAN (19633) |
| 27. | *Ceraceomyces borealis* (Rom.) J. Erikss. & Ryv. | PAN (19533) |
| 28. | *Ceraceomyces cystidiatus* (J. Erikss. & Hjortstam in Jülich) Hjortstam | PUN (4842) |
| 29. | *Ceraceomyces sublaevis* (Bres.) Jülich | PUN (1849, 3416) |
| 30. | *Ceratobasidium obscurum* D.P. Rogers | PUN (4542) |
| 31. | *Clavulicium delectabile* (Jacks.) Hjortstam | PAN (19392) |
| 32. | *Clavulicium macounii* (Burt) J. Erikss. & Boidin ex Parmasto, | PUN (6058, 6059) |
| 33. | *Conferticium ochraceum* (Fr.) Hallenb. | PUN (5015, 5016) |
| 34. | *Coniophora arida* (Fr.) Karst. | PAN (19420) |
| 35. | *Coniophora betulae* Karst. | PAN (19575) |
| 36. | *Coniophora cordensis* Rattan | PAN (19546) |
| 37. | *Coniophora deflectens* (Bres. & Sydow) Parmasto | PUN (6025, 6026) |
| 38. | *Coniophora fusispora* (Cooke & Ell.) Cooke | PAN (19427, 19583) |
| 39. | *Coniophora olivacea* (Fr.) Karst. | PAN19355, 19544 |
| 40. | *Conohypha terricola* (Burt) Jülich | PUN (4504) |
| 41. | *Corticium roseum* Pers. | PUN (3419, 3579) |
| 42. | *Cristinia helvetica*(Pers.) Parm. | PAN (19477) |
| 43. | *Crustoderma corneum* (Bourdot and Galzin) Nakasone | PUN (4444,4447) |
| 44. | *Crustoderma dryinum* (Berk. & M.A. Curtis)Parmasto | PUN (3491, 4450) |
| 45. | *Crustoderma testatum* (H.S. Jacks. and Dearden) Nakasone | PUN (3816, 4451) |
| 46. | *Cylindrobasidium evolvens* (Fr. : Fr.) Jülich | PAN (19462) |
| 47. | *Dacryobolus karstenii* (Bres.) Oberw. | PAN (19641) |
| 48. | *Dendrothele alliacea* (Quél.) P.A. Lemke | PUN (4902) |
| 49. | *Dendrothele Mexicana* (P.A. Lemke) P.A. Lemke | PUN (6088, 6089) |
| 50. | *Dendrothele seriata* (Berk. & M.A. Curtis) P.A. Lemke | PUN (4906) |
| 51. | *Dendrothele strumosa* (Fr.) P.A. Lemke | PUN (6090, 6091) |
| 52. | *Deviodontia pilaecystidiata* (Parmasto) Hjortstam & Ryvarden | PUN (6103, 6104) |
| 53. | *Dichostereum kenyense* Boidin & Lanq. | PUN (4971) |
| 54. | *Dichostereum peniophoroides* (Burt) Boidin & Lanq. | PUN (5145) |
| 55. | *Duportella Miranda* Boidin, Lanq. & Gilles | PUN (6266, 6267) |
| 56. | *Fibriciellum silvae-ryae* J. Erikss. & Ryvarden | PUN (1408, 1834) |
| 57. | *Fibricium rude* (Karst.) Jülich | PUN (1835, 1837) |
| 58. | *Fibrodontia gossypina* Parm. | PAN (19669) |

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| 59. | *Fibulomyces cystoideus* Dhingra | PAN (19365) |
| 60. | *Fibulomyces mutabilis* (Bres.) Jülich | PUN (1468, 3511) |
| 61. | *Galzinia incrustans* (Höhn. & Litsch.) Parmasto | PUN (4907) |
| 62. | *Gloeocystidiellum clavuligerum* (Höhn. & Litsch.) Nakasone | PUN (6209, 6210) |
| 63. | *Gloeocystidiellum lactescens* (Berk.) Boidin | PAN (19502) |
| 64. | *Hastodontia halonata* (J. Erikss. & Hjortstam) Hjortstam & Ryvarden | PUN (6105, 6106) |
| 65. | *Hymenochaete luteobadia* (Fr.) Höhn. & Litsch. | PUN (3081, 3082) |
| 66. | *Hymenochaete rhabarbarina* (Berk.) Cooke | PUN (6119, 6121) |
| 67. | *Hymenochaete separate* G. Cunn. | PUN (3449) |
| 68. | *Hyphoderma argillaceum* (Bres.) Donk | PAN (19221) |
| 69. | *Hyphoderma cremeoalbum* (Höhn. & Litsch.) Jülich | PUN (4221) |
| 70. | *Hyphoderma deviatum* (S. Lundell) Parmasto | PUN (3353, 3355) |
| 71. | *Hyphoderma guttuliferum* (P. Karst.) Donk | PUN (4459, 4460) |
| 72 | *Hyphoderma luridum* (Bourdot & Galzin) J. Erikss. & Hjortstam | PUN(5134) |
| 73. | *Hyphoderma macedonicum* (Litsch.) Donk | PUN (4463, 4464) |
| 74. | *Hyphoderma medeoburiense* (Burt) Donk | PUN (1883, 3752) |
| 75. | *Hyphoderma obtusum* J. Erikss. | PUN (4465) |
| 76. | *Hyphoderma occidentale* (D.P. Rogers) Boidin & Gilles | PUN (4051) |
| 77. | *Hyphoderma orphanellum* (Bourdot & Galzin) Donk | PUN (4619) |
| 78. | *Hyphoderma pallidum* (Bres.) Donk | PAN (19609) |
| 79. | *Hyphoderma praetermissum* (Karst.) J. Erikss. & Strid | PAN (19501) |
| 80. | *Hyphoderma puberum* (Fr.) Wallr. | PAN (19512) |
| 81. | *Hyphoderma rude* (Bres.) Hjortstam and Ryvarden | PAN (19837) |
| 82. | *Hyphoderma sambuci* (Pers.) Jülich | PAN (19753, 19871) |
| 83. | *Hyphoderma setigerum* (Fr.) Donk | PAN (19534) |
| 84. | *Hyphoderma sibiricum* (Parmasto) J. Erikss. & Å. Strid | PUN (4203, 4615) |
| 85. | *Hyphoderma terricola* (Burt) K.J. Martin and Gilb. | PUN (4504) |
| 86. | *Hyphoderma tsugae* (Burt) J. Erikss. and Å Strid | PAN (19106) |
| 87. | *Hyphodontia abieticola* (Bourdot & Galzin) J. Erikss. | PUN (6184) |
| 88. | *Hyphodontia alutacea* (Fr.) J. Erikss. | PAN (19625) |
| 89. | *Hyphodontia aspera* (Fr.) J. Erikss. | PAN (19433) |
| 90. | *Hyphodontia barbajovis* (Bull.) J. Erikss. | PUN (4948) |
| 91. | *Hyphodontia hastata*(Litsch.) J. Erikss. | PUN (4950) |
| 92. | *Hyphodontia juniperi* (Bourdot & Galzin) J. Erikss. & Hjortstam | PUN (4952) |
| 93. | *Hyphodontia nespori* (Bres.) J. Erikss. & Hjortstam | PAN (19745) |
| 94. | *Hyphodontia pallidula* (Bres.) J. Erikss. | PAN (19475) |
| 95. | *Hyphodontia propinqua* Hjortstam | PAN (19267) |
| 96. | *Hypochnicium caucasicum* Parm. | PAN (19011) |
| 97. | *Hypochnicium cremicolor* (Bres.) H. Nilsson & Hallenb. | PUN (4516) |
| 98. | *Hypochnicium erikssonii* Hallenb. & Hjortstam | PUN (4508) |
| 99. | *Hypochnicium geogenium* (Bres.) J. Erikss. | PAN (19121), PUN (4509) |
| 100. | *Hypochnicium punctulatum* (Cooke) J. Erikss. | PUN (3824, 4507) |
| 101. | *Hypochnicium sphaerosporum* (Höhn. & Litsch.) J. Erikss. | PAN (19005) |
| 102. | *Hypochnicium subrigescens* Boidin | PUN (4520) |
| 103. | *Intextomyces contiguus* (Karst.) J. Erikss. & Ryvarden | PAN (19550) |
| 104. | *Kneiffiella microspora* (J. Erikss. & Hjortstam) | PUN (6188, 6189) |
| 105 | *Kneiffiella subalutacea* (P. Karst.) Julich and Stalpers | PUN (6190, 6191) |
| 106. | *Lauriliasulcata* (Burt) Pouzar | PAN (19642) |
| 107. | *Laxitextum incrustatum* Hjortstam & Ryvarden | PUN (4174, 4966) |
| 108. | *Leptosporomyces galzinii* (Bourdot) Jülich | PUN (4864) |

109. *Leptosporomyces raunkiaerii* (Christ.) Jülich PAN (19431)
110. *Leptosporomyces roseus* Jülich PAN (19557), PUN (4866)
111. *Leptosporomyces septentrionalis* (J. Erikss.) Krieglst. PUN (5088)
112. *Licrostroma subgiganteum* (Berk.) Lemke PAN (19520), PUN (4909)
113. *Lopharia crassa* (Lev.) Boidin PAN (19768)
114. *Megalocystidium luridum* (Bres.) Jülich PUN (3663, 3664)
115. *Odonticium flavicans* (Bres.) Nakasone PUN (6130, 6131)
116. *Pachykytospora tuberculosa* (Fr.) Kotl. and Pouzar PUN (4578, 4579)
117. *Paullicorticium delicatissimum* (Jacks.) Liberta PUN (4899)
118. *Peniophora limitata* (Fr.) Cooke PAN (19615)
119 *Peniophora ovalispora* Boidin, Lanq. & Gilles PUN (6279)
120. *Peniophora pithya* (Pers.) J. Erikss. PUN (3839)
121. *Peniophora rufomarginata* (Pers.) Litsch. PAN (19002)
122. *Peniophorella clavigera* (Bres.) K.H. Larss. PUN (4459, 4460)
123. *Peniophorella echinocystis* (J. Erikss. &Å. Strid) K.H. Larss. PUN (6162)
124. *Peniophorella praetermissa* (P. Karst.) K.H. Larss. PUN (1880, 3432)
125. *Phanerochaete calotricha* (P. Karst.) J. Erikss. & Ryvarden PUN (4200)
126. *Phanerochaete filamentosa* (Berk. & Curt.) Parm. PAN (19414)
127. *Phanerochaete galactites* (Bourdot and Galzin) J. Erikss. & Ryvarden PAN (19104)
128. *Phanerochaete septocystidia* (Burt) J. Erikss. and Ryvarden PUN (4552)
129. *Phanerochaete sordida* (Karst.) J. Erikss. & Ryvarden PAN (19464)
130. *Phanerochaete tropica* (Sheng H. Wu) Hjortstam PUN (4222)
131. *Phanerochaete velutina* (DC. : Fr.) Karst. PAN (19581)
132. *Phlebia cornea* (Bourdot & Galzin) Parmasto PUN (3445)
133. *Phlebia deflectens* (P. Karst.) Ryvarden PUN (4548)
134. *Phlebia expallens* (Bres.) Parmasto PUN (4908)
135. *Phlebia gaspesica* PUN (4624)
136. *Phlebia griseoflavescens* (Litsch.) J. Erikss. & Hjortstam PUN (3682)
137. *Phlebia livida* (Fr.) Bres. PAN (19618)
138. *Phlebia ochraceofulva* (Bourdot & Galzin) Donk PUN (4529)
139. *Phlebia rufa* (Pers. : Fr. ) M.P. Christ. PUN (4156)
140. *Phlebia segregata* (Bourdot & Galzin) Parmasto PUN (4531)
141. *Phlebia singularisa* Dhingra PAN (19612)
142. *Phlebiella ardosiaca* (Bourdot and Galzin) K.H. Larss. and Hjortstam PUN (4622)
143. *Phlebiella allantospora* (Oberw.) Larss. & Hjortstam PAN (19631)
144. *Phlebiella grisella* (Bourd.) Larss. & Hjortstam PAN (19263)
145. *Phlebiella subflavido-grisea* (Litsch.) Oberw. PAN (19245)
146. *Phlebiella tulasnelloidea* (Höhn. & Litsch.) Oberw. PAN (19713)
147. *Phlebiopsis gigantea* (Fr.) Jülich PAN (19410)
148. *Pseudotomentella atrofusca* M.J. Larsen PUN (1766)
149. *Pseudotomentella tristis* (P. Karst.) M.J. Larsen PUN (5037)
150. *Punctularia atropurpurascens* (Berk. & Br.) Petch PUN (6100)
151. *Radulomyces confluens* (Fr.) M.P. Christ. PUN (4362)
152. *Resinicium friabile* Hjortstam & Melo PUN (4922)
153. *Scotoderma viride* (Sacc.) Jülich PUN (3493)
154. *Scytinostroma albocinctum* (Berk. & Broome) Boidin & Lanq. PUN (4979)
155. *Scytinostroma alutum* Lanq. PUN (4980)
156. *Scytinostroma galactinum* (Fr.) Donk PUN (6257)
157. *Scytinostroma hemidichophyticum* Pouzar PUN (6259)
158. *Scytinostroma ochroleucum* (Bres. & Torrend.) Donk PAN (19147)

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| 159. | *Scytinostromella heterogena* (Boud. & Galz.) Parm. | PAN (19442) |
| 160. | *Serpula himantoides* (Fr.)Cunn., | PAN (19688) |
| 161. | *Sidera lunata* (Romell ex Bourd. & Galz.) Miettinen & K.H.Larss. | PUN (4859) |
| 162. | *Sistotrema binucleosporum* Hallenb. | PUN (1404) |
| 163. | *Sistotrema heteronemum* (J. Erikss.) Å. Strid | PUN (6062) |
| 164. | *Sistotrema octosporum* (J. Schröt. ex Höhn. & Litsch.) Hallenb. | PUN (6064) |
| 165. | *Sistotrema porulosum* Hallenb. | PUN (1406) |
| 166 | *Sistotrema subtrigonospermum* D.P. Rogers | PUN (4900) |
| 167. | *Sistotremastrum niveocremeum* (Höhn. & Litsch.) J. Erikss. | PAN (19542) |
| 168. | *Sistotremastrum suecicum* Litsch. ex J. Erikss. | PUN (6352) |
| 169. | *Stereuma canthophysatum* Rehill & Bakshi | PAN (19895) |
| 170. | *Stereum australe*Lloyd | PUN (3453) |
| 171. | *Stereum gausapatum* Fr. : Fr. | PAN (19132) |
| 172. | *Stereum hirsutum* (Willd. : Fr.) Gray | PAN (19664) |
| 173. | *Stereum ostrea* (Blume & Nees : Fr.) Fr., | PAN (19804) |
| 174. | *Stereum sanguinolentum* (Alb. & Schw.) Fr. | PAN (19071) |
| 175. | *Stereum subtomentosum* Pouzar | PUN (5026) |
| 176. | *Subulicystidium meridense* Oberw. | PAN (19857) |
| 177. | *Suillosporium cystidiatum* (D.P. Rogers) Pouzar | PUN (1845) |
| 178. | *Thelephora atra* Weinm. | PUN (1747, 3709) |
| 179. | *Thenatophorus obscures* (D.P. Rogers) P. Roberts | PUN (4923) |
| 180. | *Thenatophorus ochraceus* (Massee) P. Roberts | PUN (1840) |
| 181. | *Tomentella asperula* (Karst.) Höhn. & Litsch. | PUN (1748) |
| 182. | *Tomentella bicolor* (Atk. & Burt) Bourdot & Galzin | PUN (5040) |
| 183. | *Tomentella brevispina* (Bourd. & Galz.) M.J. Larsen | PUN (5041) |
| 184. | *Tomentella chlorina*(Mass.) Cunn. | PAN (19519) |
| 185. | *Tomentella cinereoumbrina* (Bres.) Stalpers | PUN (6331) |
| 186. | *Tomentella cladii* Wakef. | PUN (5048) |
| 187. | *Tomentella clavigera* Litsch. | PUN (5042) |
| 188. | *Tomentella fibrosa* (Berk. & M.A. Curtis) Kõljalg | PUN (6332) |
| 189. | *Tomentella galzinii* Bourdot | PUN (5043) |
| 190. | *Tomentella griseoviolacea* Litsch. | PUN (5044) |
| 191. | *Tomentella lapida* (Pers.) Stalpers | PUN (1741, 5045) |
| 192. | *Tomentella muricata* (Ellis & Everh.) Wakef. | PUN (1737) |
| 193. | *Tomentella nitellina* Bourdot & Galzin | PUN (3412) |
| 194. | *Tomentella olivascens* (Berk. & M.A. Curtis) Bourdot & Galzin | PUN (1764, 1765) |
| 195. | *Tomentella puberula* Bourdot & Galzin | PUN (1754) |
| 196. | *Tomentella pyrolae* (Ellis & Halst.) M.J. Larsen | PUN (5046) |
| 197. | *Tomentella radiosa* (P. Karst.) Rick | PUN (6338) |
| 198. | *Tomentella scobinella* G. Cunn. | PUN (1757) |
| 199. | *Tomentella stuposa* (Link) Stalpers | PUN (5050) |
| 200. | *Tomentella subalpine* M. J. Larsen | PAN (19322) |
| 201. | *Tomentella subclavigera* Litsch. | PUN (5051) |
| 202. | *Tomentella subilacina* (Ellis & Holw.) Wakef. | PUN (5038) |
| 203. | *Tomentella terrestris* (Berk. & Broome) M.J. Larsen | PUN (5052) |
| 204. | *Tomentella testaceogilva* Bourdot & Galzin | PUN (5053) |
| 205. | *Tomentella variecolor* Malençon | PUN (5054) |
| 206. | *Tomentella viridula* Bourd. & Galz. | PUN (6350) |
| 207. | *Trechispora praefocata* (Bourdot & Galzin) Liberta | PUN (5066) |
| 208. | *Trechispora coharens* (Schw.) Jülich& Stalpers | PUN (1841, 3623) |

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| 209. | *Trechispora fastidiosa* (Pers.) Liberta | PUN (5064) |
| 210. | *Trechispora microspora* (P. Karst.) Liberta | PUN (1472, 1842) |
| 211. | *Trechispora mutabilis* (Pers.) Liberta | PUN (3112) |
| 212. | *Tubulicrinis borealis* J. Erikss. | PUN (4604) |
| 213. | *Tubulicrinis confusus* K.H. Larss. & Hjortstam | PUN (4607) |
| 214. | *Tubulicrinis effugiens* (Bourdot & Galzin) Oberw. | PUN (4610) |
| 215. | *Tubulicrinis orientalis* Parmasto | PUN (4612) |
| 216. | *Tubulicrinis subulatum* (Bourd. & Galz.) Donk | PAN (19626) |
| 217. | *Tubulirinis calothrix* (Pat.) Donk | PUN (4605) |
| 218. | *Vararia ambigua* Boidin | PUN (6260) |
| 219. | *Vararia minidichophysa* Boidin & Lanq. | PUN (4990) |
| 220. | *Vararia rugosispora* Boidin, Lanq. & Gilles | PUN (4991) |
| 221. | *Varariasphaericospora* Gilbertson | PUN (3637) |
| 222. | *Vararia trinidadesis* Welden | PUN (6261) |
| 223. | *Veluticeps abietina* (Pers.) Hjortstam & Tellería | PUN (4911) |
| 224. | *Xylobolus frustulatus* (Pers. : Fr.) Boidin | PAN (19012) |
| 225. | *Xylobolus subpileatus* (Berk. & Curt.) Boidin | PAN (19678) |

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# CONSERVATION AND CHARACTERIZATION OF *PLEUROTUS* VARIABILITY OF INDIA

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## ABSTRACT

The present investigation was undertaken to conserve, characterize, domesticate and commercialize the *Pleurotus* variability prevalent in different geographical regions of India.The species /isolates varied greatly in their cultural, nutritional, morphological and agronomical characters. All the species/isolates were tropical in nature requiring 25-30 °C for both vegetative growth and sporophore induction. Among the five species/isolates studied, the Madurai isolate showed molecular resemblance to *P. floribundus*, the isolate from Western Ghats showed resemblance to *P. djamor* var. *roseus*, pink isolate from Balaghat forest region of Madhya Pradesh belonged to *P. djamor*, the white isolate from the same region also showed 70% resemblance to *P. djamor* group. The Balaghat isolates showed very high contents of iron and zinc content and hence can play an important role in mitigation of malnutrition in India.

**Keywords:** conservation, India, *Pleurotus,* variability

## INTRODUCTION

*Pleurotus* species commonly called as ‘oyster mushrooms’ are one of the most gregarious of the cultivated mushrooms with wide adaptability in terms of environment and growing substrate. India, due to its varied geography and forest types is rich in mushroom diversity which awaits its conservation and domestication. The present investigation was undertaken to conserve and characterize the variability of *Pleurotus* species found in India for a better scope of diversification of the Indian mushroom industry.

## MATERIALS AND METHODS

### Explorations

Explorations were undertaken in the western Ghats of Karnataka (Shimoga district) lying between the latitudes 13°27' and 14°39' N and between the longitudes 74°38' and 76°04' E at a mean altitude of 640 meters above sea level. Shimoga district is a part of the Malnad region of Karnataka and is also known as the ‘Gateway to Malnad’ or ‘MalenaadaHebbagilu’ in Kannada. A pink *Pleurotus djamor* was collected from this region. The second exploration was undertaken in the forest of Madhya Pradesh (Balaghat district) located at 21°48’ N 80°11’ E. It has an average elevation of 288 meters. A pink and a white *Pleurotus* spp./isolates were collected from these forests. The third exploration was done in the Hessaraghatta campus of IIHR in Bangalore district lying in the southeast of Karnataka. It is positioned at 12.97° N 77.56° E at an average elevation of 920 meters. Bangalore is located in the heart of the Mysore Plateau (a region of the larger Deccan Plateau). The blackish gray *P. cystidiosus* was collected growing on Singapur cherry tree (*Muntingia calabura)* from this area. The fourth exploration was undertaken in the forest region of Madurai circle in Tamil Nadu. Madurai is located between latitude: 9°55’ 59” N and longitude: 78°07’ 00” E at an elevation of 136 meters. A white *Pleurotus* spp/isolate was collected from this region.

### Pure culture

Tissue culture of the species/isolates were raised from sporophores collected from wild on malt extract agar medium (MEA) comprising of 50 g malt extract agar powder (Himedia Pvt. Ltd. Mumbai) dissolved in 1000 ml of distilled water,

sterilized at 121 °C, 15 psi pressure for 20 minutes in glass culture tubes. The cultures were purified by sub culturing in Petri plates (90 mm diameter) on the same medium. The pure cultures were stored in refrigerator for further studies.

**Validation studies**

Mother spawn was made on half boiled sorghum grains in 500 ml glass bottles by the traditional grain spawn method and incubated at 27±2 °C. The fully colonized mother spawn was used for further inoculation of planting spawn. The substrate for planting spawn was similar as mother spawn but the container used was 800mm x 1200mm, 150 gauze polypropylene (PP) bags and plugged with non absorbent cotton. Spawn substrate preparation, sterilization and incubation were done as described earlier. After complete colonization, the planting spawn was used for sporophore induction studies.

Sporophore induction was studied on sterilized (121°C, 15 psi pressure for 15 minutes) paddy straw having 65±2% moisture filled in polypropylene (PP) bags (1000mm x 1200mm, 150 gauze thickness). Each bag contained 1000 g of wet paddy straw and inoculated with 50 g of planting grain spawn. The bags were incubated at 26±2 °C for spawn running after which they were shifted to cropping rooms and pierced with 25 mm diameter holes (4 holes per bag). Temperature of 26±2°C, humidity of 80-85% and light (two 40 w fluorescent tubes for 8 hour per day) was maintained in the cropping rooms. Ventilation was through 4 mesh (insect proof) open ventilators (590 x 450mm) in each cropping room. The sporophores of the first flush were harvested and fresh tissue cultures raised and conserved as described earlier.

**Cultural characterization**

Cultural characteristics were investigated by evaluating various physiological factors (media, temperature and pH) required for optimal vegetative growth and conservation. Media tested for optimal culture growth included: malt extract agar (MEA), potato dextrose agar (PDA), potato malt agar (PMA), potato carrot agar (PCA) and oatmeal agar (OMA), ready-to-use dehydrated powder from Himedia Laboratories Pvt Ltd, Vadhani Industrial Estate, LBS Marg, Mumbai, India) and Raper's Complete medium (RC) containing 20.0 g glucose, 2.0 g peptone, 2.0 g yeast extract, 0.50 g magnesium sulphate, 0.46 g potassium dihydrogen phosphate and 20.0 g agar powder per liter of distilled water. Wheat extract agar (WEA) and rice bran agar (RBA) was prepared by boiling 30.0 g of whole wheat grain or rice bran powder in 500 ml of distilled water for one hour, followed by decanting and filtering through several thicknesses of cheese cloth padded with cotton. The final volume was made up to one liter with distilled water and 20.0 g agar powder was added. All the media were sterilized in autoclave at 15 lb pressure for 20 minutes. The effect of temperature on mycelial growth was conducted on MEA. Plates of MEA media were inoculated with 7 mm mycelial discs taken from the periphery of 10 days old pure culture. Plates were incubated at variable temperatures (15-40 °C at intervals of 5 °C) and the radial growth was recorded at an interval of 2 days. The effect of pH was studied in malt extract broth (malt extract powder 30.0 g, peptone 5.0 g per litre of distilled water) of variable pH (3.0-8.0, at intervals of 0.5 pH) and the mycelial dry weight was recorded as the measure of mycelial growth. Microscopic studies of the vegetative mycelium and basidiospores were also undertaken.

**Agronomical characterization**

Fruiting trials for agronomical characterization were conducted as described under validation trials. Monthly crop evaluation was done for three years.

**Nutritional characterization**

The nutritional analysis included estimation of protein, fat, carbohydrate, vitamin B and minerals. The proximate nutritional composition was determined by the methods of AOAC [1]. Proximate analysis studies included the determination of crude fat (A.O.A.C. 7.056, 1980), crude fiber (A.O.A.C. 7.061, 1980) and crude protein (A.O.A.C.47.021, 1980) using the conversion factor (N X 6.25). Carbohydrate was determined by phenol-sulphuric method of Dubois [2]. Mineral nutrient analysis was done with oven dried (70p C) samples as per the method described by Piper [3] using KjeltekAut-Analyzer, Gerhardt, Germany and atomic absorption spectrophotometer (AAS). Water soluble vitamins biotin (B7), niacin (B3), pyridoxine (B6), pantothenic acid (B5), folic acid (B9), cyanocobalamine (B12), thiamine (B1) and riboflavin (B2) were analyzed by UPLC-MS/MS as per methodology given by Esteve *et al.*[4]

## RESULTS AND DISCUSSION

Tables1 (a-c) represents the observations on cultural characterization of different *Pleurotus* species/isolates collected from different geographical regions. All the species/isolates could grow in a temperature range of 15-35 °C except *P. cystidiosus,* which showed mere initiation of growth at 15 °C. The optimum temperature for mycelial growth of all the species/isolates was 25-30 °C. The upper limit of temperature tolerance was 40 °C except for *P. cystidiosus,* which could not be revived after exposure to this temperature. The other species/isolates could be revived when brought back to optimal temperature after high temperature (40 °C) exposure for 12 days (Table 1a).

**Table 1a**. Effect of temperature on mycelia growth of wild *Pleurotus* isolates/species

| **Temperature (°C)** | **Radial growth in mm/day** | | | | |
|---|---|---|---|---|---|
| | ***Pleurotus djamor*** **(Shimoga isolate)** | **Pink *Pleurotus* spp. (Balaghat isolate)** | **White *Pleurotuss* pp. (Balaghat isolate)** | ***P. cystidiosus*** **(Bangalore isolate)** | **White *Pleurotus*** **(Madurai isolate)** |
| 15 | 5.54 | 2.46 | 1.82 | Only initiation | 7.39 |
| 20 | 8.03 | 9.00 | 8.18 | 4.34 | 10.54 |
| 25 | 12.85 | 15.0 | 15.0 | 6.84 | 12.70 |
| 30 | 11.07 | 15.0 | 14.91 | 6.25 | 14.26 |
| 35 | 7.69 | 7.68 | 3.21 | 1.57 | 10.97 |
| 40 | No growth | 0.00 | 0.00 | No growth (lethal) | 0.00 |
| CD at 1% | 0.90 | 0.86 | 0.71 | 0.22 | 0.35 |

**Table 1b.** Effect of pH on myceial growth of wild *Pleurotus* isolates/species

| **pH** | **Mycelial dry weight (mg)** | | | | |
|---|---|---|---|---|---|
| | ***Pleurotus djamor*** **(Shimoga isolate)** | **Pink *Pleurotus* spp. (Balaghat isolate)** | **White *Pleurotus* spp. (Balaghat isolate)** | ***P. cystidiosus*** **(Bangalore isolate)** | **White *Pleurotus*** **(Madurai isolate)** |
| 3 | No growth | No growth | No growth | 5.2 | 0.00 |
| 3.5 | 73.96 | 67.2 | 88.4 | 6.02 | 0.00 |
| 4 | 66.1 | 42.2 | 81.6 | 5.2 | 52.34 |
| 4.5 | 61.23 | 102.4 | 130.2 | 91.8 | 67.40 |
| 5 | 146.26 | 157.6 | 108.8 | 102.14 | 103.62 |
| 5.5 | 237.40 | 229.8 | 127.4 | 331.58 | 132.08 |
| 6 | 202.2 | 184.6 | 151.4 | 348.34 | 139.94 |
| 6.5 | 275.43 | 220.4 | 208.2 | 436.9 | 143.80 |
| 7 | 250.23 | 258.2 | 232.2 | 382.36 | 147.28 |
| 7.5 | 272.2 | 132.6 | 233.6 | 381.3 | 138.98 |
| 8 | 250.63 | 289 | 250 | 364.38 | 134.36 |
| 8.5 | 234.9 | 164.2 | 220.8 | 357.06 | 133.86 |
| 9 | 285.3 | 98.4 | 234.6 | 350.2 | 130.70 |
| CD at 1% | 46.5342 | 150.72 | 84.86 | 57.45 | 4.15 |

**Table 1c.** Effect of different media on mycelia growth of wild *Pleurotus* isolates/species

| Media | Mycelial dry weight (mg) | | | | |
|---|---|---|---|---|---|
| | ***Pleurotus djamor* (Shimoga isolate)** | **Pink *Pleurotus* spp. (Balaghat isolate)** | **White *Pleurotus* spp. (Balaghat isolate)** | ***P. cystidiosus* (Bangalore isolate)** | **White *Pleurotus* (Madurai isolate)** |
| MEA | 12.85 | 18.0 | 18.0 | 3.94 | 12.79 |
| RC | 12.21 | 18.0 | 18.0 | 4.61 | 12.48 |
| PDA | 11.25 | 17.0 | 18.0 | 3.74 | 12.5 |
| OMA | 9.21 | 14.33 | 12.33 | 3.21 | 7.76 |
| WEA | 9.4 | Not studied | Not studied | 4.91 | 8.79 |
| RBA | 10.75 | Not studied | Not studied | 4.39 | 12.33 |
| PMA | 12.08 | 17.86 | 18.0 | 2.91 | 8.33 |
| PCA | 10.41 | 18.0 | 18.0 | 3.75 | 7.08 |
| CD at 1% | 0.99 | 1.89 | 1.21 | 0.48 | 0.29 |

MEA=Malt extract agar, RC= Rapers complete medium, OMA=Oat meal agar
WEA=Wheat extract agar RBA=Rice bran agar, PDA=Potato dextrose agar
PMA = Potato malt agar, PCA = Potato carrot agar

The pH tolerance of *P. djamor* (Shimoga isolate), pink and white *Pleurotus* spp. (Balaghat isolates) was 3.5-9.0 optimum being 5.5-9.0 (Shimoga isolate), 5.5 – 7.0 (pink *Pleurotus* spp, balaghat isolate), 6.5 – 9.0 (white *Pleurotus* spp, balaghat isolate), 5.5 – 9.0 (*P. cystidiosus*) and 5.5 – 9.0 (white *Pleurotus* spp, Madurai isolate). All the species/isolates could grow on the commonly used mycological media like MEA, PDA and RC medium. However, *P. cystidiosus* showed better growth on WEA.

Phenotypic variability among the species/isolates has been shown in Table 2. The sporophore color was pink (Shimoga & Balaghat isolates), white (Balaghat & Madurai isolates) and black (*P. cystidiosus* from Bangalore). The sporophore color gradually became lighter on maturity. The pileus margin varied from smooth to lobed. Stipe varied from rudimentary to well formed cylindrical among the isolates. Position of the stipe varied from eccentric to sub eccentric to central.The agronomical variability has been shown in table 3. All species/isolates could be grown on sterilized/pasteurized paddy straw. *P. djamor* (Shimoga isolate) and pink *Pleurotus* spp. (Balaghat isolate) were short duration crops with a cropping cycle (period from spawning to getting 80% harvest) of 18-32 days. White *Pleurotus* spp. (Balaghat isolate) required 25-62 days, white *Pleurotus* spp. (Madurai isolate) required 35-48 days and *P. cystidiosus* (Bangalore isolate) required 35-48 days. *Pleurotus djamor* (Shimoga isolate), white *Pleurotus* spp. (Madurai isolate) and white *Pleurotus* spp. (Balaghat isolate) gave higher sporophore yield compared to pink *Pleurotus* spp. (Balaghat isolate) and *P. cystidiosus* (Bangalore isolate). The post harvest shelf life of *P. cystidiosus* (Bangalore isolate) was superior than other isolates. It could be stored for 3-4 days at room temperature (27-29 °C) and for 15-30 days at 4-5 °C depending on maturity at the time of harvest. The other species/isolates showed poor to moderate shelf life (Table 4).

Tables 5 a & b show the nutritional status of different species/isolates. Highest crude protein was observed in white *Pleurotus* (Madurai isolate), followed by pink *Pleurotus* isolates (Balaghat & Shimoga isolates). Highest carbohydrate content was in pink *Pleurotus* (Shimoga isolate) and lowest in *P. cystidiosus* (Bangalore isolate). The highest crude fiber content was in *P. cystidiosus* (Bangalore isolate). Among the minerals both white and pink *Pleurotus* (Balaghat isolates) and white *Pleurotus* (Madurai isolate) showed highest iron content. Highest zinc content was observed in Balaghat isolates followed by Shimoga isolate. In general all the isolates were low on calcium and high on potassium (Table 5a). Highest biotin content was found in pink *Pleurotus* spp. (Balaghat isolate) followed by white *Pleurotus* spp from the same region and *P. cystidiosus* (Bangalore isolate), highest niacin was in white *Pleurotus* spp. (Balaghat isolate) followed by *P. cystidiosus* (Bangalore isolate). Pyridoxine was higher in pink *Pleurotus* spp. (Balaghat isolate) followed by *P. djamor* (Shimoga

**Table 2.** Phenotypic variability among wild *Pleurotus* isolates/species

| Phenotypic characters | *Pleurotus djamor* (Shimoga isolate) | Pink *Pleurotus* spp. (Balaghat isolate) | White *Pleurotus* spp. (Balaghat isolate) | *P. cystidiosus* (Bangalore isolate) | White *Pleurotus* (Madurai isolate) |
|---|---|---|---|---|---|
| Color of pileus at pinhead stage | Pink | Pink | White | Black | White |
| Color of pileus at mature stage | Pink (lighter as compared to pinhead stage) | Pink (lighter as compared to pinhead stage) | White | Black, sometimes fading to brown | White |
| Shape of pileus | Pleurotoid | Pleurotoid like flower due to central stipe | Pleurotoid becoming funnel shaped on maturity | Pleurotoid like on maturity | Pleurotoid becoming flattened plate |
| Margin of pileus | Smooth | Lobed becoming like a flower | Wavy | Smooth | Smooth |
| Pileus height (point of attachment to stipe to the edge of pileus) (mm) | Range - 30.19-66.34 Average - 49.9 | Range – 35.27 Average – 26.95-52.70 | Range – 25.8 – 38.5 Average – 31.5 | Range -56.84 - 130.5 Average – 73.92 | Range 8.6 – 23.5 Average -15.46 |
| Pileus width (from one edge of the pileus to the other edge)(mm) | Range- 25.18-40.19 Average - 33.94 | Range -19.50-56.32 Average-32.21 | Range – 27.5 – 62.7 Average – 37.22 | Range -72.07-100.5 Average – 82.60 | Range -17.7 – 31.6 Average – 25.26 |
| Color and texture of stipe at pinhead stage | Pink, tough | Whitish pink | White | Gray brown, hard | White |
| Color of stipe at harvest stage | Same as above | Same as above | Same as above | Same as above | Same as above |
| Position of stipe | lateral | Eccentric to central | Eccentric to central | Lateral | Eccentric to central |
| Stipe length (mm) | Range-12.79-39.61 Average - 25.40 | Range- 24.79 – 47.47 Average - 32.47 | Range – 33 - 57 Average – 41.79 | Range – 33.05-84 Average – 45.92 | Range – 8.6 - 34.35 Average -25.75 |
| Stipe thickness (mm) | Range - 3.21-7.29 Average - 5.56 | Range -5.86 – 11.37 Average - 8.75 | Range – 8.66 - 12 Average – 10.33 | Range –9.05 – 16.2 Average – 11.90 | Range– 3.83 – 6.23 Average -4.63 |
| Color of gills | Pink | Pink | White | Creamish white | White |
| Coremia | No | No | No | Yes, black heads with white stalks, single celled | No |
| Color of spore print | White with some pink shade | White with some pink shade | White | White with grayish tinge | White |
| Microscopic features | Hyphae dimitic, basidiospore cylindrical (18.50 x 11.73 µ) with numerous sterile spores (22.01x 12.46 µ) | Hyphae dimitic, basidiospore cylindrical (19.48 x 10.94 µ) | Hyphae dimitic, basidiospore cylindrical (19.56 x 9.79 µ) | Hyphae monomitic, conidia one celled, basidiospores cylindrical, (11-14 x 4-5µ) | Hyphae dimitic, basidiospore cylindrical (23.41 x 11.20 µ) |

**Table 3.** Agronomical characters of wild *Pleurotus* isolates/species

| Agronomical parameters | *Pleurotus djamor* (Shimoga isolate) | Pink *Pleurotus* spp. (Balaghat isolate) | White *Pleurotus* spp. (Balaghat isolate) | *P. cystidiosus* (Bangalore isolate) | White *Pleurotus* (Madurai isolate) |
|---|---|---|---|---|---|
| Spawn running period (days) | 18-19 | 18-19 | 23-24 | 24-32 | 20-22 |
| Time taken for 1st harvest after opening of bags (days) | 6-7 | 6-7 | 9-10 | 13-27 | 19-20 |
| Average yield of 1st flush (g per kg wet substrate) | 106.37 | 74.38 | 117.51 | 42.10 | 120.68 |
| Average yield of 2nd flush (g per kg wet substrate) | 33.62 | 26.00 | 48.15 | 59.76 | 55.16 |
| Average yield of 3rd flush (g per kg wet substrate) | 32.70 | 16.82 | 51.33 | 44.73 | 46.66 |
| Period to obtaining 80% or more of total yield after opening of bags (days) | 4-10 | 4-10 | 7-33 | 19-60 | 15-31 |
| Cropping period from spawning to getting 80% or more of total yield (days) | 18-32 | 19-30 | 25-62 | 42-90 | 35-48 |
| Cropping cycle from spawning to cleaning of rooms (days), 2 cleaning days common for all | 20-34 | 21-32 | 27-64 | 44-92 | 37-50 |
| Number of possible crops in 365 days in a given space | 18.25-10.73 | 17.38-11.40 | 13.51-5.70 | 8.29-3.96 | 9.86-7.3 |

**Table 4.** Post harvest storage studies of wild *Pleurotus* isolates/species

| Species/isolate | Post harvest storage in trays wrapped with antifog film (27-29 °C) | Post harvest storage in trays wrapped with antifog film (4-6 °C) |
|---|---|---|
| *Pleurotus djamor* (Shimoga isolate) | Shelf life 1-2 days depending on the harvesting stage, sporophore color fades faster on storage. No aerial hyphae visible on sporophores even after 48 hours, not soggy | Shelf life 4-6 days depending on the harvesting stage, color fades slowly on storage, No aerial hyphae visible on sporophores, not soggy |
| Pink *Pleurotus* spp. (Balaghat isolate) | Similar to pink isolate from Western ghats | Similar to pink isolate from Western ghats |
| White *Pleurotus* spp. (Balaghat isolate) | Same as for pink isolate from the same region | Same as for pink isolate from the same region |
| *P. cystidiosus* (Bangalore isolate) | Shelf life 3-4 days depending on the harvesting stage, color fades on storage. No aerial hyphae visible on sporophores even after 3-4 days. The black sporophores show better shelf life than lighter colored sporophores, not soggy | Shelf life 20-30 days depending on sporophore maturity, no odor or softening. No aerial hyphae visible on sporophores even after 25-30 days storage at low temperature, not soggy |
| White *Pleurotus* (Madurai isolate) | Shelf life 1-1½ days, browning starts after 24 hours, aerial growth visible on stipe by 36 hours, becoming soggy | Shelf life 3-5 days, aerial growth visible on stipe by 5th day, becoming soggy |

**Table 5a.** Proximate composition and mineral content of wild *Pleurotus*species/isolates

| Nutritional parameters | *Pleurotus djamor* (Shimoga isolate) | Pink *Pleurotus* spp. (Balaghat isolate) | White *Pleurotus* spp. (Balaghat isolate) | *P. cystidiosus* (Bangalore isolate) | White *Pleurotus* (Madurai isolate) |
|---|---|---|---|---|---|
| Crude Protein (% dw) | 29.81 | 30.87 | 24.56 | 24.68 | 36.25 |
| Carbohydrates (% dw) | 50.59 | 51.41 | 53.22 | 33.43 | UI |
| Fat(% dw) | 4.5 | 5.1 | 4.9 | 5.04 | UI |
| Crude fiber(% ) dry weight basis | 13.2 | 15.9 | 14.2 | 27.80 | UI |
| Nitrogen (%) dry weight basis | 4.77 | 4.94 | 3.93 | 3.95 | 5.8 |
| Calcium (%) dry weight basis | 0.001 | 0.12 | 0.005 | 0.21 | 0.002 |
| Potassium (%) dry weight basis | 2.23 | 1.66 | 1.71 | 2.1 | 2.87 |
| Phosphorus (%) dry weight basis | 1.09 | 0.60 | 0.60 | 0.55 | 1.49 |
| Magnesium (%) dry weight basis | 0.09 | 0.102 | 0.085 | 0.12 | 0.11 |
| Iron (µg/g) dry weight basis | 192 | 350 | 284 | 59 | 232 |
| Zinc (µg/g) dry weight basis | 156.56 | 201 | 169 | 111 | 130 |

UI = under investigation

isolate), white *Pleurotus* spp. (Balaghat isolate) and *P. cystidiosus* (Bangalore isolate). Pantothenic acid was highest in white *Pleurotus spp.* (Balaghat isolate) followed by pink *Pleurotus* spp. (Balaghat isolate), *P. cystidiosus* (Bangalore isolate) and *P. djamor* (Shimoga isolate). Folic acid was highest in the white and pink *Pleurotus* spp (Balaghat isolates) followed by *P. djamor* (Shimoga isolate) and *P. cystidiosus* (Bangalore isolate). Cyanocobalamine was highest in *P. cystidiosus* (Bangalore isolate) followed by white and pink *Pleurotus* spp (Balaghat isolates) and *P. djamor* (Shimoga isolate). Thiamin was highest in *P. cystidiosus* (Bangalore isolate) and in white *Pleurotus spp.* (Balaghat isolate) followed by pink *Pleurotus* spp. (Balaghat isolate) and *P. djamor* (Shimoga isolate). Riboflavin was highest in pink and white *Pleurotus* spp (Balaghat isolates) followed by other isolates/spp (table 5b).

**Table 5b.** Water soluble vitamins of wild *Pleurotus* species/isolates

| Vitamin | *Pleurotus djamor* (Shimoga isolate) | | Pink *Pleurotus* spp. (Balaghat isolate) | | White *Pleurotus* spp. (Balaghat isolate) | | *P. cystidiosus* (Bangalore isolate) | | White *Pleurotus* (Madurai isolate) |
|---|---|---|---|---|---|---|---|---|---|
| | FW | DW | FW | DW | FW | DW | FW | DW | |
| Biotin (B7) mg/100g | 0.236 | 1.269 | 0.324 | 2.160 | 0.274 | 1.724 | 0.198 | 1.081 | UI |
| Niacin (B3) mg/100g | 1.968 | 10.585 | 1.321 | 8.807 | 3.659 | 23.021 | 3.216 | 17.551 | UI |
| Pyridoxin (B6) mg/100g | 0.102 | 0.549 | 0.124 | 0.827 | 0.094 | 0.591 | 0.071 | 0.387 | UI |
| Pantothenic acid (B5) mg/100g | 0.949 | 5.104 | 1.159 | 7.727 | 1.959 | 12.325 | 1.312 | 7.160 | UI |
| Folic acid (B9) µg/100 gm | 3.200 | 17.212 | 4.800 | 32.000 | 5.200 | 32.717 | 2.100 | 11.460 | UI |
| Cyanacobalamine (B12) µg/100 gm | 0.761 | 4.093 | 0.954 | 6.362 | 1.742 | 10.962 | 2.612 | 14.257 | UI |
| Thiamine (B1) mg/100g | 0.106 | 5.753 | 0.103 | 6.879 | 0.117 | 7.402 | 0.139 | 7.594 | UI |
| Riboflavin (B2) mg/100g | 0.226 | 1.216 | 0.654 | 3.693 | 0.286 | 1.799 | 0.214 | 1.168 | UI |

FW = Fresh weight DW = Dry weight UI = under investigation

Numerous studies in India have been conducted earlier on the occurrence of indigenous *Pleurotus* species [5, 7]. However, none of these studies were focused on end to end study from conservation to domestication and commercialization. This is the first attempt to present a complete investigation on *Pleurotus* variability of India with a view to conserve and develop a complete technology for the utilization of the wild species. Similar studies were conducted by Lechner *et al*. in Argentina [8]. The authors reported the occurrence of three varieties of *P. djamor*: var *djamor*, var. *cyathiformis* and var. *roseus* and *P. cystidiosus*. These two species seem to be widely distributed in the tropical and subtropical regions. The wild species were very high in Vitamin B, protein, iron and zinc content. Hence these wild species can play a pivotal role through fortification in mitigating mineral malnutrition and for breeding nutritionally rich commercial strains suitable for India.

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# MEDICINAL MUSHROOMS IN ITALY AND THEIR *EX SITU* CONSERVATION THROUGH CULTURE COLLECTION

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## ABSTRACT

A wide variety of mushrooms, including the edible, nutraceutical and medicinal ones, are present in Italy, a small country characterized by a great habitat richness. Inevitably, the extensive action of many mycophiles collecting sporophores, mainly for culinary uses, can impact on this important source of biodiversity. In order to protect the rare *taxa*, also collecting their biological material for application purposes, conservation *ex situ* is suggested.

Wood-inhabiting fungi were collected from Italian Alps, Apennines, wood plains and Mediterranean areas. Pure culture isolation was carried out from fruiting bodies in experimental sterile conditions. The culture collection has been registered at the Mycological Laboratory of Pavia University (Italy). In few years about 150 species were isolated and, among them, some rare and precious *taxa* such as *Ganoderma pfeifferi* Bres., *Laricifomes officinalis* (Batsch) Kotl. & Pouzar, *Lenzites warnieri* Durieu & Mont. and *Perenniporia meridionalis* Decock & Staplers deserve to be mentioned. It is well known that medicinal mushroom bioactivities could depend not only on biological characteristics (*i.e.* the genetic profile) but also on the geographical origin and the substrate. Therefore, Italy could really become a new resource of different fungal ecotypes, characterized by different and potentially useful properties worthy to be investigated.

**Keywords**: medicinal mushrooms, mycelia, sporophores, wood-inhabiting fungi

## INTRODUCTION

Italy is located in Southern Europe and comprises the boot-shaped Italian Peninsula and a number of islands including the two largest, Sicily and Sardinia. Most of the land is surrounded by Mediterranean sea. It is crossed by Apennine Mountains, which are like a backbone, while the Alps form most of its northern boundary, where Italy's highest point is located on Mont Blanc (4,810 m/15,782 ft). The longest river is the Po, that flows in the Padan plain, bounding area of the continental climate from the Mediterranean one. In the north a few lakes, due to glacial deposits, lead to Mediterranean microclimates too. The country is situated at the meeting point of the Eurasian Plate and the African Plate, leading to considerable seismic and volcanic activity: there are 14 volcanoes in Italy, four of which are still active.

Thanks to the longitudinal extension of the peninsula and to the mostly mountainous internal conformation, the climate of Italy is highly diverse. In most of the inland northern and central regions, the climate ranges from humid subtropical to humid continental and oceanic. The coastal areas generally fit the Mediterranean climate stereotype (Köppen climate classification Csa). Conditions on peninsular coastal areas can be very different from the interior's higher ground and valleys, particularly during the winter months when the higher altitudes tend to be cold, wet, and often snowy. The coastal regions have mild winters and warm and generally dry summers, although lowland valleys can be quite hot in summer.

Therefore, even if Italy is a relatively small country it is characterized by numerous different habitats where many fungal species are present [1, 2] as well as many mycophiles collecting fruiting bodies mainly for culinary uses. Anyway the use of wild fungi also for other purposes dates back to ancient civilizations, for instance *Amanita caesarea*, Caesar's mushroom [1]. Despite the long tradition the toxicity or edibility of some non-timber forest product is still dubious. Keeping on this topic, it is well known that the genus *Amanita* includes various species edible or not, poisonous and sometimes even deadly: among the group of the white ones, *Amanita ovoidea* is described as both edible and with a good taste, on the

other hand this mushroom seems to have caused several cases of poisoning, at least in Italy, classified as allenic norleucine syndrome. Phytochemical screening shows a very low presence of amino acids and for this the poisoning by allenic norleucine seems to be excluded. In respect to the environment, *A. ovoidea*, like the edible *A. caesarea*, can accumulate high amounts of heavy metals and could therefore be harmful to humans [2]. Finally, as said before, there are still to solve numerous questions even if scientific and technological progress is increased. In spite of the numerous activities of Italian mycologists, belonging to scientific research centres or amateur groups, concerning different aspects such as biodiversity and mapping [3, 4] the importance of fungi in nature conservation is still limited and it has to be vigorously promoted.

Besides, during the last decade, the interest in medicinal mushrooms has been increasing, especially because almost all the better-known species having officinal properties grow in the Italian areas. In order to protect these fungal species from an extensive collection threatening the rare *taxa*, conservation *ex situ* could be suggested [5, 6]. Culture collections play a key role in preservation and maintenance of fungal genetic resources and they are an important tool to get biological material for application purposes [7].

Aims of the present work are: a) to investigate selected areas favorable to the development of wood-inhabiting basidiomata; b) to identify the species by means of the morphological approach; c) to isolate the mycelia in pure culture; d) to confirm the strain identification by molecular analysis.

## MATERIALS AND METHODS

### Sampling sites

According to the specific research activities of the authors, some Italian environments were investigated more in detail and for a longer time than others. Wood-inhabiting fungi were collected from different habitats of Italian Alps, Apennines, wood plains and Mediterranean areas. The attention has been particularly focused on both "polyporoid" fungi and *Corticiaceae* [8-11].

### Pure culture isolation

Isolation of mycelia in pure culture was performed according to the methodology provided by [12] and [13]. Each one of the following steps presupposes high attention in preventing fungal material and culture medium from any external contaminants; decayed and/or infested specimens were avoided as well. 1) The sporophores were opened aseptically to make the context accessible; 2) few $mm^3$ of context were drawn by means of lancet; 3) drawn material was inoculated by penetration into Petri dishes containing culture medium (e.g. MEA 2% or PDA 4%); 4) most species grew well at 25 °C, during their initial phase. At first, antibiotics were added to the cultural medium. Each strain was registered at the Mycotheque of Pavia University (DSTA) and to the Culture Collection of Miconet Srl (Pavia University-academic spin off). Cultures were maintained at 4 °C on 2% Malt Extract Agar (MEA) plates (Biokar Diagnostics).

### Molecular analyses

Cultures were grown in 200-ml Erlenmeyer flasks containing 50 ml of malt extract broth on a rotary shaker (100 rpm) for 10 d at 25 °C. Biomass was collected in microcentrifuge tube and DNA was extracted with CTAB method [14].

CTAB (hexadecyltrimethylammonium bromide) lysis buffer (2%) were added to fresh biomass and mycelium was ground with sterile pestle. Extraction was performed with a phenol-chloroform- isoamyl alcohol (25:24:1) solution. Afterward the phases were separated by centrifugation and DNA was precipitated from the aqueous phase with an equal volume of isopropanol. Total nucleic acids were collected by centrifugation, the pellet was rinsed with 70% ethanol, and the nucleic acids were dissolved in 100 μl sterile deionized water.

Internal transcribed spacer- ITS region was amplified using the primer pair ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'–TCCTCCGCTTATTGATATGC-3' [15]. Each PCR reaction contained 1 μl of fungal DNA, 1X DNA polymerase buffer, 0.2 mM dNTP mix (dUTP, dATP, dGTP, dCTP), 0.2 ìM (each) of ITS1 and ITS4, and 0.025 U/ìl of *Taq* DNA polymerase (5 PRIME, Germany) in a total volume of 25 μl.

The amplification reactions were run in a BIORAD thermocycler with an initial denaturation of 5 min at 95 °C, followed by 39 cycles of 35 sec at 95 °C, 35 sec at 55 °C, and 50 sec at 72 °C, with a final extension step of 72 °C for 8 min. PCR products were analyzed by electrophoresis in a 1% agarose gel in 1X TAE buffer [(40mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8)], stained with ethidium bromide. Furthermore, the PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sent to Macrogen (The Netherlands) for sequencing. The obtained sequences were assembled, corrected and subsequently analyzed by BLAST searches using the GenBank (NCBI) and Mycobank (CBS) databases. Taxonomic assignment were based on similarity to reference sequences of these databases; afterward genotypic identification was compared with phenotype identification.

## RESULTS AND DISCUSSION

In few years about 150 species were isolated, among them there are some rare and precious *taxa* both with well known medicinal properties, such as *Laricifomes officinalis* (Batsch) Kotl. & Pouzar (1.2.Lo1 & 1.2.Lo2) and *Ganoderma pfeifferi* Bres. (1.2.Gpf1), and others never investigated before, such as *Lenzites warnieri* Durieu & Mont. (1.2.Lw1, 1.2.Lw2, 1.2.Lw3, 1.2.Lw4) and *Perenniporia meridionalis* Decock & Stalpers (1.2.Pm1). To the best of our knowledge, the strains of all these species are maintained in the Mycotheque of Pavia University (DSTA) and in the culture collection of Miconet representing the first Italian isolations.

Molecular analysis were carried out in order to confirm the isolates or to supplement morphological identification of those fungal strains belonging to *taxa* whose identification was critical. The comparison of the obtained ITS sequences obtained with the ones deposited in the available databases has allowed to confirm the taxonomic assignment at the species level, with a similarity higher than 99%. *Ganoderma* resulted the genus with major discrepancies between morphological and molecular analyses; however it is well known that it presents a great variability in macroscopic characters of basidiocarps, thus leading to confusion in the taxonomy of the genus too [16]. The same authors declared that *Ganoderma* is the most difficult genus of all polypores. Almost all the species present in Europe have been collected during this study, for many of them the mycelium was successfully isolated in pure culture. The most remarkable strain resulted *G. pfeifferi*, a species that grows quite exclusively in Europe, till now poorly investigated. Some interesting data published in the last years report the identification of new chemical substances related to it, named ganomycins A, B and K [17, 18]. *G. pfeifferi* has been reported only in few Italian locations, mostly in Tuscany (Central Italy) [3].

Among the macrofungi famous for their medicinal properties, *L. officinalis* was detected, a lignicolous fungus strictly related to the holoartic mountains, on the Alps it grows exclusively on old and thick larch trees (*Larix decidua*), a species endemic for Europe. The fungus has been well known since 1st Century A.D. for its healing properties, being used to treat several diseases, especially pulmonary ones. It is nowadays recognized that *L. officinalis* produces antibiotic substances, particularly efficient against *Mycobacterium tuberculosis* [19, 20]. Due to the past extensive collection, *L. officinalis* became so rare to be supposed almost extinct. Consequently eight European countries (Italy not included) included it into the Red Lists of threatened species. In Switzerland, for example, programs of habitat protection together with a specific census let *L. officinalis* spread again [21]. In Italy, till now, the sporocarps of this species have been registered in only four locations: in the Graian Alps two reports (2000 and 2013), one report in the Retic Alps (2013) and one nearby the "Monte Rosa" (2014). Consequently, its inclusion in the Italian Red Lists as near threatened (NT) has been recently proposed (personal communication). The mycelium was isolated from Graian (Fig. 1) and Retic (Fig. 2) specimens, both collected in late summer 2013. The sporophores did not present all the morphological characters useful to identify to species level. The exact identification was obtained by DNA analysis (Fig. 4) of liquid culture of the mycelia isolated from Graian (1.2.Lo2 – Fig. 3) and Retic (1.2.Lo1) specimens. Sequences are going to be deposited in GenBank as soon as possible.

Among the uncommon or rare and poorly investigated species, *L. warnieri* and *P. meridionalis* deserve more attention. Both prefer warm environments and are considered rare in Europe. However, in recent years the reports of the former in Italy are steadily increasing. The Authors collected *L. warnieri* in different sites of Northern Italy, from humid plains to Apennines. It was registered also in other few Italian areas [3]. The strains of this species are not always are present in the International Culture Collections. In the Mycotheque of the Pavia University four different strains (all collected in Pavia

**Figure 1.** Specimen from Graian Alps (2013)

**Figure 2.** Specimen from Retic Alps (2013)

**Figure 3.** Mycelium in pure culture (1.2.Lo2)

**Figure 4.** Elettrophoretic running of PCR products: 3a and 3b respectively belong to Retic and Graian strains (1.2.Lo1 & 1.2.Lo2)

province) have been maintained till now and their identification has been confirmed by means of the analysis of ITS sequences, compared with Mycobank and GenBank databases. The genus *Perenniporia* was revised by Decock and Stalpers [22] with the assessment of *P. meridionalis* at species level, previously named *P. medulla-panis*. Despite this taxonomic problem, the species is quite rare in our Country [3, 5]. It was collected only once, in Northern Italy, nearby the Iseo lake. Also in this case, the DNA analysis agrees with the morphological identification. Finally, recent studies [23] revealed that both the detected species possess interesting enzymatic activities, when they grow on a natural substratum.

## CONCLUSION

It is well known that medicinal mushroom bioactivities could depend on different characteristics such as the genetic profile, the geographical provenance and the substrate [24]. Therefore Italy, as already happened for other countries, could become a very interesting source of different fungal ecotypes, characterized by new and different properties worthy to be investigated. Furthermore, some species collected by the Authors in Italy (for example *L. officinalis*) are classified as endangered and protected species in other European countries. Their *in situ* conservation would be consequently necessary, avoiding the destruction of the habitat and preserving these fungi from uncontrolled actions of picking. Moreover, as quoted by other authors [25 the *ex situ* conservation by means of culture collections is an indisputable tool to get biological material for application purposes and to improve sustainable agriculture and economy.

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# FAMILY PLUTEACEAE IN NORTH WEST INDIA

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## ABSTRACT

Family *Pluteaceae* the "pink spored family" falls under order *Agaricales.* It includes 4 genera spread over 346 species [7]. Presently, 30 species falling under 3 genera of this family, *Pluteus, Volvariella* and *Volvopluteus,* have been collected from various localities of North West India. Of these 7 species, 4 varieties are presented as new to science and while 11 are recorded for the first time from India. The genus *Pluteus* is defined by the presence of bilateral convergent trama and carpophore stipe lacking both the annulus and volva, while genus *Volvariella* has bilateral convergent trama with the stipe lacking an annulus, the genus *Volvopluteus* is a newly constructed genus on the basis of spore size more than 11 µm and stipe with volva and no annulus. The pluteoid mushrooms occur in abundance in the tropical region, they come up early in the monsoon season, mostly they are terrestrial, few are folicolous and lignicolous, only genus *Volvariella* was collected from coprophilous habitat. In the study area *Volvariella bombycina, V. diplasia* and *Volvopluteus gloiocephalus* are the other commonly hunted mushrooms from the wild for human consumption. These species are also being cultivated commercially. Their wild relatives are of common occurrence in North Western India. From the surveys it has become apparent that there is enough wild germplasm for utilization in strain improvement programme in paddy straw mushroom in study area. Various available wild species of *Volvariella,* namely *V. bakeri, V. terastia, V. taylorii* and *V. cubensis,* etc. possesses acceptable agronomic features with possibilities of introduction into cultivated commercial strains through breeding experiments.

**Keywords:** systematics, pluteaceae, new species, new varieties, new records, India

## MATERIALS AND METHODS

The material was collected from Punjab plains. The morphological details were recorded from fresh carpophores. The field characters pertaining to gross morphology, shape, color and size of the pileus, stipe and lamellae, presence or absence of annulus, etc were noted down on the 'Field key' provided by Atri *et al*. [1] and the colour terminology used is that of Kornerup and Wanscher [8]. The specimens were hot air dried and packed in cellophane paper bags containing 1-4 dichlorobenzene. The microscopic details were studied by cutting free hand sections of revived part of the dried specimen and staining them in 1% Cotton blue or 2% Congo red. The spores were studied from the spore print as well as from the crush mounts of the lamellae, amyloid reaction was checked in Melzer's Reagent. The dried specimens were deposited in the Herbarium, Department of Botany, Punjabi University, Patiala, (Punjab), India under PUN for further reference.

## RESULTS AND DISCUSSION

### *Pluteus transitus* sp. nov. [Figs. 1(A) & 3]

**Etymology:** Epithet name refer to transition phase between two sections of genus *Pluteus.*

Carpophores 4.7-7.2 cm in height. Pileus 2.9-4.6 cm broad, plano-convex to flattened depressed; with acute umbo; surface pastel yellow (3A4) with grayish yellow (4C7) at centre; moist; margin regular, splitting at maturity, striated;; cuticle not peeling flesh white, unchanging, up to 0.3 cm broad; odor mild. Lamellae free, close, broad (up to 0.6 cm), yellowish white (4A2), changing to pinkish white (7A2) or reddish white; lamellulae unequal; gill edges smooth. Stipe central to somewhat excentric, 4.2-6.3 cm long, up to 0.3 cm broad above, up to 0.6 cm broad at the base, almost equal in diameter, with somewhat bulbous base, white above pale yellow (4A3) below, unchanging; smooth; solid; exannulate; volva absent.

Basidiospores [20/2/1] (4.0-) 4.8-7.2 x (4.0-) 4.8-6.4 µm, (**L** = 5.6-6.4 µm;**L'** = 5.9 µm; **W** = 4.8-5.6 µm; **W'** = 5.2 µm; Q = 1.0-1.28 (-1.33); **Q'** = 1.15); globose, subglobose to broadly ellipsoid; inamyloid; wall smooth, thick, double;

apiculate, apiculus up to 0.8 µm long, open pore type. Basidia 19.2-32.0 x 5.6-8.0 µm, clavate to sub cylindrical, granular, tetrasterigmate; sterigmata up to 3.2 µm long. Pleurocystidia metuloid, 43.2-69.0 x 11.2-19.2 µm, clavate, fusoid with two to five hooks or prongs at the apex, granular, thin walled. Cheilocystidia 27.2-64.0 x 13.6-25.6 µm, broadly clavate to fusoid, blunt tipped, granular, thin walled. Pileus cuticle subcellular, made up of 16.0-32.0 x 16.0-29.0 µm, globose, subglobose to ellipsoid, thin walled cells, intermixed with thin walled, septate, branched, 1.6-8.0 µm broad hyphae; pileus context made up of loosely arranged, tangled, septate, 1.6- 12.8 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle made up of longitudinally tangled, septate, 2.0-4.1 µm broad hyphae; stipe context made up of septate, thin walled, 8.2-16.4 µm broad hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Pathankot (600 m), Dhar, growing scattered, on dead wood, in angiospermic forest, Yadwinder Singh, PUN 6448, September 01, 2011.

**Distribution and Ecology:** Present collection has been found growing scattered on dead angiospermic wood from Punjab in late monsoon.

**Remarks:** Under genus *Pluteus* the presence of metuloid pleurocystidia with hooks or prongs is the character of section *Pluteus*, while the cellular pileus cuticle is a distinctive feature in section *Celluloderma*, as described by Fyod [30, 31]. The presently worked out collection has a unique combination of both these characters of different sections of genus *Pluteus* i.e. it has a metalloid pleurocystidia with hooks and prongs as in section of genus *Pluteus* whereas in having a cellular pileus cuticle. It seems to fall under section *Celluloderma*. Although, it is having a yellow cap but it does not match with any of the known yellow capped species of this genus. The present collection was compared with *Pluteus conizitus* which is a synonym with *P. chrysagius* according to Pradeep *et al.* [24] and as in Mycobank. But in *P. conizatus* the pleurocystidia are with simple tips lacking prongs and pileus cuticle is trichodermal. Further the present collection was matched with *P. subcervinus* which possesses pleurocystidia with 3-6 prongs at the tips but the cap color in this species is greyish to fuscous brown rather than pastel yellow of the present collection. From *P. agaeotheles* the present collection differs as in this species the cap is off white although the pileus cuticle is trichodermal, pleurocystidia and cheilocystidia are with 2-5 prongs. In *P. leoninus* the cap color is somewhat similar to the present collection but the cuticle is hyphal and the pleurocystidia are simple. The present collection was further compared with another yellow capped *Pluteus* i.e. *P. castri* but in *P. castri* the pleurocystidia are with very small finger like excrescences and the prongs & hooks are lacking. This species seems to be representing a transition phase between section *Pluteus* and section *Celluloderma*. Thus, a new species *Pluteus transitus* sp. nov. has been proposed to accommodate this collection.

### *Pluteus calvitio-stipus* sp. nov. [Fig. 1(B) & 4]

**Etymology:** epithet name refers to the white stipe of carpophore.

Carpophore up to 10.0 cm in height. Pileus up to 6.0 cm broad, planoconvex with uplifted margins; umbonate; surface greyish brown (5D3), covered with appressed fibrillose scales, white flesh seen through dissociating fibres near margin; dry; margin irregular, splitting at maturity; cuticle fully peeling; flesh white, unchanging, up to 0.4 cm broad; odor disagreeable. Lamellae free, close, broad (up to 0.7 cm); light brown (6D4), changing to dark brown on bruising; gill edges serrate; lamellulae unequal. Stipe central, up to 7.5 cm long, up to 0.8 cm above, up to 1.3 cm at the base; tapering upward; white, changing to yellowish white (4A2); smooth; solid.

Basidiospores [20/1/1] 6.4-8.0 x 5.6-6.4 µm, (**L** = 6.4-8.0 µm; **L'** = 7.1 µm; **W** = 5.6-6.4 µm; **W'** = 6.0 µm; Q =1.12-1.25 (-1.28); **Q'** = 1.18); subglobose to broadly ellipsoid; inamyloid; wall smooth, thick, double; apiculate, apiculus up to 1.6 µm long, open pore type. Basidia 19.2-32.0 x 6.4-8.0 µm, clavate, granular, tetrasterigmate to bisterigmate; sterigmata up to 3.2 µm long. Pleurocystidia 35.2-69.0 x 11.2-24.0 µm, clavate to fusoid-ventricose, blunt tipped or sometimes with finger like projections on the apex. Cheilocystidia 43.2-72.0 x 9.6-27.2 µm, clavate to narrowly clavate to fusoid, blunt tipped, granular, hyaline, thin walled. Pileus cuticle made up of radially arranged, thin walled, septate, 8.0-19.2 µm broad cystidioid hyphae, with acute to subacute or few are broadly rounded tips; pileus context made up of loosely arranged, intermingled, septate, 11.2-22.4 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle made up of

longitudinally tangled, septate, 2.4-6.4 µm broad hyphae; stipe context made up of septate, thin walled, 4.8-24.0 µm broad hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Hoshiarpur (295 m), Khadiala Saniyan, growing solitary, on humicolous soil, Yadwinder Singh, PUN 6450, July 16, 2008.

**Distribution and Ecology:** The present collection has been found growing solitary, on humicolous soil in mid July.

**Remarks:** The present collection falls under section *Hispidoderma* as it has hyphal pileus cuticle. It was compared with closely allied species of this section i.e. *Pluteus spilopus* (Berk. & Br.) Sacc.and *P. albostipitatus* (Dennis) Singer. But, *P. spilopus* differ from the present collection by possessing dark fuscous brown cap with a stipe covered with black fibrillose-squamules and internally the pleurocystidia and cheilocystidia do not exceeded 52.0 µm in length as compared to 72.0 µm long in present collection and habitat on wood rather than terrestrial in the present collection. Further, from *P. albostipitatus* it differs in *P. albostipitatus* the habit is lignicolous and the cap is striate at the margin the spores are larger i.e. 7.5-9.0 x 5.5-7.0 µm in comparison to 6.4-8.0 x 5.6-6.4 µm in presently examined collection and the pleurocystidia are with subapical constrictions which is not in the case in the present collection. The present collection is recognized by greyish brown cap, which is covered with appressed fibrillose scales, white flesh seen through dissociating fibres near margin, non striate cap margin and stipe white, changing to yellowish white on handling, non fibrillose, basidiospores subglobose to broadly ellipsoid; pleurocystidia and cheilocystidia clavate, fusoid to fusoid-ventricose in shape. The present collection does not fit into any of the known species of *Pluteus* in section *Hispidoderma*. Therefore, a new species *Pluteus calvitio-stipus* sp. nov. have been proposed to accommodate present collection.

### *Pluteus ortonii* sp. nov. [Fig. 1(C) & 5]

**Etymology:** epithet name dedicated to the P.D. Orton for his monumental work on genus *Volvariella* and *Pluteus.*

Carpophores 7.5-7.8 cm in height. Pileus 5.0- 5.8 cm broad, convex; broadly umbonate; surface pinkish white (10A2), covered with reddish grey (11B2) to brownish grey (7C2) to greyish brown (7D3) appressed fibrillose scales; moist; margin irregular, splitting at maturity, seems to be striated may be formed due to dissociation of fibres along margin; cuticle fully peeling; flesh white, unchanging, up to 0.4 cm broad; taste mild to disagreeable and odor mild to disagreeable. Lamellae free, close, broad (up to 0.7 cm); creamish white to orange brown (5D6), unequal; gill edges serrate; lamellulae present. Stipe central, 6.8-7.3 cm long, up to 0.7 cm broad, tapering upward, with marginate bulb; off white, longitudinally striated, twisted, with greyish white scales, solid.

Basidiospores [61/2/2] (4.0-) 5.6-6.4 x (4.0-) 4.8-5.6 µm, (**L** = 5.6-6.4 µm; **L'** = 6.0 µm; **W** = 4.8-5.6 µm; **W'** = 5.0 µm; Q =1.14-1.33; **Q'** = 1.19); subglobose to broadly ellipsoid; inamyloid; wall smooth, thick, double; apiculate, apiculus up to 0.8 µm long, open pore type. Basidia 22.4-33.6 x 6.4-8.8 µm, clavate, granular, tetrasterigmate; sterigmata up to 2.4 µm long. Pleurocystidia 33.6-70.4 x 8.0-24.0 µm, clavate, lageniform to fusoid to fusoid-ventricose, blunt to tubular tips, densely granular at apex, covered with a very conspicuous mucilaginous deposition at the tips, thin walled. Cheilocystidia 35.2-83.2 x 8.0-38.4 µm, clavate to fusoid, blunt tipped, hyaline, thin walled. Pileus cuticle hyphal, made up of thin walled, horizontally tangled, septate hyphae giving rise to radially arranged, 8.2-16.4 µm broad, granular, septate hyphae; pileus context made up of loosely arranged, intermingled, septate, 12.3-27.0 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle made up of longitudinally tangled, septate, 2.0-12.3 µm broad hyphae; stipe context made up of septate, thin walled, 8.2-24.5 µm broad hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Ropar (394 m), water house, growing solitary, on humicolous soil, Yadwinder Singh, PUN 6443, July 18, 2010; growing solitary, on humicolous soil, Yadwinder Singh, PUN 6444, July 19, 2011.

**Distribution and Ecology:** This *Pluteus* species has been collected growing solitary, on humicolous soil from Punjab, India.

**Remarks:** The present collection falls under section *Hispidoderma* as it has a hyphal pileus cuticle. It is recognized by pinkish white cap, having reddish grey to brownish grey to greyish brown appressed fibrillose scales, stipe is with a marginate bulb and its terrestrial habitat. It was compared with a closely allied species *Pluteus pearsonii* Orton but differs from present as in *P. pearsonii* olivaceous black colored cap is present. Further, it differs from *P. depauperatus* Romagnesi as in this species the cap is white with pale clay buff centre, with strongly striated margin and the stipe lacking marginate bulb. Due to the smaller sized carpophore, lacking marginate bulb, presence of cellular pileus cuticle and lignicolous habit of *P. semibulbosus* (Lasch apud Fries) Gillet, the present collection differs from it. *P. boudieri* Orton differs in having white cap with olivaceous-buff or grey-olivaceous at centre, habitat on wood and larger (6-8 x 5-7 µm) spore size. *P. granulates* Bresadola possesses a cinnamon or cinnamon-buff cap, scaly at centre, smooth and striate at margin. *P. murinus* Bresadola has a date brown or umber cap but with plane to slightly depressed cap centre and larger (6-8 x 5-7 µm) spore size. It does not fit into any of the known species of section *Hispidoderma*. Therefore, a new species have been proposed to accommodate this collection.

### *Volvariella bumelia* sp. nov. [Fig. 1(D) & 6]

**Etymology:** Epithet name refers to large size of carpophore.

Carpophore up to 13.5 cm in height. Pileus 9.0 cm broad, convex; broadly umbonate; surface white; covered with white, cottony fibers; margin irregular, splitting at maturity; cuticle fully peeling; flesh up to 1.0 cm thick, white, unchanging; watery latex on cutting; odor mild. Lamellae free, unequal, crowded, broad (up to 1.4 cm), yellowish white (4A2), unchanging; gill edges smooth; lamellulae present. Spore deposit white. Stipe central, up to 11.5 cm long, up to 1.5cm broad above, up to 2.0 cm broad at the base; cylindrical; surface white unchanging, watery latex on cutting; scaly above and smooth towards base; solid; volva up to 3.5 cm, saccate, 5 lobed, large, fleshy, white, light orange (5A4) at areola.

Basidiospores [37/1/1] (4.0-) 4.8-6.4 x 4.0-5.6 (-6.4) µm, (**L** = 4.8-6.4 µm; **L'** = 5.8 µm; **W** = 4.0-5.6 µm; **W'** = 4.3 µm; Q = 1.0-1.33 (-1.40); **Q'** = 1.14); globose, subglobose to broadly ellipsoid; inamyloid; wall smooth, thick; apiculate, apiculus up to 0.8 µm long, open pore type. Basidia 30.4-43.2 x 6.4-9.6 µm, clavate, granular, tetrasterigmate occasionally bisterigmate; sterigmata up to 4.0 µm long. Pleurocystidia 35.2-86.4 x 12.8-32.0 µm, claviform to fusoid to fusoid ventricose,with long tubular tips to sometimes blunt tips, densely granular at tips.Cheilocystidia 36.8-88.0 x 11.2-30.4 µm, claviform to fusoid to fusoid-ventricose,with long tubular to sometimes blunt tips, granular. Pileus cuticle hyphal made, up of thin walled, granular, septate, horizontally tangled hyphae, giving rise to turf of radially arranged, septate, granular, 4.1-20.45 µm broad hyphae; pileus context made up of loosely arranged, intermingled, septate, 8.2-31.0 µm broad hyphae. Hymenophoral trama convergent. Stipe cuticle made up of longitudinally tangled, granular, septate, 6.1-12.3 µm broad hyphae, with no projecting elements; stipe context made up of septate, thin walled, smooth, 8.2-20.4 µm broad hyphae. Volva hyphal made up of closely septate, thin walled, granular, gelatinized, 4.1-10.2 µm broad entangled hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Patiala (250 m), growing solitary, on soil, under *Duranta sp.*, Yadwinder Singh, PUN 6477, August 25, 2011.

**Distribution and Ecology:** Present collection has been found growing solitary, on soil, under *Duranta* sp. from Punjab.

**Remarks:** The above examined collection is somewhat similar in its appearance to *Volvariella bombycina* (Schaeff. : Fr.) Sing. as it possessing white cap, covered with white cottony, shiny fibrils and the volva is yellowish white as in *V. bombycina*. But, the present collection differs from *V. bombycina* in possessing a robust carpophore, which gives watery latex on cutting of the cap or stipe, there is no yellowish tinge at the cap surface, stipe is cylindrical, lacking bulbous base where as in *V. bombycina* it is not so. Further, the basidiospores are smaller in size (4.0-) 4.8-6.4 x 4.0-5.6 (-6.4) µm) in comparison to 6.6-10.4 x 4.4-6.7 µm basidiospores of *V. bombycina*, even the cystidia are smaller in size in present collection in comparison to the size of cystidia described by Shaffer [28] for *V. bombycina*. Further, *V. bombycina* is always known to be lignicolous in habitat while the present collection is terrestrial in habitat. The present collection was also compared with another white capped *Volvariella* i.e. *V. smithii* Shaffer, as *V. smithii* also has 5-lobed volva and terrestrial in habitat. But,

in *V. smithii* cap has pinkish buff centre, densely pubescent stipe and ochraceous volva with cinnamon touch. Also the spore size given for *V. smithii* by Shaffer [28] is 4.7-7 x 3.1-3.9 µm. Based upon the above discussion a new species *Volvariella bumelia* sp. nov. is proposed. *V. bumelia* sp. nov. is characterized by in possessing a white cap with no pink or yellowish tinge, all parts of carpophores exuding watery latex, a cylindrical stipe without bulbous base and the spore size varying from (4.0-) 4.8-6.4 x 4.0-5.6 (-6.4) µm.

***Volvariella albida* sp. nov. [Figs 1(E&F) & 7]**

**Etymology:** Epithet name refer to white color of carpophore.

Carpophores 8.0-8.3 cm in height. Pileus 6.5-6.6 cm broad, convex to planoconvex; with or without umbo; surface white (1A1), whitish grey at centre; covered with white, appressed fibrillose scales; margin regular, fimbriate, splitting at maturity; cuticle fully peeling; flesh up to 0.7 cm broad, white, unchanging; odor mild to disagreeable. Lamellae free, crowded, broad (up to 0.6 cm); unequal; white to pinkish white (7A2), unchanging; gill edges serrate; lamellulae truncate. Stipe central, 5.0-7.5 cm long, up to 0.7 cm above, up to 1.3 cm broad at the base; obclavate, with bulbous base; surface white, unchanging; smooth; solid; volva up to 3.4 cm, large, less fleshy, saccate, 3-lobed, white to creamish white, with areolate surface, brown at areola.

Basidiospores [34/2/2] 4.8-5.6 x 4.0-4.8 µm, (**L** = 4.8-5.6 µm; **L'** = 5.0 µm; **W** = 4.0-4.8 µm; **W'** = 4.5 µm; Q = 1.0-1.20 (-1.40); **Q'** = 1.14); ovoid, globose,subglobose to broadly ellipsoid, occasionally ellipsoid; inamyloid; smooth, thick walled; apiculate, apiculus up to 0.8 µm long, open pore type. Basidia 19.2-28.8 x6.4-8.8 µm, clavate, granular throughout, tetrasterigmate; sterigmata up to 3.2 µm long. Pleurocystidia 40-67.2 x 9.6-32 µm, claviform to fusoid with small tips, Cheilocystidia 29-90 x 11.2-32 µm, claviform to fusoid. Pileus cuticle hyphal, made up of thin walled, granular, septate, radially arranged 8.2-20.4 µm broad projecting hyphae, arising from subhorizontally tangled hyphae; pileus context made up of loosely arranged, intermingled, septate, 8.2-29.0 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle hyphal, made up of longitudinally tangled,septate, 4.1-12.3 µm broad hyphae; stipe context made up of loosely interwoven,septate, thin walled, 8.2-37 µm broad hyphae. Volva hyphal, made up of closely septate, thin walled, 4.1-20.4 ìm broad tangled hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Patiala, Punjabi University Campus (250 m), growing solitary, on soil, near *Azadirachta indica*, Yadwinder Singh, PUN 6460, July 7, 2008; Punjabi University Campus (250 m), growing solitary, on soil, near *Azadirachta indica*, Yadwinder Singh, PUN 6461, July 22, 2008.

**Distribution and Ecology:** This new species has been found solitary under *Azadirachta indica* during July at an altitude of 250 m from Patiala.

**Remarks:** This collection morphologically looks very similar to *Volvariella bombycina* (Schaeff. : Fr.) Sing. However, it differs from *V. bombycina* in lacking yellow tinge at the umbo of the cap, spores comparatively smaller i.e. 4.8-5.6 x 4.0-4.8 µm instead of 6.6-10.4 x 4.4-6.7 µm of *V. bombycina*. Pleurocystidia 40-67.2 x9.6-32 µm and cheilocystidia 29-90 x 11.2-32 µm of present collection are smaller in comparison to 26-122 x 8- 57 µm pleurocystidia and 26-144 x 8-46 µm cheilocystidia described for *V. bombycina* by Shaffer [28]. Further, even the shape of pleurocystiadia and cheilocystidia is mostly clavate to fusoid with small blunt tips in this collection as compared to fusoid to fusoid-ventricose with tubular tips in *Volvariella bombycina*. Present collection has been found growing terrestrially instead of lignicolous habitat which is known for *V. bombycina* [28]. Thus, based on these differences, a new species *V. albida* sp. nov.have been proposed to accommodate this collection.

***Volvopluteus shafferii* sp. nov. [Figs. 2(G) & 8]**

**Etymology:** Epithet name dedicated to R.L. Shaffer for his remarkable work on genus *Volvariella*.

Carpophores 3-18 cm in height. Pileus 1-11.2 cm broad, convex to flattened; broadly umbonate; surface greyish orange (5B3), orange grey (5B2) at centre, with pinkish tinge towards margin; scales appressed fibrillose, greyish brown, more

concentrated near the centre; margin regular to irregular, splitting at maturity; dry to moist; cuticle fully peeling; flesh up to 0.4 cm thick, white, unchanging. Lamellae free, subdistant, broad (up to 1.5 cm); orange grey (6B2) to pinkish brown, unchanging; edges serrate, white, flocculose; lamellulae present. Spore deposit light brown (6D5) to brownish orange ($5C_3$). Stipe central, 7.0-16.5 cm long, up to 1.8 cm broad above, up to 2.5 cm broad at the base; yellowish white (4A2); narrowing upward, with slightly bulbous base, fibrillose scaly; hollow; exannulate; volva lobed only in young carpophores then become membranous, tightly packed with stipe base as broken patches, creamish white.

Basidiospores [120/4/4] 11.2-16 x 7.2-12.8 µm, (**L** = 11.2-12.8 µm; **L'** = 12.3 µm; **W** = 8.0-10.4 µm; **W'** = 9.7 µm; Q = 1.14-1.55 (-1.60); **Q'** = 1.28); ovoid, broadly ellipsoid to ellipsoid, occasionally subglobose; inamyloid; smooth, thick walled; guttulate; apiculate, apiculus up to 1.6 µm long, open pore type. Basidia 17.6-34.0 x 8.9-16.0 µm, clavate, granular, tetrasterigmate, occasionally bisterigmate; sterigmata up to 4.8 µm long. Pleurocystidia and cheilocystidia absent.Gill edges sterile. Pileus cuticle hyphal, gelatinized, made up of horizontally tangled septate, granular hyphae, giving rise to turf of radially arranged, septate, granular, 2.4-12.3 µm broad hyphae; pileus context made up of loosely interwoven, septate, thin walled 8.2-20.4 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle hyphal, made up of longitudinally tangled, 1.6-6.4 µm broad, septate hyphae interspersed caulocystidia present, caulocystidia elongated, narrowly clavate, granular, 12.8-48.0 x 3.2-11.2 µm; stipe context made up of thin walled, septate, 3.2-19.2 µm broad hyphae. Volval elements made up of septate, 3.2-11.2 µm broad hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Hoshiarpur (295 m), growing soliatry on leaf litter of *Eucalyptus perriniana* and under *Cannabis sativa*, Yadwinder Singh, PUN 4026, July 19, 2008; Ludhiana, Punjab Agriculture University (254 m), growing scattered, on humicolous soil under *Ficus*, Baljit Kaur, PUN 3934, July 18, 2009; Sangrur, Nadampur village (231m), growing scattered, on humicolous soil. Jagdeep Kaur, PUN 4094, July 25, 2009.

**Distribution and ecology:** Present collection was commonly found growing solitary to scattered in groups, on humicolous soil or on leaf litter of *Eucalyptus* from different localities of Punjab during July-August at an altitude varying from 231-295 m.

**Remarks:** In the presently worked out collections the spores are more than 11 µm and pileus cuticle is gelatinized thus, they fall under genus *Volvopluteus* Vizzini, Contu & Justo. In gross morphological and anatomical characters the presentcollections are in some features similar to *Vp. speciosa* as described by Justo *et al.* [6]. But, they differ from *Vp. speciosa* in possessing smaller spore size range i.e.8-16 x 6.8-12.8 µm, instead of 11.7-20.9 × 7.2-12.4 µm reported for *Vp. speciosa* by Justo *et al.* [25], pleurocystidia and cheilocystidia both are present in *Vp. speciosa*, while are absent in the present collections. The present collections also share some characters with *Vp. gloiocephalus* (DC.) Vizzini, Contu & Justo and *Vp. earlei* (Murrill) Vizzini, Contu & Justo [6] in having spores more the 11 µm, gelatinized pileus cuticle, but it differs from these species in possessing larger carpophore size up to 18 cm, lacking lobed volva and lacking pleurocystidia and cheilocystidia. The presently worked out collection is unique in its characters as it possesses a broadly umbonate greyish orange cap with orange grey centre, with a pinkish tinge towards the margin, the volva is lobed only in the young carpophores at maturity it becomes membranous, tightly packed around the stipe base in the form of broken, creamish white patches, basidiospores 8-16 x 6.8- 12.8 µm. The present collection does not completely matched with any known species of genus *Volvopluteus*, thus based upon the above discussion, a new species *Vp. shafferii* sp. nov. is being proposed to accommodate this collection.

***Volvopluteus diversisporus* sp. nov. [Figs. 2(H) & 9]**

**Etymology:** Epithet name refers to different shapes of basidiospores i.e. *diversi* = varied + *sporus* = *spores*.

Carpophores 3.7-8.7 cm in height. Pileus 4.1-5.9 cm broad, convex; umbonate; surface orange white (6A2); dry; atomate, glabrous; margin irregular, splitting at maturity, striated along margin; cuticle fully peeling; flesh up to 0.3 cm broad, white, unchanging; taste sour or slightly peppery; odor mild. Lamellae free, subdistant, moderately broad (up to 0.3 cm); greyish orange (6B3), unchanging; gill edges smooth; lamellulae attenuate. Stipe central, 3.6-8.5 cm long, up to 0.4 cm broad above, up to 0.6 cm broad at base; tapering upward, with slightly bulbous base; white (2A1), unchanging; smooth; solid; exannulate; volva, up to 1.5 cm wide, small, membranous, tightly packed at base, light grey.

Basidiospores [20/1/1] (8.8-) 11.2-16.0 x 7.2-14.4 µm, (**L** = 9.6-16.0 µm; **L'** = 12.1 µm; **W** = 8.0-14.4 µm; **W'** = 9.1 µm; Q = 1.1–1.78; **Q'** = 1.35); subglobose, ovoid, obovoid, oblong, amygdaliform some with a short blunt snou, broadly ellipsoid, ellipsoid, elongated, or more broader and shorter in length 7.2-14.4 x 9.6-19.2 µm, triangular to typically heart shaped;inamyloid; double walled, smooth; vacuolated; apiculate, apiculus up to 1.6 µm long, open pore type. Basidia 25.6-46.4 x 9.6-17.6 µm, clavate, granular, tetrasterigmate, occasionally bisterigmate, rarely unisterigmate; sterigmata up to 8.0 µm long; lamellae edges heteromorphous. Pleurocystidia 20.8-86.4 x 8.8-34.0 µm, broadly clavate, fusoid to fusoid -ventricose, granular, with blunt to long tubular tips, abundant; cheilocystidia 32.0-80.0 x 9.6-40.0 µm, clavate, fusoid to fusoid-ventricose, granular, densely granular at tips, with blunt, rounded to small knob like to tubular tips, thin walled, abundant. Pileus cuticle hyphal, gelatinized, made up of thin walled, septate, horizontally tangled hyphae, giving rise to radially tangled, gelatinized, 4.1-16.4 µm broad hyphae; pileus context made up of loosely and irregularly interwoven, septate, 8.2-20.4 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle hyphal, made up of longitudinally tangled, septate, 4.1-12.3 µm broad hyphae, with no projecting element on the surface; stipe context made up of septate, thin walled, 8.1-29.0 µm broad hyphae. Volva hyphal, composed of septate, granular, 1.6-14.4 µm broad tangled hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Patiala, Bahadurgarh (250 m), growing scattered, among grasses, along road side, Yadwinder Singh, PUN 6480, May 28, 2008.

**Distribution and Ecology:** The above examined collection has been found growing solitary to scattered among grasses in the month of May from Punjab.

**Remarks:** The macroscopic and microscopic details of the presently worked out collection of *Volvo pluteus* are not very convincing when matched with the allied species *Vp. gloiocephalus* (DC.) Vizzini, Contu & Justo and *Vp. earlei* (Murrill) Vizzini, Contu & Justo as both these species are with large sized carpophores, the basidiospores are either ovoid, broadly ellipsoid to ellipsoid and are with comparatively lesser breadth, where as in the present collection the carpophores are smaller, basidiospores are having diverse shapes. On the basis of diverse shapes of the basidiospores this collection was matched with *Volvariella heterospora* Menolli & Capelari as described by Menolli & Capelari [10] and has two different types of basidiospores but, it differs from the present collection in possessing dark grey cap color and lacking angular or heart shaped spores. Thus, based upon the above observations a new species i.e. *Vp. diversisporus* sp. nov. is proposed. *Vp. diversisporus* sp. nov. is characterized in possessing an orange white, glabrous, umbonate cap, without any scales on the surface, basidiospores of diverse shapes i.e. some smaller subglobose, ovoid, obovoid, oblong, amygdaliform with short snout to larger spores which are much broader angular to typically heart shaped, both pleurocystidia and Cheilocystidia present.

***Volvariella bombycina*** (Schaeff. : Fr.) Sing. *Lilloa* **22**: 401, 1951 var. ***parva*** **var. nov. [Fig. 2(I) & 10]**

**Etymology:** Variety name refers to small size of carpophore.

Carpophore up to 5.4 cm in height. Pileus up to 2.8 cm broad, convex; lacking umbo; surface white; decorated with white appressed fibrillose, shiny scales; margin regular, fimbriate or toothed; cuticle fully peeling; flesh white, unchanging; taste and odour mild. Lamellae free, crowded, unequal, white, unchanging; gill edges smooth; lamellulae truncate, spore deposit white (2A1). Stipe central, up to 4.0 cm long, up to 0.8 cm broad; subcylindric; white, unchanging; smooth; exannulate; volva saccate, large, fleshy, 2-lobed, white from inner side and yellowish white (4A2) from outside.

Basidiospores [32/1/1] 4.8-6.4 x 3.2-5.6 µm, (**L** = 4.8-5.6 µm; **L'** = 5.4 µm; **W** = 3.2-4.8 µm; **W'** = 4.3 µm; Q = 1.17-1.50; **Q'** = 1.30); ovoid, broadly ellipsoid to ellipsoid; inamyloid; smooth, thick walled; guttulate; apiculate, apiculus up to 0.8 µm long, open pore type. Basidia 20.8-27.2 x 6.4-8.0 µm, clavate, granular, tetrasterigmate to bisterigmate; sterigmata up to 2.4 µm long. Pleurocystidia 32.0-72.0 x 9.6-19.2 µm, claviform to fusoid. Cheilocystidia 22.4-62.4 x 11.2-27.2 µm, fusoid, clavate, blunt tipped. Gill edges sterile. Pileus cuticle hyphal, made up of thin walled, septate, horizontally tangled hyphae, giving rise a turf of radially tangled, 2.4-9.6 µm broad hyphae; pileus context made up of loosely arranged, intermingled, septate, 3.2-14.4 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle made up of

longitudinally tangled, septate, 4.1-12.3 µm broad hyphae; stipe context made up of septate, thin walled, smooth, 8.2-24.5 µm broad hyphae. Volva consists of septate, branched, thin walled, 4.1-20.4 µm broad hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Patiala, Punjabi University, Botanical Garden (251m), growing solitary, on sandy soil, Yadwinder Singh, PUN 6454, August 3, 2007.

**Distribution and Ecology:** The above examined collection has been found growing solitary, on humicolous soil from Punjab.

**Remarks:** The present collection has a white cap, covered with white shiny appressed hair like scales, the stipe is white with yellowish white volva. Thus, in its gross morphology and the microscopic details it falls under *Volvariella bombycina* (Schaeff. : Fr.) Sing. But, the present collection having smaller basidiocarp with small sized cap of up to 2.5 cm as compared to cap 5-20 cm broad cap reported by Shaffer [28] for *V. bombycina*. The cap also lacking yellowish tinge and the spore size, pleurocystidia and cheilocystidia size is also smaller than the size reported for *V. bombycina* by Shaffer [28]. The present collection was then compared with another white colored species i.e. *V. smithii* Shaffer, but, this differs in having white cap with pinkish buff centre, while stipe is densely pubescent having 5-lobed volva, which is fibrillose externally, ochraceous to light cinnamon in color, and spores are much narrower 3.1-3.9 µm rather than 3.2-5.6 µm of present collection. The present collection is similar to *V. bombycina*, but, due to the presence of smaller carpopohore somewhat smaller spore range, smaller size of cystidia, it is proposed as a variant of *V. bombycina*. Therefore, a new variety *V. bombycina* var. *parva* var. nov. is proposed to accommodate present collection.

***Volvariella bombycina*** (Schaeff. : Fr.) Sing. *Lilloa* **22**: 401, 1951 var. ***terricola*** var. nov. **[Figs. 2 (J) & 11]**

**Etymology:** Variety name refer to the habitat from which present taxon have been collected.

Carpophores 4.8-13.5 cm in height. Pileus 3.5-8.4 cm broad, companulate to convex to flattened; umbonate; surface white (1A1) to yellowish white (4A2) with pale yellow (4A3) to yellowish brown (5D4) at centre with maturity; covered with white, appressed fibrillose, shiny scales; margin regular, fimbriate; cuticle fully peeling; flesh up to 1.0 cm, white, unchanging; taste mild to spicy; odor agaricoid to disagreeable. Lamellae free, close to crowded, broad (up to 1.4 cm); unequal; yellowish white (4A2), or pinkish white (7A2), or orange white (6A2), or orange grey (6B2), unchanging; gill edges serrate; lamellulae truncate. Stipe central, 4.2- 11.8 cm long, up to 1.1 cm above, up to 1.3 cm broad at middle, up to 2.0 cm at the base, obclavate, with bulbous base; surface white to yellowish white (4A2), to pale yellow (4A3), unchanging; scaly; hollow; volva up to 3.3 cm, saccate, large, fleshy, 3-lobed, yellowish white to creamish white, with areolate surface.

Basidiospores [121/3/3] 6.4-10.4 x 4.8-6.4 µm, (**L** = 6.4-8.0 µm; **L'** = 7.7 µm; **W** = 4.8-6.4 µm; **W'** = 5.6 µm; Q = (1.11-) 1.14-1.67; **Q'** = 1.37); ovoid, obovoid, broadly ellipsoid to ellipsoid, occasionally subglobose, rarely elongate; inamyloid; smooth, thick walled; apiculate, apiculus up to 0.8 µm long, open pore type. Basidia 19.2-43.2 x 6.4-11.2 µm, clavate, granular throughout, tetrasterigmate; sterigmata up to 3.2 µm long. Pleurocystidia 28.8-118.4 x 9.6-54.4 µm, claviform to fusoid-ventricose with long to small tubular tips, granular with crystalline depositions inside. Cheilocystidia 32-118 x 9.6-45 µm, similar with pleurocystidia.Gill edges fertile. Pileus cuticle hyphal, made up of thin walled, granular, septate, radially arranged 1.6-20.4 µm broad hyphae; pileus context made up of loosely arranged, intermingled, septate, 3.2-27.2 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle hyphal, made up of longitudinally tangled, septate, 1.6-16.4 µm broad hyphae, granular with yellowish pigment; no projecting elements; stipe context made up of loosely interwoven, septate, thin walled, 3.2- 37.0 µm broad hyphae. Volva hyphal, made up of closely septate, thin walled, granular, 1.6-28.6 µm broad entangled hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Patiala, University Campus (250 m), growing solitary, in open grassy lawn, Yadwinder Singh, PUN 6451, June 27, 2008; Hoshiarpur, Bhagana (295 m), growing solitary, on soil, Yadwinder Singh, PUN 6452, July 20, 2008; Mansa, Gagowal (217 m), growing solitary, on humicolous soil, Harwinder Kaur, PUN 6453, August 6, 2010.

**Distribution and Ecology:** Present collections have been found growing on humicolous soil among grasses from different localities in Punjab in the monsoon season between June to August.

**Remarks:** The diagnostic characters with regard to their morphological and anatomical details are in conformity with *Volvariella bombycina* (Schaeff. : Fr.) Sing. except of its terrestrial habitat. It is characterized by large sized white carpophore pileal surface covered with white shiny hairs, margin exceeding the lamellae. It is interesting to note that *V. bombycina* has always been collected from various countries in lignicolous habitat. It has been collected solitary to scattered or gregarious on decaying trunks or large logs or stumps of maple, beech, elm, etc, by Smith [31], Coker [3] from North Carolina, Shaffer [23] from North America, Orton [14] from England, Monoson *et al.* [11] from Illinois, Seok *et al.* [27] from Korea, and Menolli and Capelari [10] from Brazil. Lakhanpal *et al.* [9] collected it from North West Himalayas on decorticated trunks of *Picea smithiana* while, Pradeep *et al*. [18] found this species on the stumps of dead Mango tree from Kerala. While the present collection has been found growing on humicolous soil among grasses from different localities in Punjab in the monsoon season from June to August. Thus, based on the observation of its terrestrial habit from 3 different localities of Punjab i.e. Mansa, Hoshiarpur & Patiala during monsoon season collected in three different years a new variety is proposed as *Volvariella bombycina* var. *terricola* var. nov.

***Volvariella terastia*** (Berk.& Br.)Sing., *Mushr.& Truffl*: 114, 1961 var. ***magnacystidiata*** var. nov. **[Figs. 2 (K) & 12]**

**Etymology:** Variety name refer to the large size of cystidial elements.

Carpophores 10-10.5 cm in height. Pileus 8.6-9.7 cm broad, convex to plano-convex; broadly umbonate; surface white (5A1), with brownish beige (6E3) to brown (5E4) appressed scales, more concentrated in the centre; margin regular, splitting at maturity, fimbriate; cuticle fully peeling; flesh up to 1.0 cm thick, white, unchanging; taste and odor mild. Lamellae free, close, broad (up to 1.1 cm); unequal; orange white (6A2), unchanging; gill edges serrate, white; lamellulae truncate. Spore deposit orange grey (6B2). Stipe central, 8.6-9.3 cm long, 1.3 cm above, 1.5 cm broad in middle and 2.2 cm broad at the base; narrowing upward, with bulbous base; white (5A1), unchanging; scaly; volva up to 5.3 cm, large, fleshy, saccate, 3-5 lobed, aerolate, greyish brown to yellowish brown.

Basidiospores [53/2/2] 4.0-5.6 x 4.0-4.8 (-5.6) µm, (**L** = 4.8-6.4 µm; **L'** = 5.1 µm; **W** = 4.0-4.8 µm; **W'** = 4.5 µm; Q = (1.0-) 1.17–1.20; **Q'** = 1.13); subglobose to broadly ellipsoid, occasionally globose; inamyloid; smooth, double walled; apiculate, apiculus up to 0.8 µm long. Basidia 22.4-30.4 x 6.4-8.0 µm, clavate, granular, tetrasterigmate, occasionally bisterigmate; sterigmata up to 4.0 µm long. Pleurocystidia 38.4-96.0 x 11.2-36.8 µm, clavate to fusoid to fusoid ventricose, blunt tipped to tubular tips, hyaline, thin walled. Cheilocystidia 27.2- 85.0 x 9.6-32.0 µm, clavate, fusoid-ventricose, with blunt to long tubular tips, hyaline, thin walled. Pileus cuticle hyphal, made up of granular, septate, horizontally tangled hyphae, giving rise to turf of radially tangled, thin walled, septate, 1.6-24.5 µm broad hyphae; pileus context made up of loosely intermingled, septate, 8.0-33 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle made up of longitudinally tangled, septate, granular, 4.1-14.3 µm broad hyphae; stipe context made up of septate, thin walled, 8.2-33.0 µm broad hyphae. Volva made up of closely septate, intermingled, granular, branched, 4.1-24.5 µm broad hyphae. Clamp connections absent throughout.

**Collection Examined:** Punjab, Patiala, Bahadurgarh (251 m), growing solitary, on humicolous soil, Yadwinder Singh, PUN 6463, September 3, 2008; Punjabi University campus (251 m), growing solitary, under *Azadirachta indica*, Yadwinder Singh, PUN 6464, August 16, 2008.

**Distribution and Ecology:** The above examined collections has been found growing solitary, under *Azadirachta indica* in the months of August and September from Punjab.

**Remarks:** The morphological and anatomical details of these collections are similar with the description given for *Volvariella terastia* (Berk. & Br.) Sing. as described by Pegler [17] except for the larger carpophores size, larger pleurocystidia (38.4- 96 x 11.2-36.8 µm) and cheilocystidia (27.2-85 x 9.6-32 µm) as compared to pleurocystidia (35-50 x 15-22 ìm) and cheilocystidia (32-36 x 13 µm) described by Pegler [17] for *V. terestia*. In present collection the size of the basidia

(22.4-30.4 x 6.4-8.0 µm) is also comparatively larger. On the basis of these differences from *V.terastia*, a new variety *V. terastia* var. *magna-cystidiata* var. nov. have been proposed.

***Volvariella volvacea*** (Bull. : Fr.) Sing. *Lilloa* **22**: 401, (1949) 1951 var. ***lignicola*** var. nov.

**[Figs. 2 (L) & 13]**

**Etymology:** Variety name refer to the habitat from which present taxon have been collected i.e. *lignum* = wood, *cola* = habitat.

Carpophores 8.5-13.5 cm in height. Pileus 10.5-14.0 cm broad, convex; broadly umbonate; surface brownish grey (5D2), with a paler centre, brownish orange (5C3) at maturity; covered with hairy, greyish black to shiny scales; margin regular, splitting at maturity; cuticle fully peeling; flesh up to 0.6 cm thick, white, unchanging; taste and odour mild. Lamellae free, close, broad (up to 0.8 cm); unequal; pinkish brown in young becomes brownish orange (6C5) at maturity, unchanging on bruising; gill edges serrate; lamellulae attenuate. Spore deposit brownish orange (6C3) to pale red (7A3). Stipe excentric, 9.0-12.5 cm long, up to 0.8 cm above, up to 1.1 cm broad at the base; subcylindric; creamish white, unchanging; smooth; solid; volva up to 2.5 cm, saccate, 2-lobed, large, fleshy, dark greyish.

Basidiospores [34/1/1] 5.6-9.6 x 4.8-6.4 µm, (**L** = 6.4-8.0 µm; **L'** = 7.2 µm; **W** = 4.8-7.2 µm; **W'** = 5.3 µm; Q = 1.14–1.50 (-1.57); **Q'** = 1.24); broadly ellipsoidto ellipsoid, occasionally subglobose; inamyloid; smooth, thick walled; apiculate, apiculus up to 0.8 µm long, open pore type. Basidia 19.2-40.0 x 6.4-9.6 µm, clavate, granular, tetrasterigmate, occasionally bisterigmate; sterigmata up to 4.0 µm long. Pleurocystidia 37.0-109.0 x 11.2-33.6 µm, claviform to fusoid with blunt to elongate, tubular tips. Cheilocystidia 29.0-101.0 x 9.6-24.0 µm, fusoid, clavate, blunt to elongate, tubular tipped, densely granular near the tips.Gill edges fertile. Pileus cuticle hyphal, made up of horizontally tangled, septate, thin walled, granularhyphae, giving rise to turf of radially tangled, thin walled, granular, septate, 4.1- 16.4 µm broad hyphae; pileus context made up of loosely interwoven, septate, 4.1- 24.5 µm broad hyphae. Hymenophoral trama bilateral convergent. Stipe cuticle hyphal, made up of longitudinally tangled, granular, septate, 4.1-12.3 µm broad hyphae, with granular, closely septate, projecting hyphae, as if volval elements,19.2-49.6 x 3.2-9.6 µm; stipe context made up of septate, thin walled, smooth, 8.18- 20.45 µm broad hyphae. Volval elements hyphal, granular, with pointed tips, branched, closely septate, projecting up ward, possessing brownish to olive greenish pigment, 4.1-29.0 µm broad. Clamp connections absent throughout.

**Collection Examined:** Punjab, Ropar (250 m), growing scattered in small groups,on decaying trunk of *Ficus religosa*, Yadwinder Singh, PUN 6478, July 18, 2010.

**Distribution and Ecology:** The present collection has been found growing scattered in groups on dead trunk of *Ficus religosa* in mid July.

**Remarks:** The present collection is similar with the description given by Shaffer [28] and Orton [14] for *Volvariella volvacea* (Bull. : Fr.) Sing. except pileus brownish grey to brownish orange, paler at centre, covered with greyish black, hairy, shiny scales, stipe with dark greyish volva and spores smaller in length (5.6- 9.6 x 4.8-6.4 µm) in comparison to fuliginous to greyish brown cap, blackish brown at centre and brownish volva and larger spores (6.9-10.4 X 4.6-7 µm) in *V. volvacea*. It also differs from *V. volvacea* in its lignicolous habitat. Earlier, *V. volvacea* has been reported on a variety of decaying plant material like paddy straw compost piles, saw dust etc. by Shaffer [28], Orton [14], Coker [3], Monoson *et al.* [11], Seok *et al.* [27] and Priest and Conde [25]. From India too it has been collected from similar habitat by Natarajan and Manjula [12], Bhavani Devi [2], Patil *et al.* [16] and Pradeep *et al*. [18] from South India. Pathak *et al.* [15] and Hennings [5] reported it from North West Himalyas and Garcha [4] documented this species from Ludhiana in Punjab. According to Orton [13] the substratum of species in genus *Volvariella* is most important and also stated that *V. volvacea* grows on compost and is not lignicolous, but the present collection has been collected growing on decaying tree trunk. Based upon the lignicolous habitat of present collection and the difference in cap color and comparatively smaller spore size from *V. volvacea*, a new variety of *V. volvacea* i.e. var. *legnicola* var. nov. have been proposed.

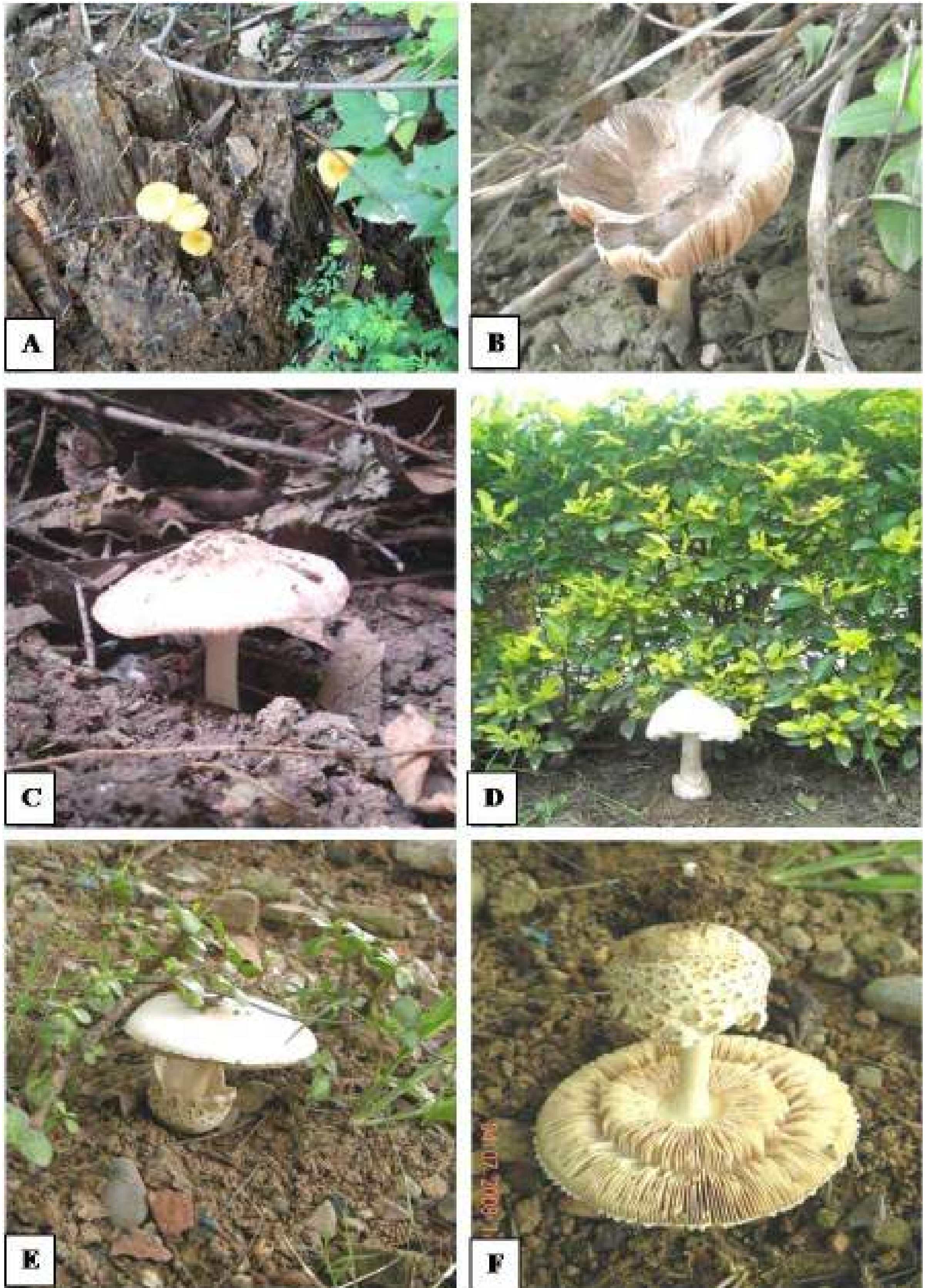


**Figure 1. A.** Carpophore of ***Pluteus transitus* sp.nov.** growingon dead wood. **B.** Carpophore **of *P. calvitio-stipus* sp. nov.** growing in soil and cap surface greyish brown, covered with appressed fibrillose scales. **C .** Carpophore of ***P. ortonii* sp. nov.**growing in its natural habitat. **D.** Carpophore o f ***Volvariella bumelia* sp. nov.** growing in its natural habitat. **E.** Carpophore of ***Volvariella albida* sp. nov.** in its natural habitat. **F.** Underside of cap of ***V. albida* sp. nov.** with free, crowded, yellowish white lamellae and stipe with saccate, white to creamish white volva with areolate surface, brown at areola

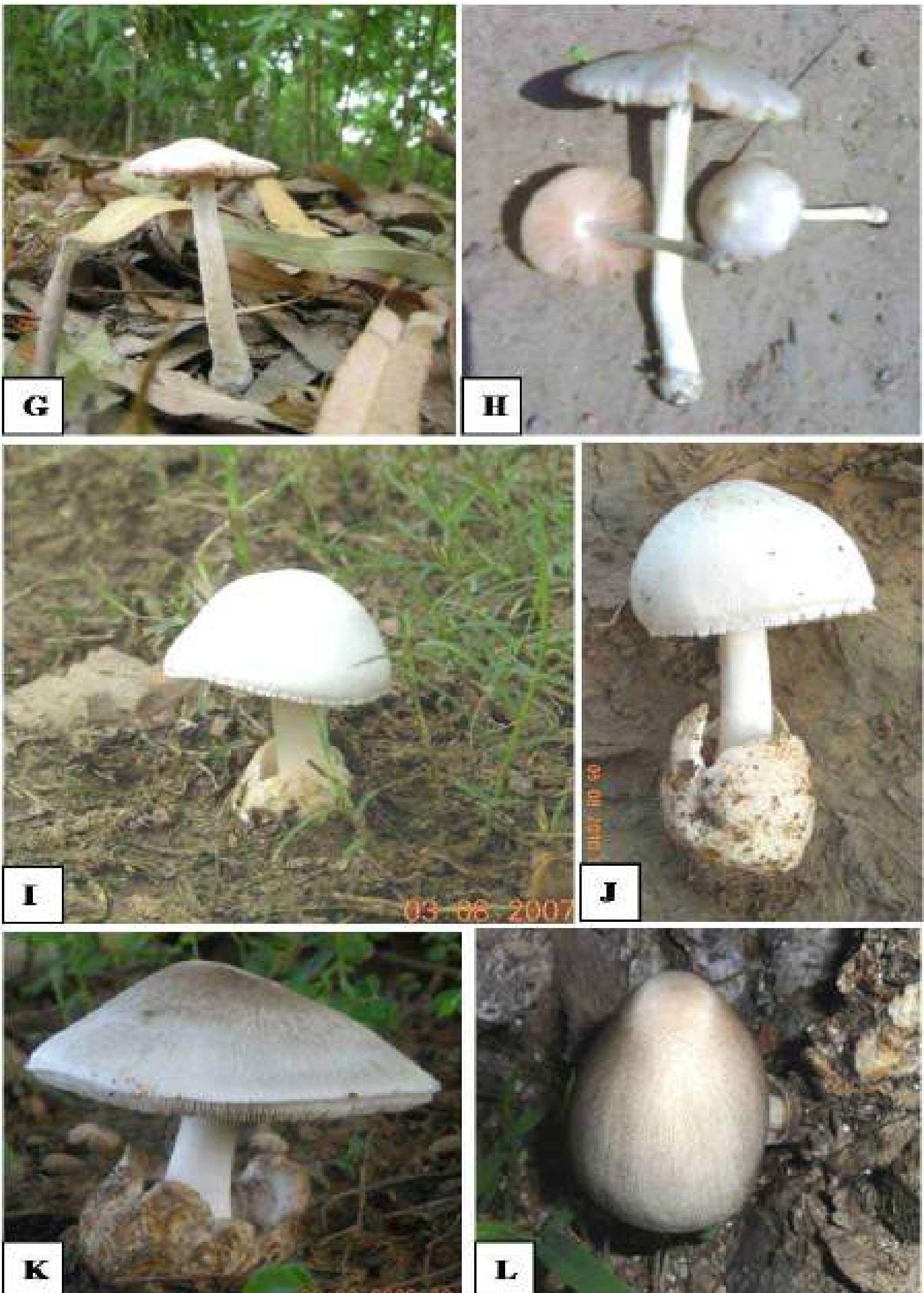


**Figure 2. G.** Carpophore of ***Volvopluteus shafferii* sp. nov.** growing in its natural habitat on leaf litter. **H. *Vp. diversi-sporus* sp. nov.** Cap surface orange white, atomate, glabrous and underside of cap showing subdistant, greyish orange lamellae. **I. *Volvariella bombycina* var. *parva* var. nov.** White carpophore growing in its natural habitat. **J.** Carpophore of ***V. bombycina* var. *terricola* var. nov. K. *V. terastia* var. *magna-cystidiata* var. nov.** with white cap having brownish beige appressed scales and with fimbriate margins. **L.** Carpophore of ***V. volvacea* var. *lignicola* var. nov.** growing on decaying trunk of *Ficus religosa*

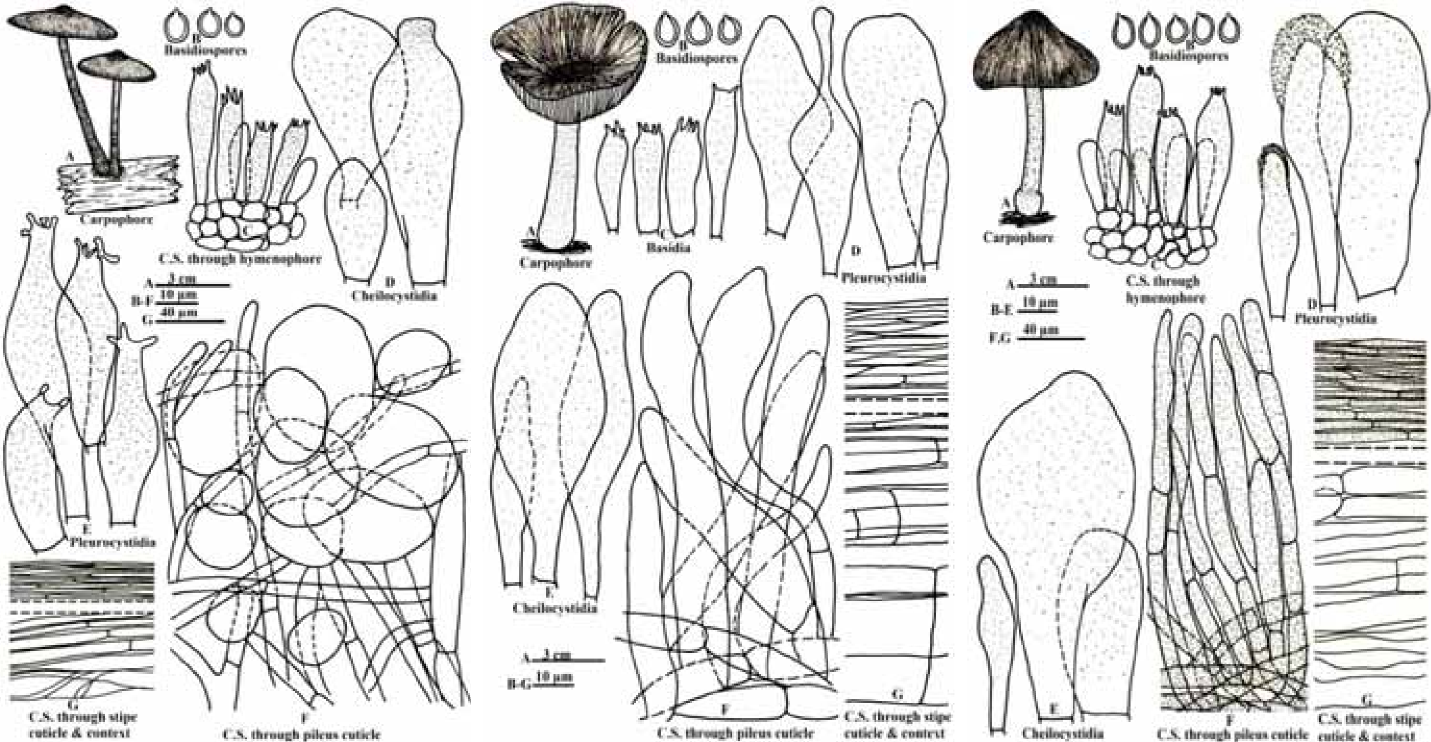


**Figure 3.** *Pluteus transitus* sp. nov. **Figure 4.** *Pluteus calvitio-stipus* sp. nov. **Figure 5.** *Pluteus ortonii* sp. nov.

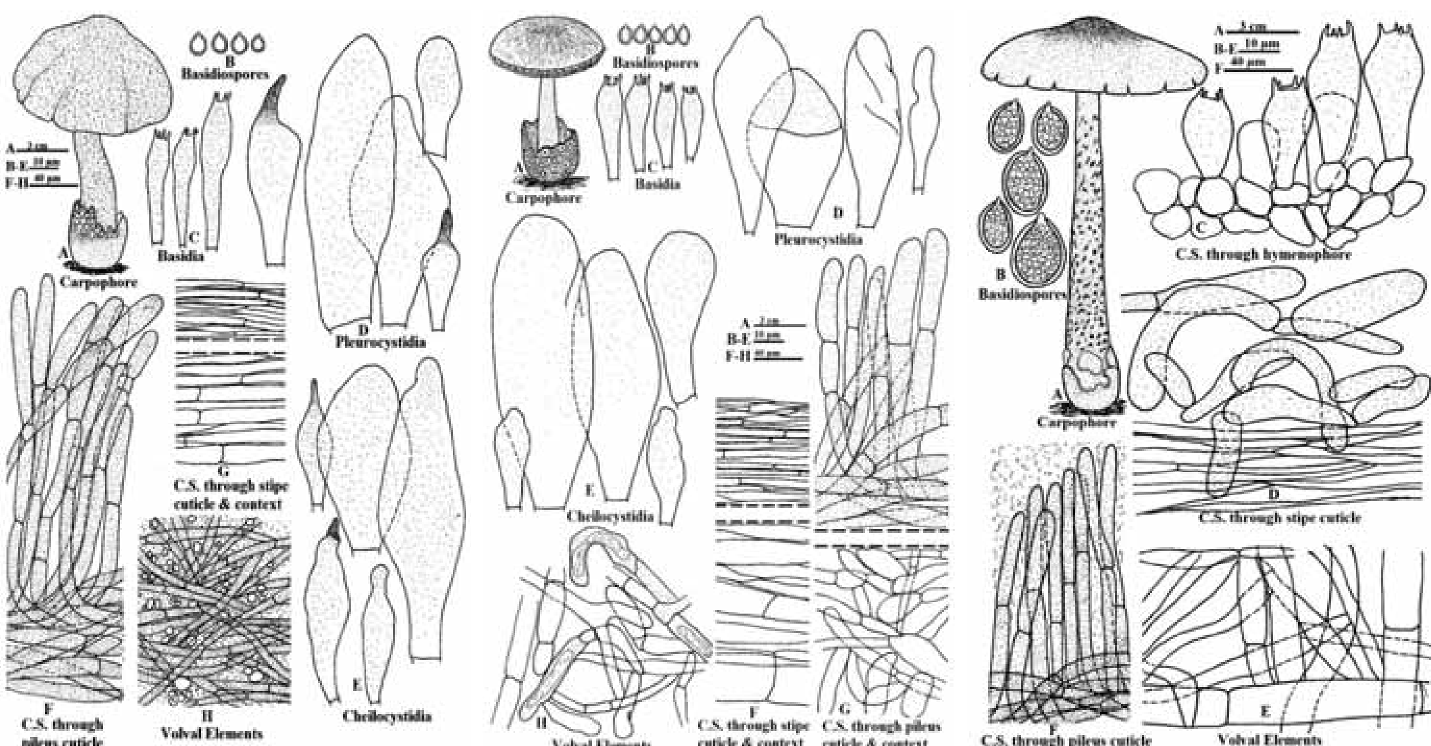


**Figure 6.** *Volvariella bumelia* sp. nov. **Figure 7.** *Volvariella albida* sp. nov. **Figure 8.** *Volvopluteus shafferii* sp. nov.

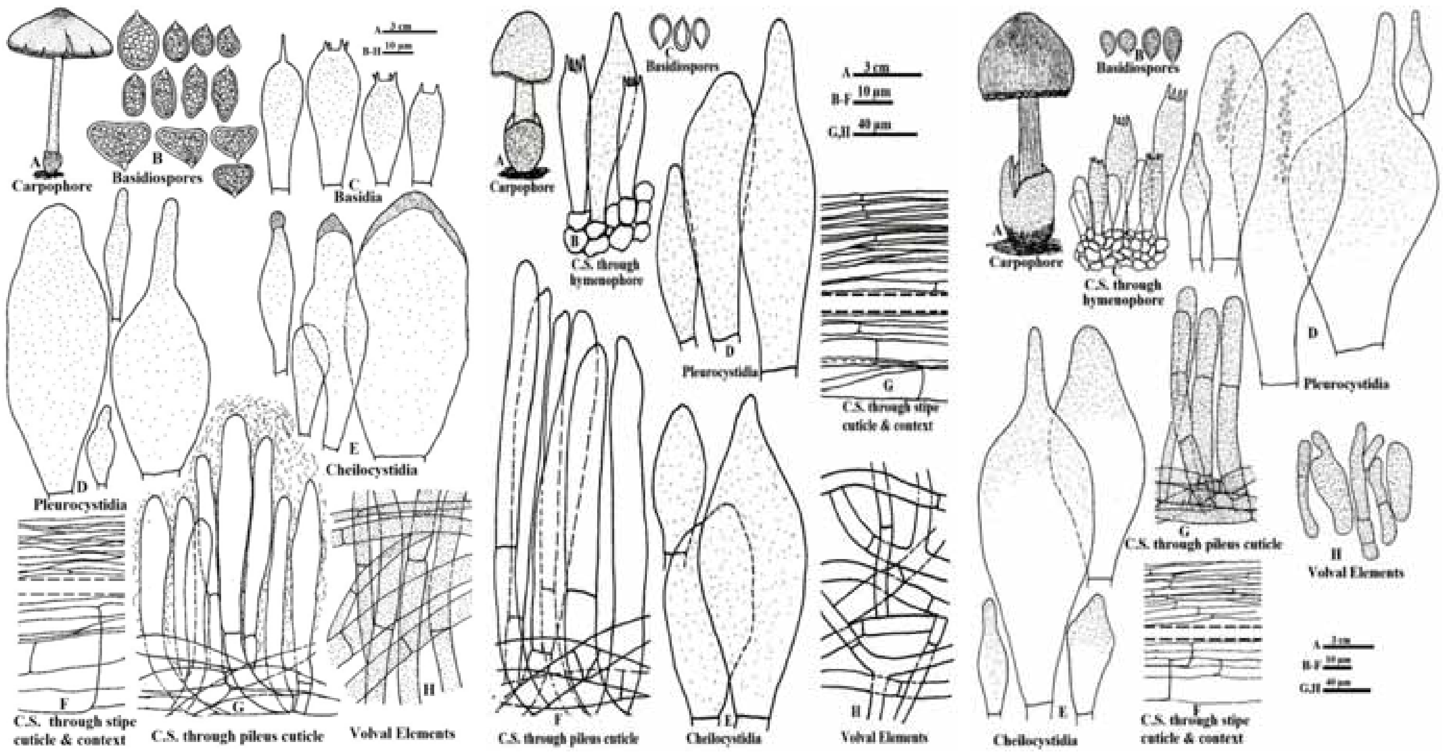


**Figure 9.** *Volvopluteus diversisporus* sp. nov.

**Figure 10.** *Volvariella bombycina* (Schaeff. : Fr.) Sing. var. *parva* var. nov.

**Figure 11.** *Volvariella bombycina* (Schaeff. : Fr.) Sing. var. *terricola* var. nov.

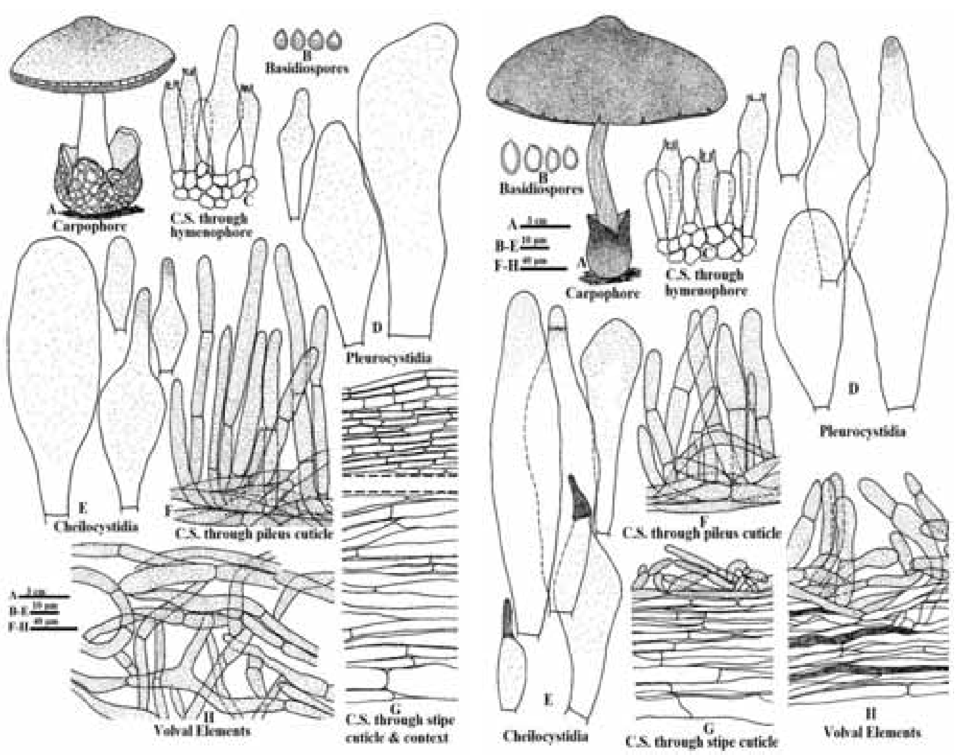


**Figure 12.** *Volvariella terastia* (Berk.& Br.) Sing. var. *magna-cystidiata* var. nov.

**Figure 13.** *Volvariella volvacea* (Bull.: Fr.) Sing. var. *lignicola* var. nov.

During fungal forays to different localities of North-Western India 10 taxa were recorded for the first time from India viz. *Pluteus dryophiloides* P.D. Orton (PUN 6447), *Pluteus ephebeus* (Fr.) Gillet (PUN 6445, PUN 6446), *Volvariella volvacea* var. *nigricans* Kawam. ex Hongo (PUN 6476), *Volvariella volvacea* var. *masseei* Singer & Wasser (PUN 6473), *Volvopluteus earlei* f. *acystidiatus* (N.C. Pathak) Vizzini & Contu. (PUN 6491), *Volvariella jamaicensis* (Murr.) Shaff. (PUN 6465), *Volvariella nullicystidiata* Menolli & Capelari (PUN 6474), *Volvariella lepiotospora* Singer (PUN 6472), *Volvariella peckii* (G.F. Atk.)Shaffer (PUN 6470), *Volvariella bakeri* (Murr.) Shaff. (PUN 4025), *Volvopluteus medius* (Schumach.) comb.nov. (PUN 6490).

## CONCLUSION

Earlier from India 28 taxa of *Volvariella*, 24 taxa of *Pluteus* and 2 taxa of *Volvopluteus* are recorded till date (Pradeep & Vrinda [19-21, 23, 24, 26, 32-34]. As a result of the present work 3 species of *Pluteus*, 2 species each of *Volvariella* and *Volvopluteus* are introduced as new to science, while 4 varieties of *Volvariella* are described as new to science. 6 species of *Volvariella* and 2 species each of *Pluteus* and *Volvopluteus* are recorded for the first time from India. Although there is no clear cut test for determining the edibility of mushrooms however, investigated taxa were screened into edible and inedible types based upon the information gathered from the field and available literature. In the study area *Volvariella bombycina, V. diplasia*, *V. volvacea* and *Volvopluteus gloiocephalus* are the commonly hunted mushrooms from the wild for human consumption. These species are also being cultivated commercially and have an established edibility and nutraceutical potential as confirmed from the literature. Sometimes the edible species like *Volvariella volvacea* causes gastrointestinal disorders in some people. Most of *Volvariella* and *Volvopluteus* species were found growing on grasses in the rainy season. *Volvariella bakeri*, *V. bombycina*, *V. diplasia*, *V. terastia*, *V. volvacea* var. *nigricans*, *Volvopluteus gloiocephalus* were edible taxa as reported in literature and of the 15 taxa reported to form putative ectomycorrhizal associations 10 were recorded for the first time in putative ectomycorrhizal association. The wild relatives Pleutoid mushrooms are of common occurrence in North Western India. From the surveys it has become apparent that that there is enough wild germplasm for utilization in strain improvement programme in paddy straw mushroom in study area. Various available wild species of *Volvariella,* namely *V. bakeri, V. terastia, V. taylorii* and *V. cubensis,* etc. possesses acceptable agronomic features with possibilities of introduction into cultivated commercial strains through breeding experiments.

## AKNOWLEDGEMENTS

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# DIVERSITY OF MACROFUNGAL COMMUNITIES IN CHIKMAGALUR DISTRICT OF WESTERN GHATS, INDIA

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## ABSTRACT

Macrofungi form an integral part of all forest systems. The main goal of the present study is to expand the baseline database for macrofungal documentation, diversity and its distribution in the Western Ghats of Chikmagalur district for establishing an inventory of the macrofungi species.Twenty transects each measuring 50 x 20 m were laid in sampling stations of Chikmagalur district. The study sites were selected randomly and macrofungi were collected during 2007 to 2011. A total of 6,950 sporomas were collected. The relationship between macrofungal species richness and sampling plots was analyzed using linear regression. Dacrymycetaceae, Ganodermataceae, Polyporaceae and Russulaceae were found to be the most dominant families during study. *Armillaria, Calocera, Ganoderma, Panaeolus*, *Polyporus, Psathyrella* and *Russula* were the most dominating genera during five years study (2007-11). *Calocera viscosa, Ganoderma carnosum*, *Panaeolus fimicola*, *Psathyrella candolleana, Russulaatro purpurea*, *Scutellinia erinaceus, Termitomyces tylerianus* and *Trametes hirsuta* were the most predominating species. The Shannon and Simpson diversity indices were found to be highest during 2007 (H'=4.35; D=0.031), respectively.

**Keywords:** epigeous macromycetes, sporoma, diversity, dominance

## INTRODUCTION

According to Hawksworth [1], the estimated number of fungal species worldwide is 1.5 million and less than 5% have been described. The number of fungi recorded in India exceeds 27,000 species and forms the largest biotic community after insects [2]. Fungi of various taxonomic groups producing conspicuous sporocarps are collectively known as macrofungi which include gilled fungi, jelly fungi, coral fungi, stinkhorns, bracket fungi, puffballs, and bird's nest fungi [3]. They grow prolifically and are found in many parts of the world [4]. Macrofungi studies are of interest to scientists due to their important role in food industry, in medicinally effective products and in biodegradation [5]. Despite their great ecological significance, the community ecology, biogeography, and conservation status of macrofungi are poorly known. In Karnataka, knowledge about diversity, abundance, fruiting pattern of mushroom and their ecology is scarce and only documentation has been done. Ecological studies of macrofungi based on sporocarps are hampered by the erratic nature of fruiting and the short duration of sporocarps.The present work has been undertaken to study the diversity of macrofungi in Chikmagalur district of Western Ghats. The climatic condition and humus formation favours the luxuriant growth of macrofungi in the study area.

## MATERIALS AND METHODS

### Study area

The study was conducted in Chikmagalur district, Karnataka, India, a part of naturally rich biodiversity, situated between 12°54'42'' and 13°53'53'' North latitude and 75°04'46'' and 76°21'50'' East longitude. The geographical area of the districtis 7201 sq. km with a forest area of 200485 ha. The average annual rainfall in the district is 1762 mm. The annual mean air temperature is 17 °C to 20 °C in winter and 32 °C to 35 °C in summer [6].

### Survey

Macrofungi were identified by the presence-absence for sporomas and were limited to epigeous macromycetes of soil and wood-inhabiting macrofungi that were visible to the naked eye (>1mm). Survey can be performed just after the rain [7].

The difficulty was that only small proportions of macrofungi were visible on a single visit. Hence repeated surveys were done in all sampling plots during January 2007 to December 2011.

**Sampling**

The fruiting phenology of macrofungi varies all through the year, depending on temperature, relative humidity, different altitudes [8] and regions. To circumvent this, study sites were plotted in Chikmagalur district. Twenty transects were laid, each measuring 50 x 20 m. The study sites were selected randomly and macrofungi collected within transects and characterized for further analysis. The geographic coordinates of each study sites were integrated into ArcGIS9.2 software to generate a study map for spatial distribution of sampling locations in Chikmagalur district. The readings of latitude/longitude were taken using handheld GPS (Garmin GPSMAP® 76CSx).

**Collection**

Fresh specimens were collected with great care without any damage and soil debris was removed using a soft brush. Wood inhabiting macrofungi were collected along with the substratum [9]. The habitat and morphological characteristics of the macrofungi were noted [10] and photographed using NIKON D60 digital SLR camera for further diagnosis during the collection [11]. Fleshy sporomas were wrapped in waxed paper or newspaper [12] and labelled with the collection number.

**Drying and preservation**

The sporomas were dried in air-vented mushroom drier. The dehydrated collections were removed depending upon the delicate nature of specimens. Tough and pliable collections were kept for longer durations for drying and the dehydrated specimens were sealed in polythene covers containing napthalene balls which prevent the attack of mites and insects damage. The sealed sporomas were labelled with collection code and date of collection. The representative specimens were deposited in the Department of Applied Botany, Kuvempu University, Shankaraghatta, Karnataka.

**Characterization**

Macrofungi were assessed for the presence/absence of several morphological characters such as volva, annulus, gills/ pores, peridium and gleba. Accurate and consistent notation of sporomas, colour, including colour changes of mature sporomas and colours of different development stages were noted [13-18].

**Morphological characters**

Every morphological feature (basidiocarp, basidia, basidiospores, cystidia, ascocarp, asci, ascospores) has a taxonomic significance at different levels of taxonomic groups in classification. Colour notations were described from Kornerup and Wanscher [19]. They were identified in the laboratory using morphological features, appropriate keys and monographs. Taxonomic classification of species is zone according to Kirk *et al*. [20] and fungal nomenclature is based on Mycobank (http://www.mycobank.org/Biolomics.aspx? Table=Mycobank&Page =200&ViewMode=Basic).

**Microscopical characters**

The species were also identified based on observations of microscopic structures. The sporomas were cut into small fragments for free hand sectioning using a razor blade. The microscopic characters such as size, shape, ornamentation, clamp-connections were clearly observed for basidia, cystidia, asci, basidio/asco spores, capillitium, and exo/endoperedial elements. All the observations of the microscopic structures were made under an oil-immersion objective with an Olympus CX31 microscope. Microscopic measurements were calculated using calibration (with ocular and stage micrometer).

**Data analysis**

Qualitative analysis entails determining three attributes of the species in the community: density (number of sporomas per unit area), which is a measure of the numerical individuals relative to species; frequency (how many samples contain sporomas of a given species), which measures the commonness of the species and dominance, the incidence at which the species occurs most in the sampling area [21].

**Density**

Density is an expression of the numerical strength of a species where the total number of sporomas of each species in all transects is divided by the total number of transect studied. Density was calculated as follows

Density (De) = Total number of sporomas in all quadrats/ Total number of transect studied

**Dominance and Abundance**

Dominance (D) for genera and family were estimated by the highest number of species and species dominance was estimated by highest number of total sporomas. Abundance was estimated by number of sporomas of different species in the community per unit area. Sampling was done randomly at several places within transects and the number of sporomas of each species is summed for all transects divided by the total number of transects in which the species occurred. Dominance is represented by the equation

Abundance (A) = Total number of sporomas in all transects/ Number of transects of occurrence

**Frequency**

The term frequency refers to the degree of dispersion of individual species in an area and usually expressed in terms of percentage occurrence. Sampling was done randomly in the study area at several places within transects and recorded the name of sporomas that occurs in each sampling units. It is calculated by the formula.

Frequency (F) = Number of transects of species occurrence/Total number of transects studied

**Alpha Diversity**

Shannon-Weiner's diversity index, a measure of richness and evenness [22] and Simpson's diversity index of dominance [23] were measured for each year in Chikmagalur district and also each for the complete district.

Shannon-Weiner diversity Index = $H' = -\Sigma (pi \times \ln pi)$

Simpson's Diversity Index = $D = \Sigma \frac{ni\,(ni-1)}{N\,(N-1)}$

Where, 'i$^{th}$' species = one of all the enumerated species

Pi = the proportion of the 'i$^{th}$' species = (ni/N)

ni = number of individuals of the 'i$^{th}$' species

N = total number of individuals.

**Regression Analysis**

Regression analysis was determined through the statistical software (Minitab 15) in Chikmagalur district to discern whether the sampling plots influenced the species richness of sporomas. Transects are equally efficient in sampling macrofungi from a given sampling plots. Alternatively, macrofungi were strongly dispersal limited, they were sampled in a limited transects. Similarly, the dispersion-limitation predicted more species to develop in a restricted transect. The analysis was run separately

for each year of 5 year study (2007-2011) in overall Chikmagalur district. The statistical significance of the included variables was determined by R-Sq test with 0.05% significance as a criterion.

### Principle Component Analysis

Principle component analysis was peformed on top 20 abundant species from four substrates (coprophyllous, folicolous, lignicolous and terricolous). The scores of first two components from the PCAs were used to compare differences of the macrofungal communities among the four substrates. The software Xlstat 13 was used to perform the statistical analysis.

### Pearson Correlation Coefficient

The species richness of each family and genera of Chikmagalur district was subjected to analysis using Microsoft Excel 2007. The analysis was performed in terms of least significant difference ($LSD_{0.05}$) in species richness of each family and genera among the total rainfall.

## RESULTS

### Sporoma

A total of 6950 sporomas were collected in Chikamagalur district during 2007-2011 and maximum productivity was recorded during 2007 with 1832 individuals followed by 1620 individuals during 2009, 1404 during 2008 and 1175 in the year 2010. Lowest sporomas were encountered during last year of study (2011) with 905 individuals (Fig. 1)

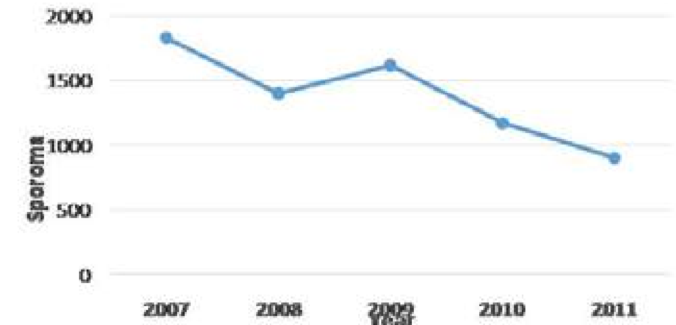


**Figure 1.** Total sporoma in Chikmagalur district during 2007-2011

### Families

A total of 29 families were enumerated from district during 2007-11. Species of 29 families were recorded during first three consecutive years (2007-2009) accounting for highest families followed by 26 and 24 families during 2010 and 2011 respectively (Fig. 2).

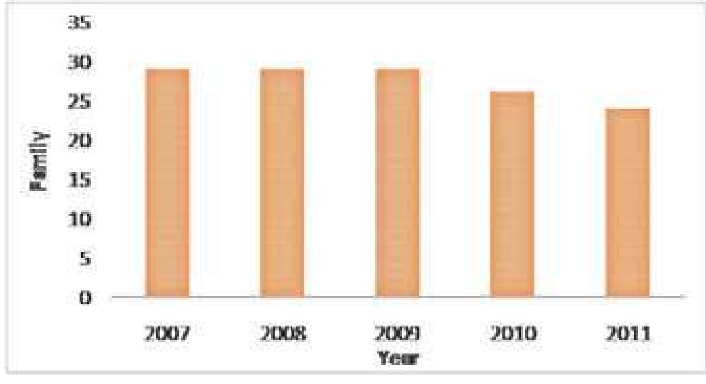


**Figure 2.** Total families occurring in Chikmagalur district during 2007-11

### Genera

A total of 64 genera were encountered during 2007-11. Sixty genera were encountered during 2009 accounting for highest and 59 in 2007 followed by 56, 52 and 48 genera during 2008, 2010 and 2011, respectively (Fig. 3).

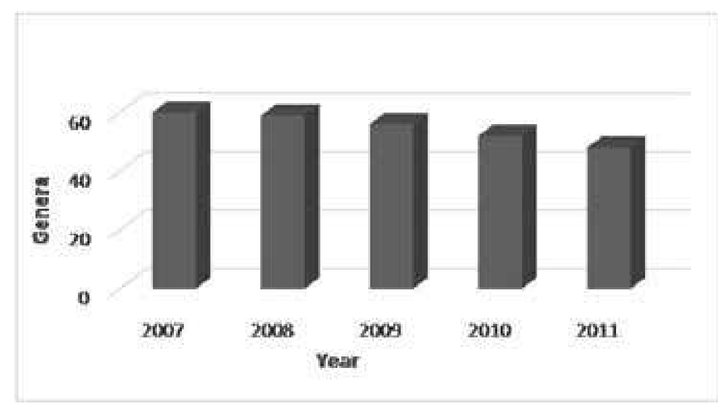


**Figure 3.** Total genera occurred in Chikmagalur district during 2007-2011

### Morpho groups

Fleshy gilled fungi ranked first with 61% followed by bracket fungi (18%) and coral fungi (6%) (Fig. 4).

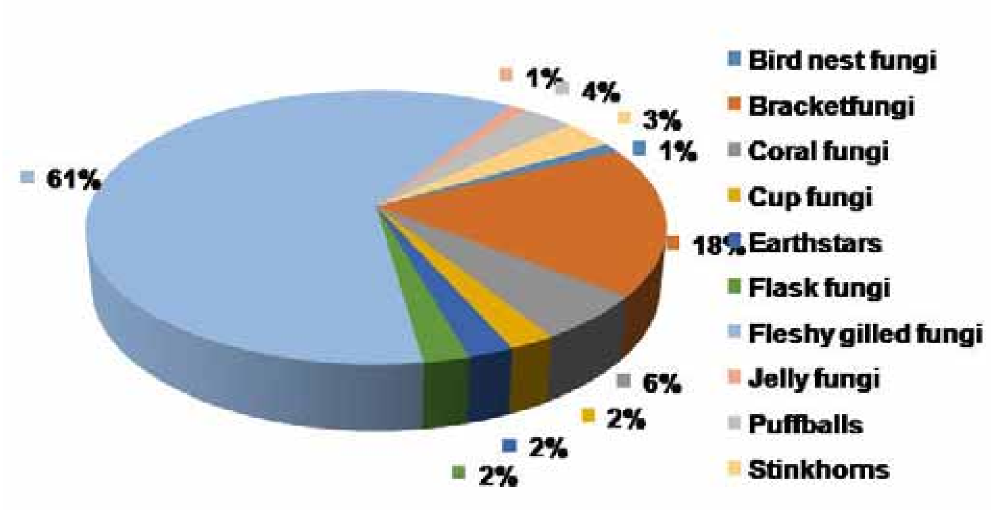


**Figure 4.** Macrofungalmorpho-group percentage of Chikmagalur district

## Statistical analysis

### Macrofungal species richness versus sampling plots

The relationship between macrofungal species richness and sampling plots of Chikmagalur district was analyzed using linear regression. The pooled data of five years were considered for analysis in Chikmagalur district. The linear regression plots indicated a strong positive association between macrofungal species richness and sampling plots The linear regression plots for macrofungal species richness versus sampling plots were analyzed for Chikmagalur district. Positive correlation was observed and found significant in the study area.

**Table 1.** Results of Linear regression analysis relating macrofungal species richness versus sampling plots surveyed in Chikmagalur district

| Study area | *p* | $R^2$ |
|---|---|---|
| Chikmagalur district | <0.000*** | 0.214 |

$p < 0.05$* $p < 0.01$** $p < 0.0001$***

**Table 2.** Results of Linear regression analysis relating macrofungal species richness versus sampling plots surveyed in Chikmagalur district

| Study area | *p* | $R^2$ |
|---|---|---|
| 2007 | <0.000*** | 0.77 |
| 2008 | <0.000*** | 0.76 |
| 2009 | <0.000*** | 0.72 |
| 2010 | <0.000*** | 0.78 |
| 2011 | <0.000*** | 0.72 |

A total of 90 species occurred with 6950 sporomas in 5 years from 2007-2011 in Chikmagalur district. The linear regression plots of macrofungal species richness versus sampling plots was generated. Significant positive relationship was observed between the components in sampling plots of Chikmagalur district (Fig. 5).

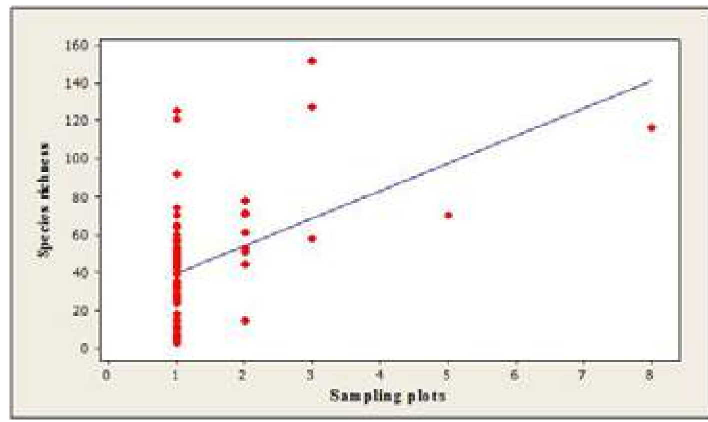


**Figure 5.** Correlation of macrofungal species richness versus sampling plots

## Density

### Family density

In Chikmagalur district, Agaricaceae was found to be the densest family during all the five years of study (2007-2011). During five years study, Agaricaceae ranked first followed by Psathyrellaceae being second and Polyporaceae third denser families in the study sites (Fig. 6).

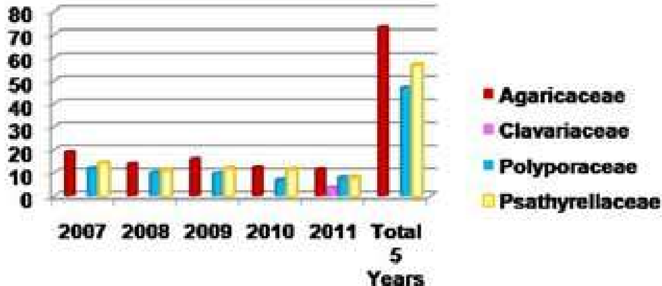


**Figure 6.** Dense families of macrofungi in Chikmagalur district (2007-2011)

### Genera density

In Chikmagalur district, *Psathyrella* ranked first during 2007 and *Coprinellus* was most densest for four years during study (2008, 2009, 2010, 2011). During five years study, *Coprinellus*, *Psathyrella* and *Polyporus* ranked first, second and third denser genera respectively in the study sites (Fig. 7).

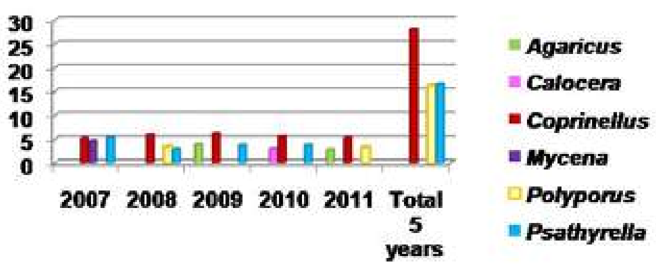


**Figure 7.** Dense genera of macrofungi in Chikmagalur district (2007-2011)

### Species Density

In Chikmagalur district, *Psathyrella candolleana* (2007), *Cyathus striatus* (2008), *Microporus xanthopus* (2008), *Coprinellus disseminatus* (2009, 2010, 2011) and *Calocera viscosa* (2010) were found to be most dense species during respective years. During five years study, *Coprinellus disseminatus* emerged as first denser species with *Cyathus striatus* and *Calocera viscosa* being second and third denser species in the study sites of Chikm galur district (Fig. 8).

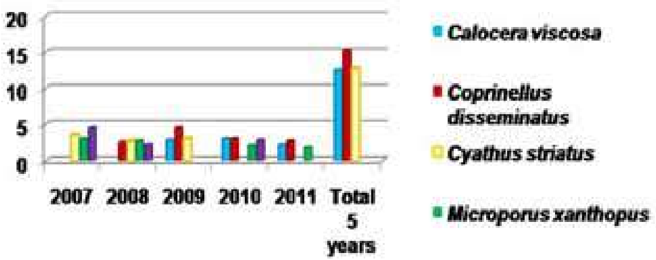


**Figure 8.** Dense species of macrofungi in Chikmagalur district (2007-2011)

## Abundance

### Family abundance

In Chikmagalur district, Dacrymycetaceae was found to be the most abundant family during 2007, 2008, 2009, 2010 and 2011. Dacrymycetaceae came forth as most abundant followed by Agaricaceae and Psathyrellaceae during five years study (Fig. 9).

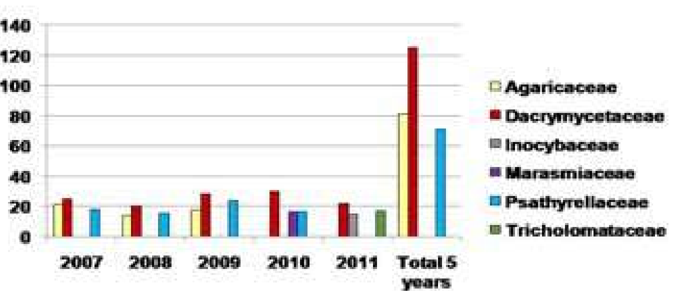


**Figure 9.** Abundant families of macrofungi in Chikmagalur district (2007-2011)

### Genera Abundance

In Chikmagalur district, *Psathyrella* (2007), *Armillaria* (2008) and *Calocera* (2008, 2009, 2010, 2011) were found to be the most abundant genera during respective years. *Calocera*, *Armillaria* and *Psathyrella* ranked first, second and third most abundant genera in the study sites (Fig. 10).

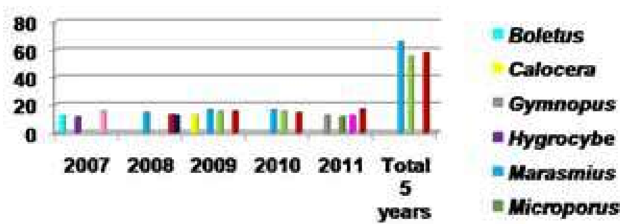


**Figure 10.** Abundant genera of macrofungi in Chikmagalur district (2007-2011)

## Frequency

### Family frequency

Polyporaceae ranked first during all the five years (2007-2011) and Agaricaceae shared first position during 2008 in the study sites of Chikmagalur district (Fig 11).

### Genera frequency

Frequent genera of macrofungi occurring in Chikmagalur district was evaluated during 2007-2011. In Chikmagalur district, *Microporus* was found to be the most frequent genera during all the five years (Table 3).

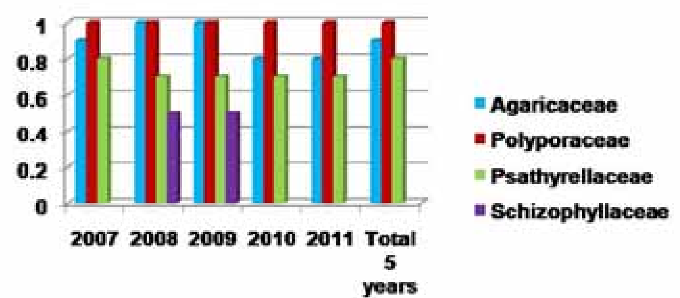


**Figure 11.** Frequent families of macrofungi in Chikmagalur district (2007-2011)

**Table 3.** Genera frequency of macrofungi in Chikmagalur district (2007-2011)

| Genera | Frequency | | | | | |
|---|---|---|---|---|---|---|
| | 2007 | 2008 | 2009 | 2010 | 2011 | Total |
| *Abortiporus* | | | 0.1 | 0.1 | | 0.1 |
| *Agaricus* | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.4 |
| *Antrodia* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Armillaria* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Bjerkandera* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Bovista* | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| *Calocera* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Cantharellus* | 0.1 | 0.1 | 0.1 | | | 0.1 |
| *Chlorophyllum* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Clavaria* | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 |
| *Clavulinopsis* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Clitocybe* | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 |
| *Collybia* | 0.1 | | 0.1 | | | 0.1 |
| *Cookeina* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Coprinellus* | 0.5 | 0.4 | 0.5 | 0.5 | 0.5 | 0.5 |
| *Coprinopsis* | 0.1 | | | | | 0.1 |
| *Coprinus* | | | 0.1 | | | 0.1 |
| *Coriolopsis* | 0.1 | 0.1 | 0.1 | | 0.1 | 0.1 |
| *Crinipellis* | 0.1 | 0.1 | 0.1 | | | 0.1 |
| *Cyathus* | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| *Daldinia* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Fomitopsis* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Ganoderma* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Geastrum* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Gloeoporus* | | | 0.1 | 0.1 | | 0.1 |
| *Gymnopus* | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 |
| *Hemimycena* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Hygrocybe* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Hypholoma* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Laccaria* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Lentinus* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Lepiota* | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 |

| Genera | Frequency | | | | | |
|---|---|---|---|---|---|---|
| | 2007 | 2008 | 2009 | 2010 | 2011 | Total |
| *Leucoagaricus* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Leucocoprinus* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Lycoperdon* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Lysurus* | 0.1 | 0.1 | 0.1 | 0.1 | | 0.1 |
| *Macrolepiota* | 0.1 | 0.2 | 0.1 | | | 0.2 |
| *Marasmius* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Melanoleuca* | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.2 |
| *Microporus* | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| *Mycena* | 0.3 | 0.3 | 0.3 | 0.3 | 0.1 | 0.3 |
| *Oudemansiella* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Panaeolus* | 0.3 | 0.3 | 0.1 | 0.3 | 0.3 | 0.3 |
| *Paxillus* | 0.1 | 0.1 | | 0.1 | | 0.1 |
| *Phallus* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Pleurotus* | 0.1 | 0.1 | 0.1 | 0.1 | | 0.1 |
| *Pluteus* | 0.1 | 0.1 | 0.1 | 0.4 | | 0.1 |
| *Polyporus* | 0.4 | 0.4 | 0.4 | | 0.4 | 0.4 |
| *Porodaedalea* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Psathyrella* | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 |
| *Pycnoporellus* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Pycnoporus* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Ramaria* | 0.1 | 0.1 | 0.1 | 0.1 | | 0.1 |
| *Rhodocybe* | | | 0.1 | | | 0.1 |
| *Schizophyllum* | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| *Scutellinia* | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| *Termitomyces* | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 | 0.3 |
| *Trametes* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Tricholomella* | 0.2 | | | 0.1 | 0.1 | 0.1 |
| *Tubaria* | 0.1 | 0.1 | 0.1 | | 0.2 | 0.2 |
| *Vascellum* | 0.1 | 0.1 | 0.1 | | | 0.1 |
| *Xeromphalina* | 0.1 | | | | | 0.1 |
| *Xerula* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *Xylaria* | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

## Species frequency

*Microporus xanthopus* ranked first during five years study (2007-2011) and in total five years in the study sites of Chikmagalur district (Fig. 12).

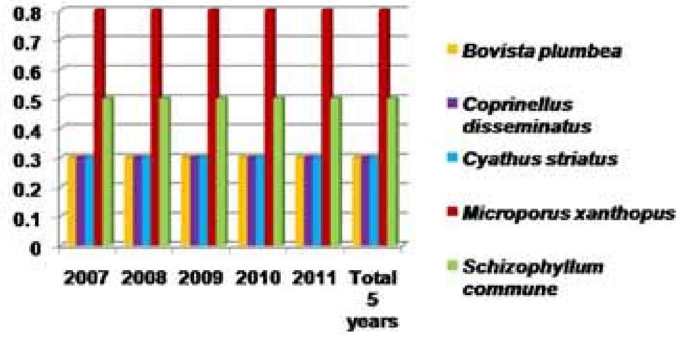


**Figure 12.** Frequent species of macrofungi in Chikmagalur district (2007-2011)

## Dominance

### Family dominance

Dacrymycetaceae, Ganodermataceae, Polyporaceae and Russulaceae comprised of highest species accounting for dominant family in Chikmagalur district during each year from 2007-11.

## Genera dominance

In Chikmagalur district, *Armillaria, Calocera,Ganoderma, Panaeolus*, *Polyporus,Psathyrella* and *Russula* were found to be dominant during the study period.

## Species dominance

In Chikmagalur district, *Psathyrella candolleana* accounted for most dominant species in for first two years during 2007 and 2008. In the next three years, *Calocera viscosa* became the most dominant species in the next three years during 2009, 2010 and 2011(Fig. 13).

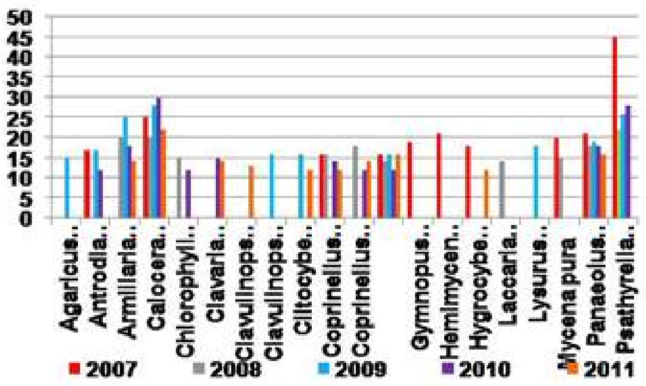


**Figure 13.** Dominant species of macrofungi in Chikmagalur district (2007-2011)

## Species richness among trophic groups of macro fungi

In Chikmagalur district, *Coprinellus disseminatus* (CDI) featured highest and *Polyporus arcularius* (POR) was found to be lowest among represented taxa in lignicolous. *Cyathus striatus* (CYS) and *Bovista plumbea* (BPL) were prominant with former being highest and latter being lowest among represented variables in terricolous. *Marasmius praecox* (MPR) was the only species among foliicolous during five years study (Fig. 14).

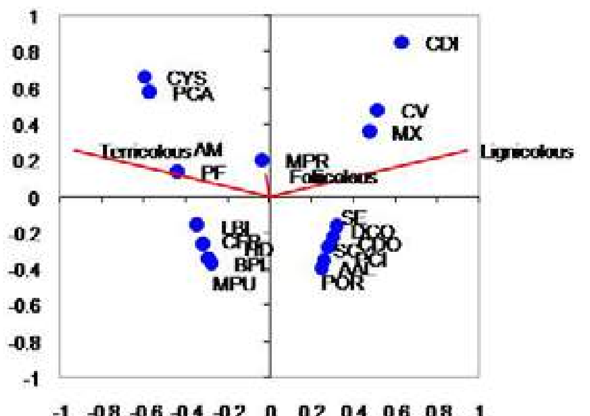


**Figure 14.** Species richness among trophic groups of macrofungi in Chikmagalur district (2007-2011)

## Shannon-Weiner's diversity index

Shannon Weiner's diversity index of Chikmagalur district was found to be 4.35, 4.18, 4.32, 4.14, 4.01 during 2007, 2008, 2009, 2010 and 2011 respectively. Diversity reached its peak in the year 2007 (H'= 4.35). The Simpson diversity index was calculated and diversity was found to be more during 2007 (D= 0.015).

## Simpson's diversity index

Simpson diversity index was calculated for Chikmagalur district for five years study. The index value was highest during 2007 (D= 0.031) showing maximum diversity followed by 2008-09, 2010 (D= 0.032) and 2011 (D= 0.033).

## Influence of rainfall on macrofungal species richness

Three species in the study sites of Chikmagalur district were found to be significant with rainfall (Table 4).

## DISCUSSION

The status and diversity of Chikmagalur was found to be found to be maximum during 2007-2011. We found that environmental factors like light, temperature and relative humidity greatly influence the growth and development of macrofungi. The reduction of the macrofungi sporulation occurs mainly due to reduction in rainfall and fruiting diversity depends on the tree populations. This diversity is due to luxuriant substrate humus and relative humidity in the study area. The Shannon diversity indices were found to be more in 2007 and gradually decreased over the years. The simpson diversity index increased with the value of 0.02 in five years. The family Agaricaceae, Polyporaceae and Psathyrellaceae was found to be dominant in all the five years (2005 to 2010). Our study revealed the loss of 29 species in Chikmagalur district. The

**Table 4.** Pearson correlation coefficient of macrofungal families in Chikmagalur district

| Sl. No. | Family | Chikmagalur district | Sl. No. | Family | Chikmagalur district |
|---|---|---|---|---|---|
| 1. | Agaricaceae | 0.98293** | 16. | Meruliaceae | 0.56429 |
| 2. | Cantharellaceae | 0.80566* | 17. | Mycenaceae | 0.86543 |
| 3. | Clavariaceae | 0.84650 | 18. | Paxillaceae | 0.97086 |
| 4. | Dacrymycetaceae | -0.0337 | 19. | Phallaceae | -0.36194 |
| 5. | Entolomataceae | 0.13200 | 20. | Physalacriaceae | -0.09704 |
| 6. | Fomitopsidaceae | 0.86416 | 21. | Pleurotaceae | -0.29383 |
| 7. | Ganodermataceae | 0.24364 | 22. | Pluteaceae | 0.16192 |
| 8. | Geastraceae | 0.83430 | 23. | Polyporaceae | 0.93821* |
| 9. | Gomphaceae | 0.80130 | 24. | Psathyrellaceae | 0.49103 |
| 10. | Hydnangiaceae | 0.79664 | 25. | Pyronemataceae | -0.02812 |
| 11. | Hygrophoraceae | 0.69342 | 26. | Sarcoscyphaceae | 0.91168* |
| 12. | Hymenochaetaceae | 0.86998 | 27. | Schizophyllaceae | 0.23390 |
| 13. | Inocybaceae | -0.09872 | 28. | Strophariaceae | 0.70525 |
| 14. | Lyophyllaceae | 0.93450* | 29. | Tricholomataceae | 0.79695 |
| 15. | Marasmiaceae | 0.97149* | 30. | | |

* Significant 0.05 % ; ** Significant 0.01 %

**Table 5.** Pearson correlation coefficient of macrofungal genera in Chikmagalur district

| Sl. No. | Genera | Chikmagalur district | Sl. No. | Genera | Chikmagalur district |
|---|---|---|---|---|---|
| 1. | *Abortiporus* | 1 | 33. | *Leucoagaricus* | 0.75823 |
| 2. | *Agaricus* | 0.49486 | 34. | *Leucocoprinus* | 0.58629 |
| 3. | *Antrodia* | 0.76826 | 35. | *Lycoperdon* | -0.14098 |
| 4. | *Armillaria* | -0.02461 | 36. | *Lysurus* | -0.16284 |
| 5. | *Bjerkandera* | 0.65707 | 37. | *Macrolepiota* | 0.80566* |
| 6. | *Bovista* | 0.42256 | 38. | *Marasmius* | 0.79787 |
| 7. | *Calocera* | -0.0337 | 39. | *Melanoleuca* | 0.64065 |
| 8. | *Cantharellus* | 0.80566* | 40. | *Microporus* | 0.75593 |
| 9. | *Chlorophyllum* | -0.04012 | 41. | *Mycena* | 0.79645 |
| 10. | *Clavaria* | 0.71219 | 42. | *Oudemansiella* | 0.22030 |
| 11. | *Clavulinopsis* | 0.26085 | 43. | *Panaeolus* | 0.82919 |
| 12. | *Clitocybe* | 0.81787 | 44. | *Paxillus* | 0.97086 |
| 13. | *Collybia* | -1 | 45. | *Phallus* | -0.53134 |
| 14. | *Cookeina* | 0.91168* | 46. | *Pleurotus* | -0.29383 |
| 15. | *Coprinellus* | -0.19256 | 47. | *Pluteus* | 0.161929 |
| 16. | *Coprinopsis* | 0.89097* | 48. | *Polyporus* | 0.150434 |
| 17. | *Coprinus* | 0.13200 | 49. | *Porodaedalea* | 0.869982 |
| 18. | *Coriolopsis* | 0.94279* | 50. | *Psathyrella* | 0.743252 |
| 19. | *Crinipellis* | 0.97471** | 51. | *Pycnoporellus* | 0.144552 |
| 20. | *Cyathus* | 0.90613* | 52. | *Pycnoporus* | 0.834348 |
| 21. | *Daldinia* | 0.54663 | 53. | *Ramaria* | 0.801306 |
| 22. | *Fomitopsis* | 0.14329 | 54. | *Rhodocybe* | 0.132004 |
| 23. | *Ganoderma* | 0.24364 | 55. | *Schizophyllum* | 0.233907 |
| 24. | *Geastrum* | 0.83430 | 56. | *Scutellinia* | 0.638913 |

| Sl. No. | Genera | Chikmagalur district | Sl. No. | Genera | Chikmagalur district |
|---|---|---|---|---|---|
| 25. | *Gloeoporus* | -1 | 57. | *Termitomyces* | 0.942689* |
| 26. | *Gymnopus* | 0.82989 | 58. | *Trametes* | 0.929818* |
| 27. | *Hemimycena* | 0.85424 | 59. | *Tricholomella* | 0.538428 |
| 28. | *Hygrocybe* | 0.69342 | 60. | *Tubaria* | -0.09872 |
| 29. | *Hypholoma* | 0.70525 | 61. | *Vascellum* | 0.997461*** |
| 30. | *Laccaria* | 0.79664 | 62. | *Xeromphalina* | 0.890978* |
| 31. | *Lentinus* | 0.19310 | 63. | *Xerula* | -0.41409 |
| 32. | *Lepiota* | 0.83328 | 64. | *Xylaria* | -0.54664 |

* Significant 0.05 %; ** Significant 0.01 %; *** Significant 0.001 %

development in *Dictyophora cinnabarina* was found to be abnormal with the reduction in height of the sporocarp, length of inducium and many eggs did not open to give rise to receptacle. The species of *Xylaria polymorpha, Stereum ostrea, Daldina concentrica, Pycnocarpus cinnabarinus*, *Termitomyces* sp. and *Daedelopsis* sp. were observed because of climatic variation. Some macrofungi were found to be medicinally important (*Ganoderma lucidum*, *Cordyceps* sp.) few were edible (*Pleurotus*, *Termitomyces*) and some were poisonous. Some were found to be new to India. The culturing and chemical constituents of macrofungi investigation is under progress.

The present study was conducted from January 2007 to December 2011, is constituted first systematically. Long-term monitoring in Karnataka, although there were differences in species richness. The present study noticed species richness as well as number of sporocarps varied between years. The decrease in number of species and sporocarps from 2007 and 2011 may be due to a variation of weather conditions. A major problem for the accurate definition of macrofungal communities in a particular site is related to sporocarp production depending on weather conditions and the sporadic fruiting of species [24]. Finding species to be deleted in every subsequent sampling year from 2007 to 2011 is in agreement with other studies that recommended long-term monitoring to assess fungal species [25]. Highest fruiting bodies recorded (species and sporocarps) during the rainy season (July to October) and some fruiting in winter (November to February). This together with the correlation analysis, suggests that fruiting phenology of macrofungal species is directly related with weather conditions, especially with rainfall and relative humidity.

## CONCLUSION

The present study conducted from January 2007 to December 2011, is first systematically constituted, long-term monitoring of macrofungi in Chikmagalur district. The contrasting patterns of macrofungal diversity are suggested to be a consequence of many macrofungal species, which is the result of fruiting of macrofungi, being unable to produce fruitbodies or to produce inconspicuous ones. Other explanations include large differences in reproductive and explorative strategies among macrofungi. In addition, the optimum fruiting conditions for some species may not have occurred during years. Despite these limitations, the current fruit body survey is still a useful way to assess the macrofungal diversity.

Among 90 macrofungi, 61 species were recovered during last year of study. Disappearance or loss of species was considered only based on visible fruitbodies and does not necessarily mean absence of mycelia of particular species in the habitat. Although, the loss of species cannot be revealed with precise lucidity, the field observation showed the disturbance in habitat was greatly affected by wood inhabiting species and human interference added to the loss of species in the study sites of Chikmagalur district.

Interpreting the results with ecological parameters has always remained a challenging task and the result has demonstrated that rainfall is statistically significant with available data and the information can be used as baseline for future prospects associated with climate change and conservation strategies owing to maintain the ecosystem health and stability. The information on common, threatened and rare macrofungi is very less in different parts of the country. Hence, the assessment and conservation of all macrofungal species has to be accomplished for local, state and national territories. The diversity,

distribution and development of macrofungi are definitely affected by the changing scenarios of the climate change. The scarcity of baseline data on the influence of climate change on fungal distribution has to be undertaken because of macrofungal interdependencies with other organisms. In this persuit, there is a need for long term monitoring of macrofungal species in Chikmagalur district for assessment of impact of ecological and other supporting parameters.

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# DIVERSITY OF POROID MUSHROOMS IN PUNJAB: FAMILY HYMENOCHAETACEAE

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## ABSTRACT

The present paper deals with the diversity of poroid members of family *Hymenochaetaceae* in Punjab. Fourteen species belonging to four genera i.e. *Fuscoporia* (*F. gilva*), *Inocutis* (*I. rheades*), *Inonotus* (*I. patouillardii*) and *Phellinus* (*P. fastuosus, P. badius, P. xeranticus, P. grenadensis, P. rimosus, P. pectinatus, P. melleoporus, P. purpureogilvus, P. rhabarbarinus*, *P. robustus* and *P. conchatus*) are being described. Ten species are being described for the first time from the study area.

**Keywords**: *Basidiomycota*, *Agaricomycetes,* Punjab

## INTRODUCTION

Family *Hymenochaetaceae* (*Agaricomycetes*, *Hymenochaetales*) is characteristic in having resupinate to pileate, smooth to poroid basidiocarps, xanthochroic tissue, hyphae without clamps, presence/absence of setae, two to four sterigmate basidia and thin- to thick-walled basidiospores. Several taxa of the family are reported to be implicated in many diseases of broad-leaved and coniferous trees, causing various types of rots and diseases. A large number of the species have medicinal and nutritional importance [1].

## OBSERVATIONS

Key to the genera

1. Hyphal system monomitic .................................................. 2

1. Hyphal system dimitic .................................................. 3

2. Setal structures present .................................................. *Inonotus*

2. Setal structures absent .................................................. *Inocutis*

3. Generative hyphae usually encrusted .................................................. *Fuscoporia*

3. Generative hyphae usually not encrusted .................................................. *Phellinus*

**1.** ***Inonotus patouillardii*** (Rick) Imazeki, Bulletin of the Tokyo Science Museum 6: 105**,** 1943. – *Polystictus patouillardii* Rick, Brotéria Série Botânica 6: 89, 1907. **Plate 1 (Figs. 1-7).**

**Basidiocarp** annual, pileate, applanate, solitary; pileus up to 15 × 7 × 4 cm; abhymenial surface smooth to tomentose, somewhat zonate, pale orange to greyish orange when fresh, brownish orange on drying; hymenial surface poroid, orange grey to brownish orange, when fresh, greyish orange to brown on drying; pores round, 3–4 per mm; dissepiments thin, entire; context up to 9 mm thick, light brown, homogeneous, fibrous; pore tubes up to 5 mm long, brownish orange; margins thinning, obtuse, wavy to irregular, sterile up to 5 mm, greyish orange on the hymenial surface, concolorous on the abhymenial surface. **Hyphal system** monomitic. Generative hyphae up to 5.0 ìm wide, branched, simple septate, thin- to thick-walled, subhyaline to yellowish brown. **Setal hyphae** up to 115.0 × 15.2 ìm, thick-walled, abundant, mostly horizontal in trama. **Setae** up to 33.0 × 8.8 ìm, ventricose, thick-walled, dark brown. **Basidia** 30.0–36.0 × 7.2–9.8 ìm, clavate to somewhat sinuous, subhyaline, with oily contents, simple septate at the base, 4–sterigmate; sterigmata up to 4.7 ìm long. **Basidiospores** 5.2–7.6 × 4.0–5.8 ìm, broadly ellipsoid, smooth, pale yellow, thick–walled, with oily contents, cyanophilous, inamyloid.

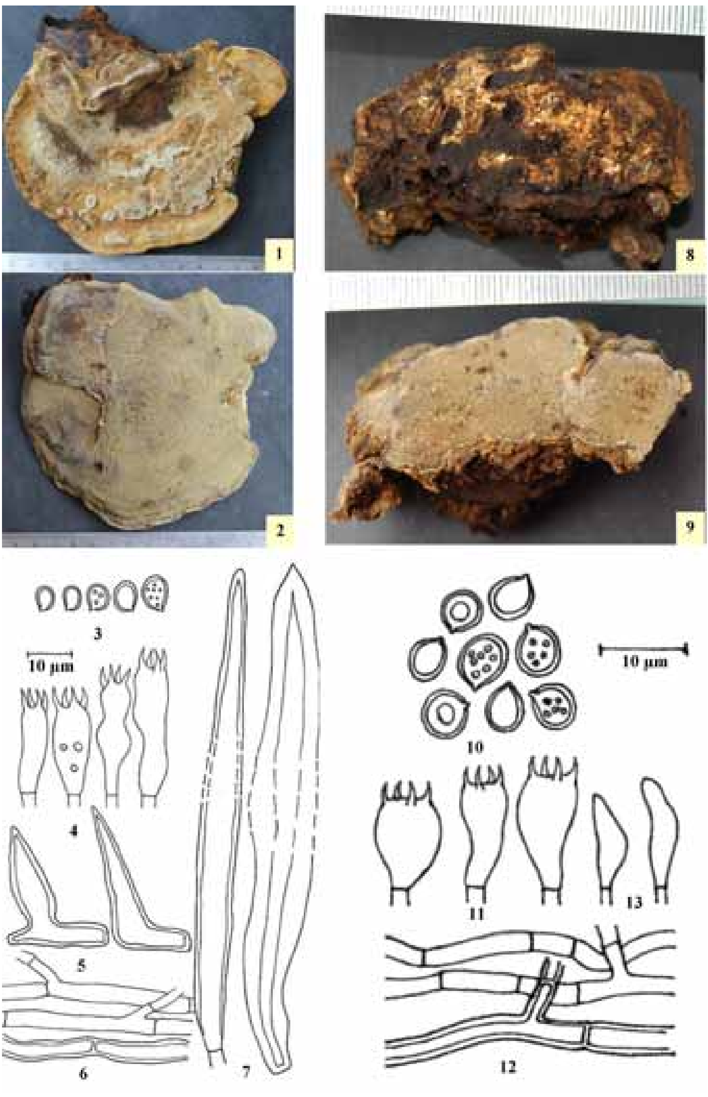


**Plate 1**: Figs. 1-7: ***Inonotus patouillardii***: 1. basidiocarp showing abhymenial surface; 2. basidiocarp showing hymenial surface; 3. basidiospores; 4. basidia; 5. setae; 6. generative hyphae; 7. setal hyphae. Figs. 8-13 ***Inocutis rheades*:** 8. basidiocarp showing abhymenial surface; 9. basidiocarp showing hymenial surface; 10. basidiospores; 11. Basidia; 12. generative hyphae; 13. cystidioles.

**Collection examined** – Punjab: Patiala, Urban Estate Phase II, on *Alstonia* sp., Gurpreet 7062 (PUN), August 13, 2013.

**Remarks** – This species is characteristic in having smooth to tomentose abhymenial surface, conspicuous setal hyphae and broadly ellipsoid basidiospores. Earlier it has been reported from India by Sharma [2-4] and Harpreet [5]. However, it is being described for the first time from Punjab.

**2. *Inocutis rheades*** (Pers.) Fiasson & Niemelä, Karstenia 24: 25, 1984 – *Polyporus rheades* Pers., Mycologia Europaea 2: 69, 1825. **Plate 1 (Figs. 8-13).**

**Basidiocarp** annual, effused-reflexed to pileate, applanate; pileus up to 5.5 × 3 × 2 cm; abhymenial surface sulcate, indistinctly zonate, brownish yellow to brown to dark brown when fresh, greyish orange to dark brown on drying, with brownish-black, hard cutis; hymenial surface poroid, orange grey to greyish orange when fresh, brownish grey on drying; pores angular, 4–5 per mm; dissepiments thin, entire; context up to 5 mm thick, homogeneous, brownish yellow; pore tubes up to 2 mm long, stratified with greyish brown zone near the context layer and dark brown zone towards hymenial surface; margins obtuse, wavy to irregular, sterile up to 1 mm, concolorous on both hymenial and abhymenial surfaces. **Hyphal system** monomitic. Generative hyphae up to 3.4 µm wide, branched, simple septate, thin- to thick–walled. **Setal hyphae** absent. **Setae** absent. **Cystidioles** 10–12.5 × 3–3.7 µm, fusoid, thin-walled, simple septate at the base. **Basidia** 10.6–13.5 × 5.6–7.5 µm, clavate to subclavate, subhyaline, simple septate at the base, 4–sterigmate; sterigmata up to 2 µm long. **Basidiospores** 5–6.5 × 4.3–5.7 µm, broadly ellipsoid to subglobose, smooth, dark brown, thick-walled, with oily contents, inamyloid, acyanophilous.

**Collection examined** – Chandigarh (UT): lake reserve forest, on angiospermous stump, Dhingra and Gurpreet 7063 (PUN), August 15, 2013.

**Remarks** – *Inocutis rheades* is characteristic in having sulcate abhymenial surface with brownish–black, hard cutis, absence of setal structures and broadly ellipsoid to subglobose basidiospoes. Earlier it has been reported from India by Sharma [6, 7] as *Inonotus rheades*. The present collection is different from the earlier descriptions from India in lacking the granular core and is a new report for the study area.

**3. *Fuscoporia gilva*** (Schwein.) T. Wagner & M. Fisch., Mycologia 94(6): 1013, 2002. – *Boletus gilvus* Schwein., Schriften der Berlinische Gesellschaft Naturforschender Freunde 1: 96, 1822. **Plate 2 (Figs. 14-20).**

**Basidiocarps** annual, pileate, imbricate; pileus 7.0 × 4 × 0.7 cm, hard; abhymenial surface strigose to hirsute, faintly zonate to azonate, light orange to orange when fresh, brownish orange to light brown on drying; hymenial surface poroid, greyish red to reddish brown when fresh, brown on drying; pores round to angular, 6–9 per mm; dissepiments entire; context up to 5 mm thick, brownish yellow to brownish orange; pore tubes up to 4 mm long, brown; margins thinning,

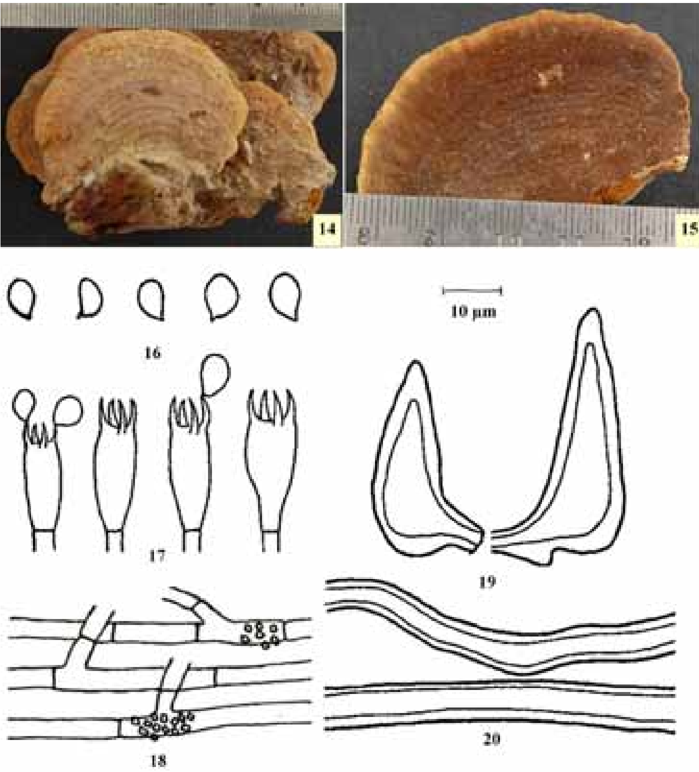


**Plate 2**: Figs. 14-20: ***Fuscoporia gilva*:** 14. basidiocarp showing abhymenial surface; 15. basidiocarp showing hymenial surface; 16. basidiospores; 17. basidia; 18. generative hyphae; 19. setae.

acute, entire to somewhat wavy, sterile up to 1 mm, concolorous on the hymenial surface, paler concolorous on the abhymenial surface. **Hyphal system** dimitic. Generative hyphae up to 3 ìm wide, simple septate, subhyaline, thin-walled, frequently branched, encrusted; thin-to somewhat thick-walled. Skeletal hyphae up to 4.1 ìm wide, rarely branched, aseptate, thick-walled, yellowish brown. **Setae** 17.0–23.0 × 7.0–7.6 ìm, subulate, acuminate, thick-walled, dark brown in 3% KOH solution, projecting up to 20 ìm out of hymenium. **Basidia** 8.0–10.5 × 3.5–4.7 ìm, clavate to subclavate, subhyaline, simple septate at the base, 4-sterigmate; sterigmata up to 2.4 ìm long. **Basidiospores** 3.5–4.7 × 2.0–3.3 ìm, ellipsoid, smooth, thin-walled, subhyaline, inamyloid, acyanophilous.

**Collection examined** – Punjab: Hoshiarpur, Panyali Khurd, on angiospermous stump, Gurpreet 7061 (PUN), August 2, 2013.

**Remarks** – A fairly common species in India, reported earlier by Bose [8-10], Banerjee [11], Thind and Chatrath [12], Dhanda [13], Singh [14], Bakshi [15], Sharma [16, 2, 7], and Leelavathy and Ganesh [17]. However, it is a new record for the study area.

## Key to the species of *Phellinus*

1. Setae present .......... 2
1. Setae absent .......... 5
2. Setal hyphae present .......... *P. conchatus*
2. Setal hyphae absent .......... 3
3. Basidiocarps pileate .......... *P. rhabarbarinus*
3. Basidiocarps resupinate .......... 4
4. Cystidioles present .......... *P. pupureogilvus*
4. Cystidioles absent .......... *P. xeranticus*
5. Basidiocarps resupinate .......... *P. melleoporus*
5. Basidiocarps pileate .......... 6
6. Pilear surface rimose .......... 7
6. Pilear surface not as above .......... 8
7. Pilear surface deeply cracked forming polygonal woody scales with age .......... *P. rimosus*
7. Pilear surface not deeply cracked, no polygonal scales .......... *P. badius*
8. Basidiospores dextrinoid .......... *P. robustus*
8. Basidiospores not dextrinoid .......... 9
9. Basidiospores subglobose, basidiocarps concentrically sulcate .......... *P. fastuosus*
9. Basidiospores broadly ellipsoid, basidiocarps not as above .......... 10
10. Cutis present .......... *P. grenadensis*
10. Cutis absent .......... *P. pectinatus*

**4.** ***Phellinus conchatus*** (Pers.) Quél., Enchiridion Fungorum in Europa media et praesertim in Gallia Vigentium: 173, 1886. – *Boletus conchatus* Pers., Annalen der Botanik (Usteri) 15: 24, 1795. **Plate 3 (Figs. 21-27).**

**Basidiocarp** perennial, pileate, suborbicular, sessile, solitary; pileus up to 27 × 21 × 15 cm; abhymenial surface sulcate, indistinctly zonate, greyish orange to orange grey to brownish orange when fresh, brownish orange to brown on drying; hymenial surface poroid, greyish orange to brownish grey to brown when fresh, light brown to brown on drying; pores round, 7–8 per mm; dissepiments thin, entire; pore tubes up to 1.6 cm long, stratified, brown, separated by thin, homogeneous, brown context layers, up to 4 mm thick; margins indeterminate. **Hyphal system** dimitic. Generative hyphae up to 3.4 ìm wide, simple septate, subhyaline, thin-walled, frequently branched. Skeletal hyphae up to 4.7 ìm wide, aseptate, thick-walled, unbranched, dark brown. **Setal hyphae** up to 16.5 ìm wide, thick-walled. **Setae** 22.0–57.0 × 7.0–8.0 ìm, abundant, subulate to ventricose, thick-walled, reddish brown. **Basidia** not seen. **Basidiospores** 5.0–6.3 × 4–5.1 ìm, ovoid to subglobose, smooth, thick-walled, pale brown, inamyloid, acyanophilous.

**Collection examined** – Punjab: Ludhiana, S.C.D. Government college, on the base of *Melia azedarach*, Gurpreet and Avneet 7071 (PUN), September 8, 2013.

**Remarks** – This species is characterized by the presence of setal hyphae, setae and ovoid to subglobose basidiospores. Earlier, this species has been reported by Bose [18], Banerjee [11], Bakshi [15] as *Fomes conchatus*, whereas by Sharma [6, 7] and Kuldeep Lalji [19] as *Phellinus conchatus*. Presently it is being reported for the first time from Punjab.

**5.** ***Phellinus rhabarbarinus*** (Berk.) G. Cunn., Bulletin of the
1965. – *Polyporus rhabarbarinus* Berk., Annals and Magaz

**Collection examined** – Punjab: Patiala, Punjabi University campus, on the trunk of *Cassia fistula*, Gurpreet 7066 (PUN), October 6, 2013.

**Remarks** – *P. rhabarbarinus* is characteristic in having perennial, solitary, applanate, sulcate, glabrous basidiocarps, brownish context and smaller (3.7–5 × 2.1–3.1 ìm) ellipsoid basidiospores. Earlier, it has been reported from Punjab by Kuldeep Lalji [19].

**6.** ***Phellinus purpureogilvus*** (Petch) Ryvarden, Norwegian Journal of Botany 19: 235, 1972. – *Poria purpureogilva* Petch, Annals of the Royal Botanic Gardens Peradeniya 6 (2): 138, 1916. **Plate 3 (Figs. 28-34).**

**Basidiocarp** annual, resupinate, not easily separable, up to 2 mm thick; pore surface light brown when fresh, brown on drying, uneven, pores round to angular, 5–6 per mm, dissepiments thin, entire, tubes not stratified, brown; margins thinning, fibrillose to irregular, whitish to paler concolorous, to indeterminate. **Hyphal system** dimitic. Generative hyphae up to 2.5 ìm wide, simple septate, hyaline, thin-walled, branched. Skeletal hyphae up to 3.7 ìm wide, aseptate, thick-walled. **Setae** 13.0–37.0 × 5.6–8.7 ìm, subulate, apex pointed, thick-walled, dark brown. **Cystidioles** 15.0–16.2 × 4.3–5.6 ìm, fusoid, subhyaline, thin-walled, simple septate at the base. **Basidia** 12.5–15.6 × 6.2–8.1 ìm, clavate, subhyaline, simple septate at the base, 4-sterigmate;

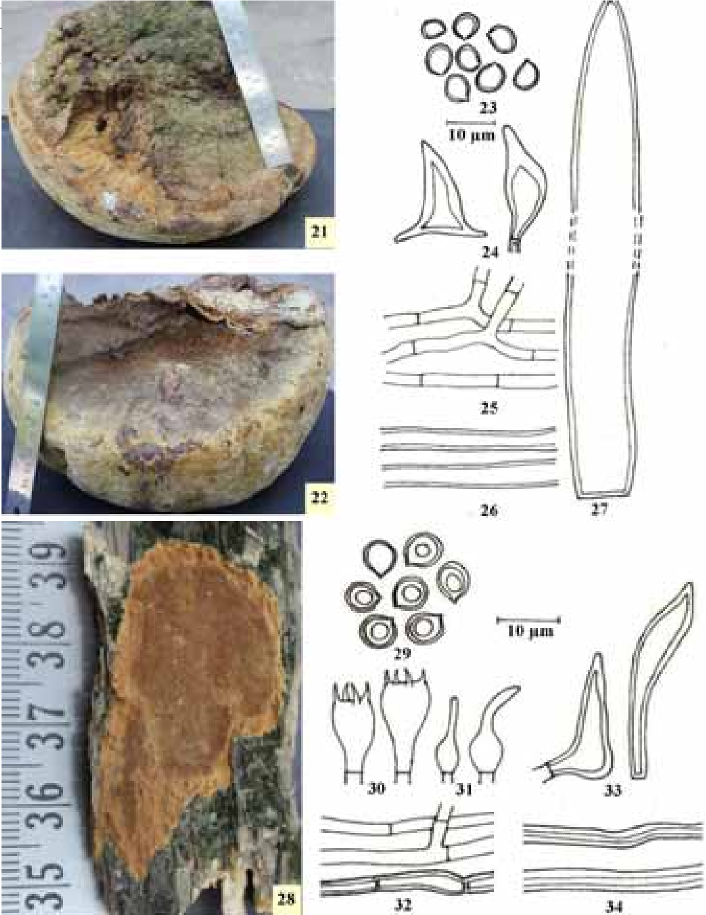


**Plate 3**: Figs. 21-27: ***Phellinus conchatus***: 21. basidiocarp showing abhymenial surface; 22. basidiocarp showing hymenial surface; 23. basidiospores; 24. setae; 25. generative hyphae; 26. skeletal hyphae; 27. setal hyphae. Figs 28-34: ***Phellinus purpureogilvus***: 28. basidiocarp showing hymenial surface; 29. basidiospores; 30. basidia; 31. cystidioles; 32. generative hyphae; 33. setae; 34. skeletal hyphae.

sterigmata up to 3.4 ìm long. **Basidiospores** 5.6–6.2 × 5.0–5.6 ìm, broadly ellipsoid to subglobose, smooth, thick-walled, inamyloid, acyanophilous.

**Collection examined** – Punjab: Patiala, Punjabi University campus, on angiospermous log, Gurpreet 7064 (PUN), August 2, 2013.

**Remarks** – This species is characterised by resupinate basidiocarps and presence of subulate setae. Earlier, it has been reported by Sharma and Ghosh [20] and Sharma [7]. However, it is being reported for the first time from Punjab.

**7. *Phellinus xeranticus*** (Berk.) Pegler, Kew Bulletin 21 (1): 44, 1967. – *Polyporus xeranticus* Berk., Hooker's Journal of Botany and Kew Garden Miscellany 6: 161, 1854. **Plate 4 (Figs. 35-40).**

**Basidiocarp** annual, resupinate, widely effused, not easily separable, up to 2 mm thick; pore surface greyish red to brown when fresh, not changing much on drying, uneven, pores angular, 5–7 per mm, dissepiments lacerate, tubes not stratified, brown, up to 2 mm long; margins thinning, whitish to paler concolorous, to indeterminate. **Hyphal system** dimitic. Generative hyphae up to 2.5 ìm wide, simple septate, subhyaline, thin-walled, branched. Skeletal hyphae up to 3.4 ìm wide, aseptate, thick-walled, yellowish brown. **Setae** 26.0–58.0 × 6.8–8.8 ìm, subulate, abundant, apex pointed, thick-walled, dark brown. **Basidia** 13.0–15.0 × 5.0–5.6 ìm, clavate, subhyaline, simple septate at the base, 4–sterigmate ; sterigmata up to 2.8 ìm long. **Basidiospores** 4.3–5.9 × 2.5–3.7 ìm, ellipsoid, hyaline, smooth, thin-walled, with oily contents, weakly cyanophilous, inamyloid.

**Collection examined** – Punjab: Ludhiana, Punjab Agricultural University, on *Dalbergia sissoo*, Gurpreet 7068 (PUN), September 8, 2013.

**Remarks** – *P. xeranticus* is peculiar in having abundant subulate setae. Earlier, from India, it has been reported by Bose [10], Banerjee [11], Pegler [21], Rattan [22], Dhanda [13], Thind and Dhanda [23, 24], Sharma [16], Singh [14], Sharma [6] and Kuldeep Lalji [19]. However, it is being reported for the first time from Punjab.

**8. *Phellinus melleoporus*** (Murrill) Ryvarden, Mycotaxon 23: 177, 1985. – *Fomitiporella melleopora* Murrill, North American Flora 9 (1): 13, 1907.

**Collection examined** – Punjab: Patiala, Botanic Gardens, Punjabi University, on bark of *Pongamia* sp., Gurpreet 7067 (PUN), September 6, 2013.

**Remarks –** It is characteristic in having resupinate, yellowish brown basidiocarp, absence of setae and small (3.2–4.2 × 2.3–3.2 ì m), pale brown basidiospores. Earlier, Dargan et al. [25] reported it from the study area.

**9. *Phellinus rimosus*** (Berk.) Pilát, Annales Mycologici 38 (1): 80, 1940. – *Polyporus rimosus* Berk., London Journal of Botany 4: 54, 1845.

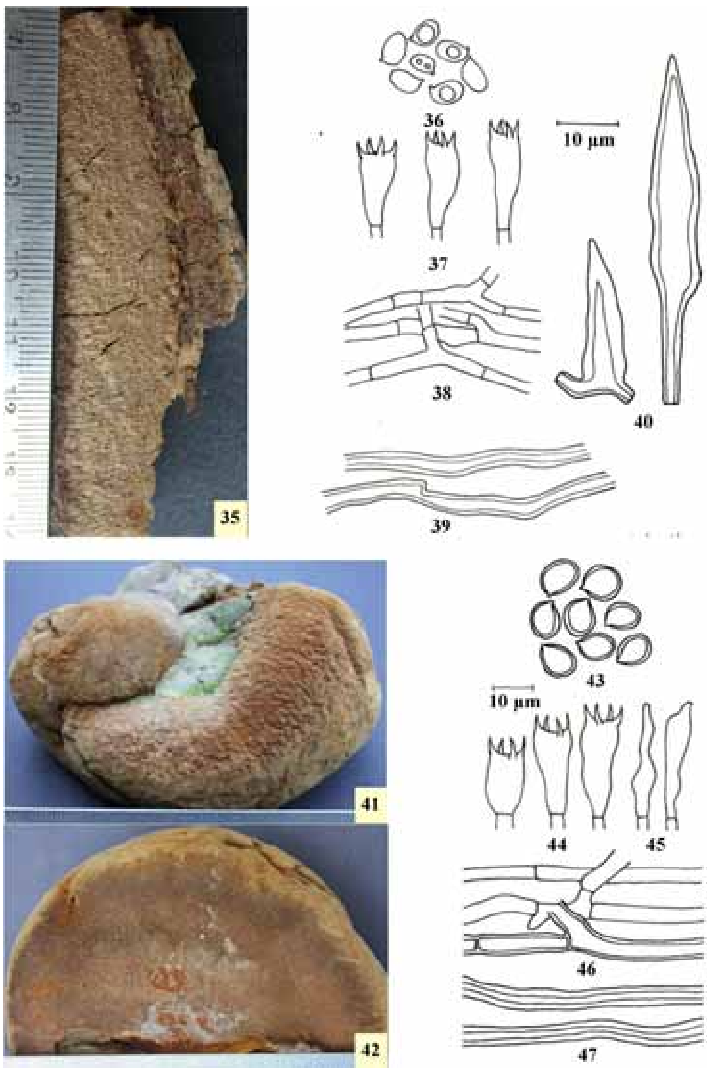


**Plate 4**: Figs. 35-40: ***Phellinus xeranticus***: 35. basidiocarp showing hymenial surface; 36. basidiospores; 37. basidia; 38. generative hyphae; 39. skeletal hyphae; 40. setae. Figs. 41-47: ***Phellinus robustus***: : 41. basidiocarp showing abhymenial surface; 42. basidiocarp showing hymenial surface; 43. basidiospores; 44. basidia; 45. cystidioles; 46. generative hyphae; 47. skeletal hyphae.

**Collection examined** – Chandigarh (UT): Sector 1, backside of Sukhna Lake, on the trunk of *Acacia* sp., Dhingra 7070 (PUN), September 19, 2013.

**Remarks –** It is characterized by pileate basidiocarps, abhymenial surface deeply cracked, forming polygonal woody scales with age. Earlier, Kuldeep Lalji [19] reported it from the study area.

**10.** ***Phellinus badius*** (Cooke) G. Cunn., Bulletin of the New Zealand Department of Industrial Research 164: 233, 1965. – *Fomes badius* Cooke, Grevillea 14 (69): 18, 1885.

**Collection examined** – Punjab: Roopnagar, Balachaur, Maili, on angiospermous log, Avneet 7060 (PUN), September 22, 2013.

**Remarks –** It is peculiar in having pileate basidiocarps with rimose pilear surface and broadly ellipsoid to subglobose basidiospores. Earlier, it has been reported from the study area by Kuldeep Lalji [19].

**11.** ***Phellinus robustus*** (P. Karst.) Bourdot & Galzin, Bulletin de la Société Mycologique de France 41: 188, 1925. – *Fomes robustus* P. Karst., Bidrag till Kännedom av Finlands Natur och Folk 48: 467, 1889. **Plate 4 (Figs. 41-47).**

**Basidiocarp** perennial, pileate, applanate, suborbicular, solitary; pileus up to 14.0 × 11 × 7.5 cm; abhymenial surface smooth to tomentose, azonate, yellowish brown to light brown when fresh, light brown to brown on drying; hymenial surface poroid, brown when fresh, not changing much on drying; pores round to angular, 4–5 per mm; dissepiments entire; pore tubes stratified, up to 5 mm long, separated by very thin layers of context, up to 5 mm thick; margins indeterminate. **Hyphal system** dimitic. Generative hyphae up to 3.1 ìm wide, branched, simple septate, thin-walled, subhyaline. Skeletal hyphae up to 3.4 ìm wide, rarely branched, aseptate, thick-walled, yellowish brown. **Setae** absent. **Cystidioles** 17.0–19.3 × 3.4–3.8 ìm, fusoid, thin-walled, with tapering apex. **Basidia** 8.7–14.3 × 5.3–5.7 ìm, clavate, subhyaline, simple septate at the base, 4-sterigmate; sterigmata up to 1.8 ìm long. **Basidiospores** 5.0–6.2 × 3.7–5.7 ìm, broadly ellipsoid to subglobose, thick-walled, smooth, yellowish brown, acyanophilous, dextrinoid.

**Collection examined** – Punjab: Patiala, Punjabi University campus, on trunk of *Cassia* sp., Gurpreet 7058 (PUN), September 18, 2013.

**Remarks** – *P. robustus* is characterised by very thick, applanate, suborbicular basidiocarp, smooth to tomentose abhymenial surface and absence of setal structures. From India, it has earlier been reported by Bakshi [15], Dhanda [13] and Sharma [16, 6, 7], Singh [14] and Harpreet [5] from different areas. However, it is being reported as a new record for the study area.

**12.** ***Phellinus fastuosus*** (Lév.) S. Ahmad, *Basidiomycetes* of West Pakistan: 56, 1972. – *Polyporus fastuosus* Lév., Annales des Sciences Naturelles Botanique 2: 190, 1844. **Plate 5 (Figs. 48-52).**

**Basidiocarps** perennial, pileate, imbricate, woody; pileus up to 15 × 12 × 1 cm; abhymenial surface sulcate, concentrically zonate, brownish grey to light brown when fresh, not changing much on drying; hymenial surface poroid, brownish orange to light brown to brown when fresh, brown on drying; pores round, 8–9 per mm, dissepiments entire; context up to 1.5 mm thick, homogeneous, brownish yellow; pore tubes up to 5 mm long, brownish orange; margins thinning, obtuse, irregular, sterile up to 9 mm, greyish brown on the hymenial surface, concolorous on the abhymenial surface. **Hyphal system** dimitic. Generative hyphae up to 3.4 ìm wide, branched, simple septate, thin- to thick-walled. Skeletal hyphae up to 4.0 ìm wide, rarely branched, aseptate, thick-walled, yellowish brown. **Basidia** not observed. **Basidiospores** 3.4–5.7 × 2.8–4.5 ìm, subglobose, thick-walled, smooth, brown, acyanophilous, inamyloid.

**Collections examined** – Punjab: Patiala, Baradari gardens, on base of *Eucalyptus* sp., Gurpreet 7065 (PUN), February, 10, 2013.

**Remarks –** Chief features of this species are sulcate, concentrically zonate abhymenial surface, absence of hymenial setae and subglobose basidiospores. Previously it has been reported from India by Bose [26], Banerjee [11], Saxena [27],

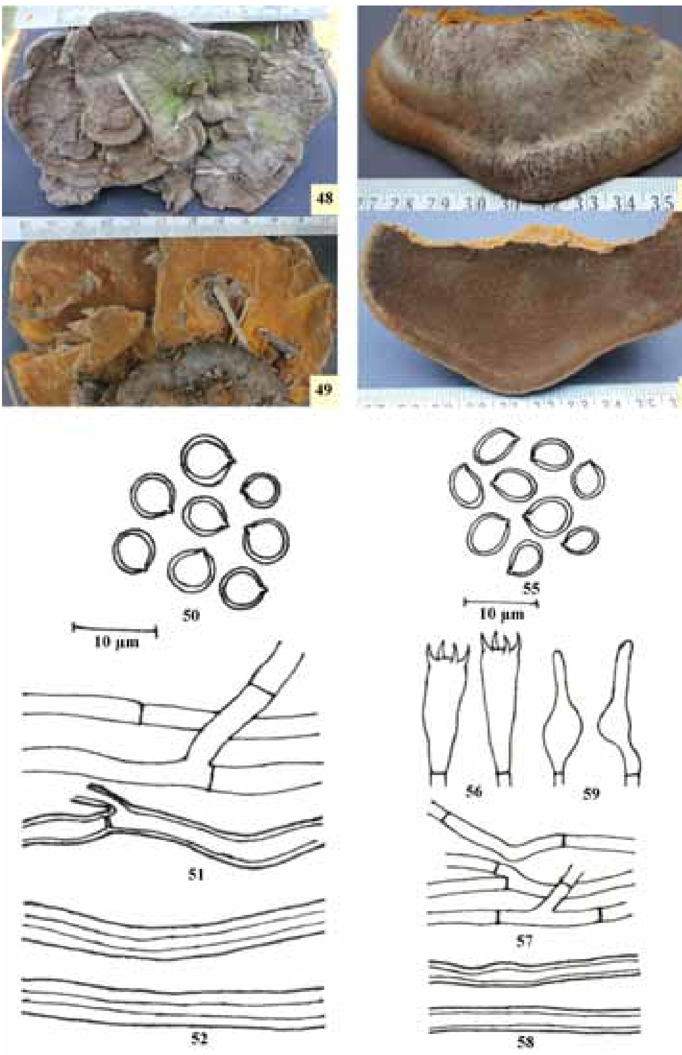


**Plate 5**: Figs. 48-52: ***Phellinus fastuosus***: 48. basidiocarp showing abhymenial surface; 49. basidiocarp showing hymenial surface; 50. basidiospores; 51.generative hyphae; 52. skeletal hyphae. Figs. 53-59: ***Phellinus grenadensis***: 53. basidiocarp showing abhymenial surface; 54. basidiocarp showing hymenial surface; 55. basidiospores; 56. basidia; 57. generative hyphae; 58. skeletal hyphae; 59. cystidioles.

Singh *et al.* [28] as *Fomes fastuosus* and by Dhanda [13], Sharma and Ghosh [20], Leelavathy and Ganesh [20] and Sharma [7] as *Phellinus fastuosus.* However, it is being reported for the first time from Punjab.

**13. *Phellinus grenadensis*** (Murrill) Ryvarden, Norwegian Journal of Botany 19: 234, 1972. – *Pyropolyporus grenadensis* Murrill, North American Flora 9 (2): 107, 1908. **Plate 5 (Figs. 53-59).**

**Basidiocarp** perennial, pileate, applanate, ungulate, solitary; pileus up to 10.5 × 7 × 4 cm; abhymenial surface smooth to rugose, zonate, greyish orange to light brown to pale red when fresh, not changing much on drying, with brown cutis; hymenial surface poroid, light brown to brown when fresh, not changing much on drying; pores round, 3–4 per mm; dissepiments entire; context up to 2.5 cm thick, zonate, fibrous, light brown; pore tubes up to 1.5 cm long, brown; margins acute, somewhat incurved, sterile up to 3 mm, light brown on the hymenial surface, concolorous on the abhymenial surface. **Hyphal system** dimitic. Generative hyphae up to 2 ìm wide, branched, simple septate, thin-walled. Skeletal hyphae up to 3.7 ìm wide, rarely branched, aseptate, thick-walled, yellowish brown. **Setae** absent. **Cystidioles** 17.8–18.7 × 4.3–5.3 ìm, fusoid, with tapering apex, thin-walled. **Basidia** 15.6–20.6 × 5.0–5.6 ìm, clavate, subhyaline, simple septate at the base, 4–sterigmate; sterigmata up to 2.2 ìm long. **Basidiospores** 5.0–6.8 × 3.4–5.3 ìm, broadly ellipsoid, thick-walled, smooth, yellowish brown, acyanophilous, inamyloid.

**Collection examined** – Punjab: Patiala, near Gol market, Punjabi University, on angiospermous stump, Gurpreet 7069 (PUN), September 15, 2013.

**Remarks** – This species is characteristic in having applanate, ungulate basidiocarp and absence of setae. From India, it has earlier been reported by Dhanda [13] and Sharma [7]. Here it is being reported for the first time from Punjab.

**14. *Phellinus pectinatus*** (Klotzsch) Quél., Enchiridion Fungorum in Europa media et praesertim in Gallia Vigentium: 173, 1886 – *Polyporus pectinatus* Klotzsch, Linnaea 8: 485, 1833. **Plate 6 (Figs. 60-66).**

**Basidiocarps** perennial, pileate, imbricate, woody; pileus up to 5.5 × 5 × 3 cm; abhymenial surface irregulary sulcate, light brown to brown to dark brown when fresh, not changing much on drying; hymenial surface poroid, light brown to brown when fresh, not changing much on drying; pores round to angular, 7–8 per mm; dissepiments entire; context up to 2.5 cm thick, homogeneous, hard, yellowish brown; pore tubes up to 5 mm long, golden yellow; margins thinning, obtuse, sterile up to 4 mm, deep yellow on hymenial surface, concolorous on abhymenial surface. **Hyphal system** dimitic. Generative hyphae up to 2.2 ìm wide, branched, simple septate; thin- to thick-walled, yellowish brown. Skeletal hyphae up to 3.1 ìm wide, aseptate, thick-walled, rarely branched, brown. **Setae** absent. **Cystidioles** 13.7–16.5 × 3.0–3.7 ìm, fusoid, subhyaline, thin-walled, simple septate at the base. **Basidia** 8.0–8.7 × 5–5.6 ìm, subovate, simple septate at the base, 4–

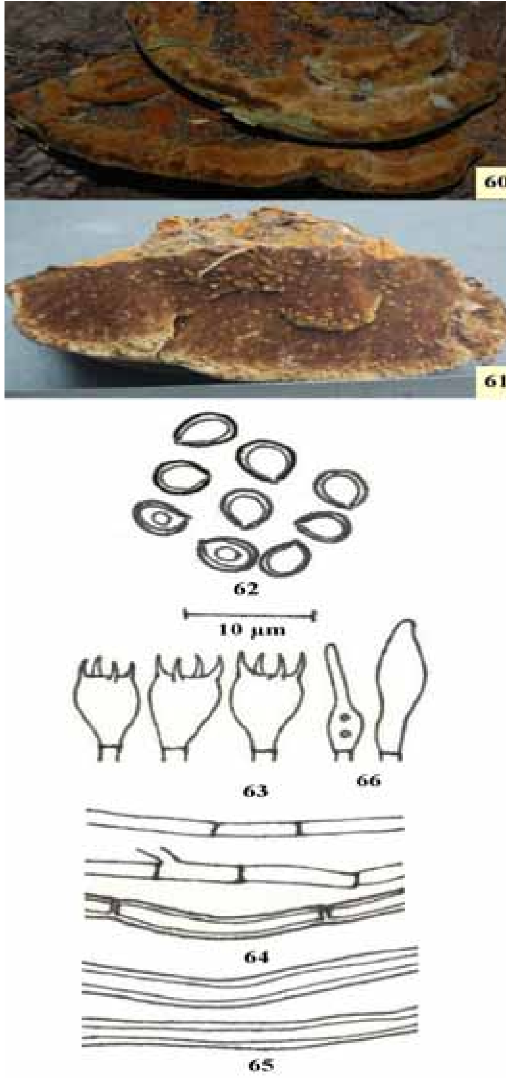


**Plate 6**: Figs. 60-66: ***Phellinus pectinatus*****:** 60. basidiocarp showing abhymenial surface; 61. basidiocarp showing hymenial surface; 62. basidiospores; 63. basidia; 64. generative hyphae; 65. skeletal hyphae; 66. cystidioles.

sterigmate; sterigmata up to 2 ìm long. **Basidiospores** 4.2–5 × 3–4 ìm, broadly ellipsoid, smooth, pale yellow to brownish, thin- to somewhat thick-walled, weakly cyanophilous, inamyloid.

**Collection examined** – Chandigarh (UT): Lake reserve forest, on base of *Acacia* sp., Gurpreet & Dhingra 7059 (PUN), September 14, 2013.

**Remarks** – It has earlier been reported by Bose [2, 9], Banerjee [1], Thind and Chatrath [12], Dhanda [13], Sharma and Ghosh [20], Sharma [7] and Harpreet [5]. Here it is being reported for the first time from the study area.

## ACKNOWLEDGEMENTS

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# DIVERSITY OF WILD MUSHROOM FLORA FROM INDIAN THAR DESERT

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## ABSTRACT

Indian Thar Desert harbours a treasure trove of fungal diversity especially wild mushrooms. Through exploration resulted in collection of 48 species belonging to 21 genera which were collected and studied. It was observed that these mushrooms were found to occur on a variety of substrates and in different habitats of Thar Desert. Threats to mushroom diversity in Thar Desert are of much concern due to ever increasing anthropogenic activities, frequent droughts and a high livestock population. Conservation of these wild mushrooms is urgent need of hour for their utilization as food, medicine and for utilizing active biomolecules in future.

**Keywords:** Basidiomycetes, Agricales, wild mushroom, Thar Desert

## INTRODUCTION

The Great Indian Desert, or Thar Desert, extends over about 0.32 million $km^2$ forming approximate 10% of the total geographic area of India. More than 60% of the Desert lies in the state of Rajasthan, followed by 20% in Gujarat. This desert from the eastern extremity of the great arid and semi arid belt of the world. It is one of the smallest deserts of the world but exhibits a wide variety of habitats and a high biodiversity due to juxtaposition of Palaeoartic, Oriental and Saharan elements. Owing to strong variations in climatic, edaphic, physiographic, topographic and geological characteristics, Thar Desert shows a wide variety of habitats and a high biodiversity. Thar Desert harbours a treasure trove of fungal diversity especially wild mushroom. In Thar Desert, wild mushrooms have an unestimated wealth of mushroom diversity, which needs to be tapped properly as there are still several undescribed species yet to be identified. Efforts need to make to identify and exploit these mushroom floras as their biodiversity and conservation and utility for the food security of a country.

The climate of Thar Desert is characterized by low rainfall with erratic distribution, extremes of diurnal and annual temperature, low humidity, high wind velocity and frequent dust storms. Soil profiles are characterized by low contents of silt, clay and humus but higher salt. Vegetation types are xerophytes thorn forest, mixed xerophytes woodland, lithophytic and psammophytic scrub desert and also halophytic vegetation.

In Thar Desert area, mushroom fungi tend to appear seasonally. The most productive months are those of the rainy season (July-September). The best conditions for the occurrence of a massive population of mushroom fruiting bodies (basidiocarps) are related with the presence of humidity in the nutritional substrate and in the air, coupled with a mild temperature. Thus during different climatic conditions (rainy, dry, sunny), different types of mushroom grow in different habitats, viz., on the ground in woodlands or associated with tree, on the ground in grasslands or open spaces, growing on trees stumps or woody debris, growing on burnt ground or brunt wood, growing on dung or enriched soil, growing on sand in sand dunes, on termite's nests and sometime parasite on living woods. This is because of a complex pattern of interaction with the climatic condition, soil profile, soil pH, soil humus, decomposed materials, plant debris and plants.

Alexopoulous *et al*. [1] classified Hymenomycetes fungi into Tremellales, Agaricales, Aphyllophorales, Auriculariales, Dacrymycetales, Ceratobasidiales, Tulasnellales and Gastromycetes lineage. The Agricales includes basidiomycetous fungi whose fruiting bodies are commonly called "mushroom". Typically the fruit body consists of an umbrella like cap on a central and vertical stipe. On the under side of the cap are radiating gills, upon which the microscopic spores are produced. Singer [2] has classified the order Agricales into 17 families as follows: Agaricaceae, Amanitaceae, Bolbitiaceae, Boletaceae, Coprinaceae, Cortinariaceae, Crepidotaceae, Entolomataceae, Gomphidiaceae, Hygrophoraceae, Paxillaceae, Polyporaceae, Russulaceae, Schizophyllaceae, Strobilomycetaceae, Strophariceae and Tricholomaceae.

## MATERIALS AND METHODS

Arid and semi arid area of Indian Thar desert viz., Jodhpur, Jaisalmer, Barmer, Bikaner, Churu, Jalore and Pali district were surveyed for wild agaricales mushroom collection during 2001 to 2010. Standard methods were followed for collection, preservation and macro and microscopic studies of mushroom [3, 4]. Macroscopic details such as shape, size, colour and odour of the fresh specimens were recorded before preservation. Fascinating feature of fruiting body, edibility, medicinal value, ectomycorrhizal association and other interesting feature were also noticed. Field photography was done using digital Nikon camera. Identification was made on the basis of critical observations of the specimens and perusal of relevant literature [2, 5-7]. The fruiting bodies were maintained in 4% formaldehyde solution in the laboratory and preserved in the herbarium division.

## RESULTS

In the present course of investigation, 48 species of 21 genera were collected from different habitat viz., grassland, pasture, roadsides, wooded area, sand dunes, over dead stumps and living trees. Detailed accounts of theses wild mushroom with their families and taxonomic status were given as according to classification given by Singer [2]. Out of seventeen families mushroom belonging to eight families viz., Hygrophoraceae, Amanitaceae, Entolomataceae, Tricholomataceae, Agaricaceae, Coprinaceae, Strophariaceae and Crepidotaceae were collected and studied (Table 1).

**Table 1.** Characteristics feature and habitat of wild mushroom occurring in IndianThar Desert

| S. No. | Name of Mushroom | Family | Characteristics and Habitat |
|---|---|---|---|
| 1. | *Hygrophoropsis aurantiaca* | Hygrophoraceae | Small orange-yellow basidiocarp with shallowly funnel shaped cap, reminiscent of a chanterelle but with true gills; in small troops on soil. |
| 2. | *Volvariella bombycina* var. *flaviceps* | Amanitaceae | Bright-flavous basidiocarp with fibrillose cap and white gills found solitary on tree trunks. |
| 3. | *Volvariella earlei* | Amantitaceae | White to pale gray basidiocarp found gregarious on soil. |
| 4. | *Volvariella hypopithys* | Amanitaceae | Medium sized white basidiocarp with long stipe found on humus rich soil. |
| 5. | *Volvariella pusilla* | Amanitaceae | Grayish white small sized basidiocarp with flattened cap and pinkish gills found on humus rich soil. |
| 6. | *Volvariella speciosa* var. *gloioceplala* | Amanitaceae | Dull white pearl gray ovoid basidiocarp, flesh pink gills, found on soil. |
| 7. | *Volvariella speciosa* var. *speciosa* | Amanitaceae | Large sized pale white to light brownish basidiocarp found growing on jowar straw on road sides |
| 8. | *Pluteus subcervinus* | Amanitaceae | Medium or large agaric with pale brown cap and pink gills, found on rotting wood. |
| 9. | *Termitomyces eurhizus* | Amanticeae | Large, white, fleshy basidiocarp with a broadly umbonate perforatorium, found solitary on termitaria. |
| 10. | *Termitomyces heimii* | Amanitaceae | Large, white basidiocarp with umbonate and long pseudorrhiza found on the boundary walls of field on termite nests. |
| 11. | *Termitomyces microcarpus* | Amantitaceae | Small white basidiocarp, found in group amongst where termite nests had been active. |
| 12. | *Termitomyces tyleranus* | Amanitaceae | Small whitish basidiocarp with tall stem and pointed perforatorium found on leaf litter. |
| 13. | *Termitomyces sp.* | Amanitaceae | Medium, white basidiocarp with long pseudorrhiza. Grow on termitaria soil. |
| 14. | *Leptonia sericella* | Entolomataceae | Very small sized basidiocarp, white cap, decurrent gills, found amongst grass. |

| S. No. | Name of Mushroom | Family | Characteristics and Habitat |
|---|---|---|---|
| 15. | *Clitocybe dealbata* | Tricholomaeae | Small fleshy basidiocarp with whitish-grey, shallowly depressed cap and white decurrent gills; trooping in rings on soil in lawns and pastures. |
| 16. | *Tricholoma lobayense* | Tricholomataceae | Large fleshy basidiocarp with solid stipe swollen at base |
| 17. | *Tricholoma sulphureum* | Tricholomataceae | Sulphur yellow coloured large fleshy basidiocarp found on soil and amongst leaf litter. |
| 18. | *Marasmiellus sp.* | Tricholomataceae | Delicate white basidiocarp, found at the base of tree |
| 19. | *Trogia infundibuliformis* | Tricholomataceae | Small brown basidiocarp with funnel shaped cap bearing deeply decurrent gills, found amongst leaf litter. |
| 20. | *Marasmius confetus* | Tricholomaceae | On soil amongest grass |
| 21. | *Marasmius oreades* | Tricholomaceae | Smallish, pale tan, fleshy basidiocarp, with blunt umbo and tough rooting stem, typically in rings on soil in short grass or lawns. |
| 22. | *Hemimycena pithya* | Tricholomataceae | Small, delicate, chalk-white basidiocarp with bell-shaped irregularly grooved cap; in large troops or some somewhat tufted on plant debris. |
| 23. | *Macrolepiota exocortiata* | Agaricaceae | Large fleshy basidiocarp with white cap, gills and stem with ring; in scattered groups on soil in pastures. |
| 24. | *Macrolepiota rachodes* | Agaricaceae | Large sized basidiocarpwith coarse brown cap scales on a white background, free gills and a bulbous base solitary to gregarious, sometimes in caespitose clusters on garden soil. |
| 25. | *Leucocoprinus cepaestipes* | Agaricaceae | Medium sized basidiocarp, white scaly campanulate cap, commonly found in parks, garden and roadsides. |
| 26. | *Leucocoprinus zeylanicus* | Agaricaceae | Pale brown basidiocarp, campanulate cap with brown umbo found growing on the trunk of tree. |
| 27. | *Agaricus alphitochrous* | Agaricaceae | Small size basidiocarp, cap creamish pale with squamutes confined to the center pink to brown gills, found in groups amongst grass. |
| 28. | *Agaricus augustus* | Agaricaceae | Medium to large basidiocarp with pale cap having fibrillose squamules, in field on sandy loam soil |
| 29. | *Agaricus bambusophillus* | Agaricaceae | Small redish brown agaric, with dark center, appendiculate margin, found on leaf litter plant debris. |
| 30. | *Agaricus benzodorus* | Agaricaceae | Medium sized basidiocarp with grayish brown cap, broadly umbonate found on soil or leaf litter. |
| 31. | *Agaricus bisporus var. hortensis* | Agaricaceae | Medium to large basidiocarp with whitish cap and stem, pink or chocolate gills and ring on stem; in trooping groups often tufted.on humus rich soil and plant debris. |
| 32. | *Agaricus purpurellus* | Agaricaceae | Cremishbasidiocarp with violaceous tins, appendiculate margin found solitary in fieldson sandy soil. |
| 33. | *Agaricus silvicola* | Agaricaceae | Medium or large basidiocarp with creamy whie cap, pink or chocolate gills, stipe with ring and bulbous base; in trooping groups on soil in leaf litter. |
| 34. | *Agaricus silvaticus* | Agaricaceae | Medium basidiocarp with reddish brown cap, with radiating scales, flesh staining deep red, found on leaf litters. |
| 35. | *Agaricus trisulphuratus* | Agaricaceae | Bright orange coloured basidiocarp, campanulate cap covered with numerous squamules found on soil |
| 36. | *Lepiota mericana* | Agaricaceae | Medium sized basidiocarp, pinkish brown to reddish brown, squamulose, umbonate cap and a fusiform to club shaped stipe on soil in grassy habitats |

| S. No. | Name of Mushroom | Family | Characteristics and Habitat |
|---|---|---|---|
| 37. | *Coprinus extinctorius* | Coprinaceae | Tall, pale brownish basidiocarp, with conical cap, blackning; solitary or in small groups, typically caespitose tufted, on stumps or wounds of broad-leaf tree. |
| 38. | *Coprinus lagopides* | Coprinaceae | Tall, fragile, grey basidiocarp covered with whitish scurf, blackening scattered or solitary on soil or charred wood. |
| 39. | *Coprinus sterquilinus* | Coprinaceae | White basidocarp with blackening gills, campanulate cap covered with white fibrillose scales, found in grassy habitats. |
| 40. | *Panaeolus fimicola* | Coprinaceae | Small grayish basidiocarp with mottled gills, becoming blackish, in trooping groups on soil in lawns parks and other grassy situation. |
| 41. | *Psathyrella magambica* | Coprinaceae | Small basidiocarp with fuscous brown cap covered by numerous white velar squamules found in tufts on wood. |
| 42. | *Psathyrella pygmaea* | Coprinaceae | Very small sized basidiocarp with brown cap, found in tufts on leaf litter. |
| 43. | *Psathyrella spadicea* | Coprinaceae | Small brown basidiocarp forming caespitose clusters on dead wood and leaf litter. |
| 44. | *Psathyrella tiarella* | Coprinaceae | Very small basidiocarp, clay brown umbonate cap, found in great numbers on soil in rainy season. |
| 45. | *Pholiota squarrosa* | Strophariaceae | Large basidiocarp, straw-yellow covered with coarse rust scales and with ring zone on stem, caespitose tufts at the base of living broad-leaf trees. |
| 46. | *Stropharia semiglobata* | Strophariaceae | Small or variable basidiocarp with domed slimy yellow cap, long slender stem with ring zone, clay or purplish brown gills; in small trooping groups, sometimes caespitose, on dung. |
| 47. | *Crepidotus herbarum* | Crepidotaceae | Very small, whitish, kidney-shaped cap with buff gills, more or less sessile on twigs, and other plant debris. |
| 48. | *Crepidotus quitensis* | Crepidotaceae | Very small, whitish to pale grayish cap, sessile, with dorsi-lateral attachment to substratum, on twigs and other debris. |

**Family Hygrophoraceae:** Hygrophoraceae family is characterized by the ability to produce white, thin walled basidiospores from rather long, cylindrical basidia. Lamellae are thick and waxy. Many members of these families are colorful producing basidiocarps with white, yellow, orange or red caps. Only one species was collected i.e. *Hygrophoropsis aurantiaca.* Repeated forked nature of lamellae is the main distinguishing character of this genus.

**Family Amanitaceae:** This family is characterized by presence of small to large basidiocarp, presence of both an annulus and a volva or these velar structures may be absent. Twelve species were studied in this family belonging to three genera viz. *Volvariella, Pluteus* and *Termitomyces. Volvariella bombycina* var. *flaviceps*, *V. earlei*, *V. hypopithys, V. pusilla, V. speciosa* var. *gloicephala*, *V. speciosa* var. *speciosa*, are well distinguished morphologically due to typical characters presence of volva, spore and cystidia morphology. *Pluteus subcervinus* is characterized by the absence of velar structure at maturity. Five strains of genus *Termitomyces* viz., *T. eurhizus, T. heimii, T. microcarpus. T. tyleranus,* and *Termitomyces* sp. *Termitomyces* was distinguished by white basidiocarp with long pseudorrhiza found on the boundary walls of field on termite nests

**Family Entolomataceae:** Only one genus in this family has been collected and studied viz., *Leptonia sericella* which is well distinguished by infundibuliform pileus, central stipe, angular spores and presence of cystidia.

**Family Tricholomataceae:** This is a very large family. It is characterized by the stipe (if present), is confluent with the pileus. Basidiocarps are minute and delicate to large and fleshy. The colour of the spores is variable and the lamellae show

all possible forms of attachment with the stipe; rarely free. In this family, eight species of six genera were studied. These are belonging to the tribes Clitocybieae, Collybieae, Marasmieae and Myceneae. In the Clitocybeae, three species were studied namely *Clitocybe dealbata, Tricholoma lobayense* and *T. sulphureum.* The *Marasmiellus* sp. and *Trogia infundibuliformis* were studied under the tribe Collybieae. Tribe Marasmieae includes *Marasmius confertus* and *M. oredes. Hemimycena pithya* was studied belonging to the tribe Myceneae.

**Family Agaricaceae:** The family is characterized by the basidiocarp that is white to brown or grey-brown cap with free gills, an annulus but no volva and a stalk that readily separates from the cap. Gills may be light in colour-often pink or white but eventually darken. 14 species belonging to 4 genera *viz*., *Macrolepiota, Leucocoprinus, Agaricus,* and *Lepiota* were studied. These species are included under the tribes Leucocoprineae, Agaricaceae and Lepioteae respectively. Tribe Leucocoprineae included *Macrolepiota exorciata, M. rachodes, Leucocoprinus cepaestipes*, *L. zeylancius*. *L. zeylanicus* is characterized by lamellar colour due to an effect of the weather. Tribe agariceae included *Agaricus alphitochrous*, *A. augustus, A. bambusophillus*, *A. benzodorus*, *A. bisporus* var. *hortensis, A. purpurellus, A. silvicola, A. silvaticus* and *A. trisulphuratus*. In tribe Lepioteae, *Lepiota americana* was studied.

**Family Coprinaceae:** The family comprises of the black or brown large spores, basidiocarps are typically fragile and bear shreds of ruptured cutis. Members of this family may be found on dead wood, dung, soil and litter. Eight species belonging to 3 genera coming under subfamilies Coprinoideae, Panaeoloideae and Psathyrelloideae were studied. In Coprinoideae *Coprinus extinctorius, C. lagopides*, *C. sterquilinus* were studied. In Panaeoloideae, *Panaeolus fimicola* was studied having deep purplish smooth spores. In *Psathyrelloideae, P. sathyrella, P. magambica, P. pygmaea, P. spadicea* and *P. tiarella* were reported

**Family Strophariaceae:** The family is characterized by the epicutis of the pileus that is composed of appressed narrow tubular often gelatinous hyphae. The basidiospores are brown and possess an apical germ pore or a minute apical discontinuity. The stipe and pileus are confluent. Two members of this family, *Pholiota squarrosa* and *Stropharia semiglobata* were collected. *S. semiglobata* is included under sub family Stropharioideae while *P. squarrosa* is included under the sub family Pholiotoideae.

**Family Crepidotaceae:** Family Crepidotaceae is characterized by fruit bodies that are shell-like, lacking a stem and laterally attached to dead and decaying wood. The mature gills and spores range in colour from cinnamon-brown to stuff-brown. Two species *Crepiodotus herbarum* and *C. quitensis* were studied.

## DISCUSSION

Considering the arid nature of this area, one may not think about the occurrence of mushroom because Thar Desert generally receives meager rains in comparison to other parts of India and this factor is coupled with a prolonged hot dry season. Therefore, it is logical not to expect the growth of mushroom which requires high humidity and low temperature. Earlier, surveys were conducted by Singh [8, 9], Nag *et al.*[10], Doshi *et al*. [11] in different areas of Rajasthan. Singh [8, 9] reported occurrence of edible mushroom from Rajasthan viz., *Agaricus spp*., *Pleurotus sp*p., and *Coprinus* spp. Nag *et al.* [10] reported 22 genera and 43 species of mushroom from Jaipur district. Doshi *et al*. [11] reported 94 species of macro-fungi of 52 genera belonging 7 families. Later Doshi and Sharma [12] enlisted 173 species from 95 genera of macro-fungi including Agricales, Aphyllophorales, Gastromycetes and Dacrymycetales from Rajasthan. Most of these genera have been collected from humid areas of southern Rajasthan but no intensive exploratory work was conducted in the arid areas of Rajasthan. Doshi *et al*. [11] collected only 13 species from 12 genera from arid zone of western Rajasthan. In the present investigation, 48 species belonging to 21 genera are reported from Thar Desert.

Maximum 14 species were recorded from the family Agaricaceae followed by 12 species of Amanitaceae, 8 species of Tricholomataceae and Coprinaceae respectively, 2 species of Strophariaceae and Crepidotaceae respectively. 1 species of Hygrophoraceae and Entolomataceae respectively. As far generic distribution is consumed maximum 6 genera were reported in and family Tricholomataceae followed by 4 genera in Agaricaceae, 3 genera in family Amanitaceae and Coprinaceae, 2 genera in family Strophariaceae, and 1 genus belongs to family Hygrophoraceae, Entolomataceae and

Crepidotaceae respectively. Maximum 9 species belong to *Agaricus* followed by 6 species of *Volvariella*, 5 species of *Termitomyces*, 4 species of *Psathyrella* and 3 species of *Coprinus* were studied while the left over genera had either 1 or 2 species.

It was observed that these mushrooms were found to occur on a variety of substrates and in different habitats. Species such as *Leucocoprinus zeylanicus, Agaricus purpurellus, Lepiota cepaestipes, Coprinus extintorius, C. strequilinus, Psathyrella magambica* and *P. pygmaea* were found to occur on diverse habitats which have not been recorded earlier. For instance, *Leucocoprinus zeylanicus* was always found from soil habitat but the during the present mycofloristic survey, it was collected from trunk of living tree of *Salvadora oleoides*. *Coprinus sterquilinus* previously reported from the mushroom beds but this is reported from grassy habitat. *Agaricus purpurellus* is found as solitary specimen but now reported gregariously on soil. *Psathyrella magambica* was reported on leaf litter but during the present studies it was found on rotting wood.

Threats of loss of mushroom diversity in the Thar Desert are of much concern since they play a significance role in human welfare. Over time, ever increasing anthropogenic activities have been accompanied by changes in the traditional pattern of land use, resulting in substantial alteration of habitats. Frequent droughts and a high livestock population are other threats. In more recent years, there has been ecological disturbances due to construction of the Rajasthan Canal (Indira Gandhi Nahar Project, IGNP).

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# ETHNOMYCOLOGY IN THE "TACANÁ VOLCANO BIOSPHERE RESERVE", CHIAPAS, MEXICO

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## ABSTRACT

In order to contribute to the rescue of the fast disappearing knowledge of mushrooms and their use held by the Mam ethnic group living within the "TacanáVolcano Biosphere Reserve", in Chiapas, Mexico, the use and knowledge of mushrooms was investigated by applying open unstructured and spontaneous interviews, to elderly speakers of the Mam language who were presented fresh biological material. Of the 50 fungi shown, informants acknowledged 16. Some names of mushrooms in Mam have no meaning in Spanish or Mam although phonetically some names are very similar to Spanish words. Most mushrooms are used for food, important mushrooms include *Lycoperdon umbrinum*, which is both edible and medicinal, *Amanita muscaria,* which is poisonous and used as cattle feed and *Agaricus sylvaticus* known as Xch'kbi lak' in the Mam language.The Mam-speaking inhabitants have developed ways of preparation and treatment for mushroom consumption and can identify mushrooms depending on the shape, appearance (mature or immature) and size they present. It is stressed that the knowledge of mushrooms possessed by this ethnic group at the studied site, is limited and on the threshold of disappearing.

**Keywords:** wild edible mushrooms, Mam, traditional use of mushrooms, toadstools

## INTRODUCTION

The knowledge and use of mushrooms inpre-Hispanic cultures located in what is now Mexico, played an important role and has been widely documented. Currently, in certain regions of the country, the mushroom-gathering tradition still persist and is carried out by the whole family for self-consumption (food, medicinal or religious purposes) and selling [1,2]. Empirical knowledge about the properties of mushrooms is part of the culture of many ethnic groups in this country. Many groups are able to differentiate between edible, poisonous and medicinal mushrooms [1].

Like other states in the country, Chiapas is known for its rich cultural and linguistic diversity. Of the 41 indigenous languages spoken, Tzotzil and Tzeltal are the most widespread, accounting for 36% and 34.4%, respectively. Mam is among the less represented languages; Mam speakers constitute 0.75% of the total indigenous population in the state [3] and live in isolated communities, in the mountains of the Sierra Madre de Chiapas,which border Guatemala [4]. The Mam ethnic group belongs to the mayatotonaco group, Mayantrunk, Mayan family, Yax sub-family and features 4 dialecticalvariants. These variants are comprehensible between each other mainly because the Mam settlements are geographically relatively close [5, 6]. Mam means father, but also has other meanings and names, including "mem" (stuttering, dumb) or "mame" (grandfather).The group is now known as Mam, Mameortokiol, all inter changeable terms [6-8].

In Chiapas, there are few studies on fungi, and even fewer on the knowledge, use and cultural significancethat mushrooms have within a community. It is therefore important to recover cultural, nutritional, medicinal and artisanal, knowledge, providing an insight that can be beneficial in everyday life. Mushrooms may be considered by certain ethnic groups as or more important than plants and /or animals. The aim of this study was to contribute to the rescue of mushroom knowledge and use by the Mam ethnic group in the Tacaná Volcano Biosphere Reserve; justified by the fact that studies of this nature have not been carried out in the reserve, and that the Mam ethnic group and culture is characteristic of this area but is fast disappearing, together with their culture and knowledge.

## TheTacaná Volcano Biosphere Reserve

The Tacaná Volcano Biosphere Reserve (TVBR) is located between coordinates15° 00´36´´- 15°12´00" LN and 92°00´17´´-92°15´00" LW; and includes the municipalities of Tapachula, Cacahoatán and Unión Juárez, covering an area that stretches from north west of the city of Tapachulato the border with Guatemala (Figure 1) (9, 10). The altitude of the study area ranges from1300-4100 m above sea level; the prevailing climate is (A) of semi-warm, humid temperate (according to Köppen), rainfall between summer and winter (May to October),with temperatures of over 18ºC, April being warmest. The mean annual temperature is 20°C, while the highest areas present a mean annual temperature of 10°C (10, 11, 12). Ecosystem types include: moorland, high and medium evergreen forest, montane cloud forest, pine and oak forest; with 56% of the area comprising of high and medium evergreen forest, 25% montane cloud forest. Montane rainforests (9,13) are present in some eco-regions of the Sierra Madre de Chiapas.

## Mushrooms in the TVBR

Previous reports indicate that there is significant mycological diversity in Chiapas. For a review of the work reported, we suggest revising the work of Andrade and Sanchez (14). Other later reports include: Ruan-Soto (15), focusing on the Ethnomycology of the Lacandon Jungle; Chanona-Gómez *et al.*(16) who studied macrofungi in Laguna Belgica Educational Park, Municipality of Ocozocoautla Espinosa, Chiapas; Alvarez-Espinosa (17) on the diversity and abundance of macrofungi in San Jose Bocomtenelté Educational Park, Municipality of Zinacantán; and a study by Alvarado-Rodriguez (18) that details Zoque ethnomycology in the town of Rayon, Chiapas. The only mycological study of the Tacaná Volcano Biosphere Reserve was carried out by Perez-Ibarra (19) consisting of a list of macroscopic fungi that grow there during the rainy season from June toNovember. In this study,42 species (37 ascomycetes and 5 basidiomycetes) were reported. Of these, fourteen species were edible. Furthermore, 13 new species for Chiapas were recorded: *Amanita fulva, A. gemmata, Armillaria ostoyae, Cantharellus cinnabarinus, Cyathus limbatus, Helvella lacunosa, H. macropus, Lycoperdon flavotictum, Ramaria botrytis, Russula emetica, R. sanguinea, Tremella mesenterica* and *Tricholomopsis formosa.*

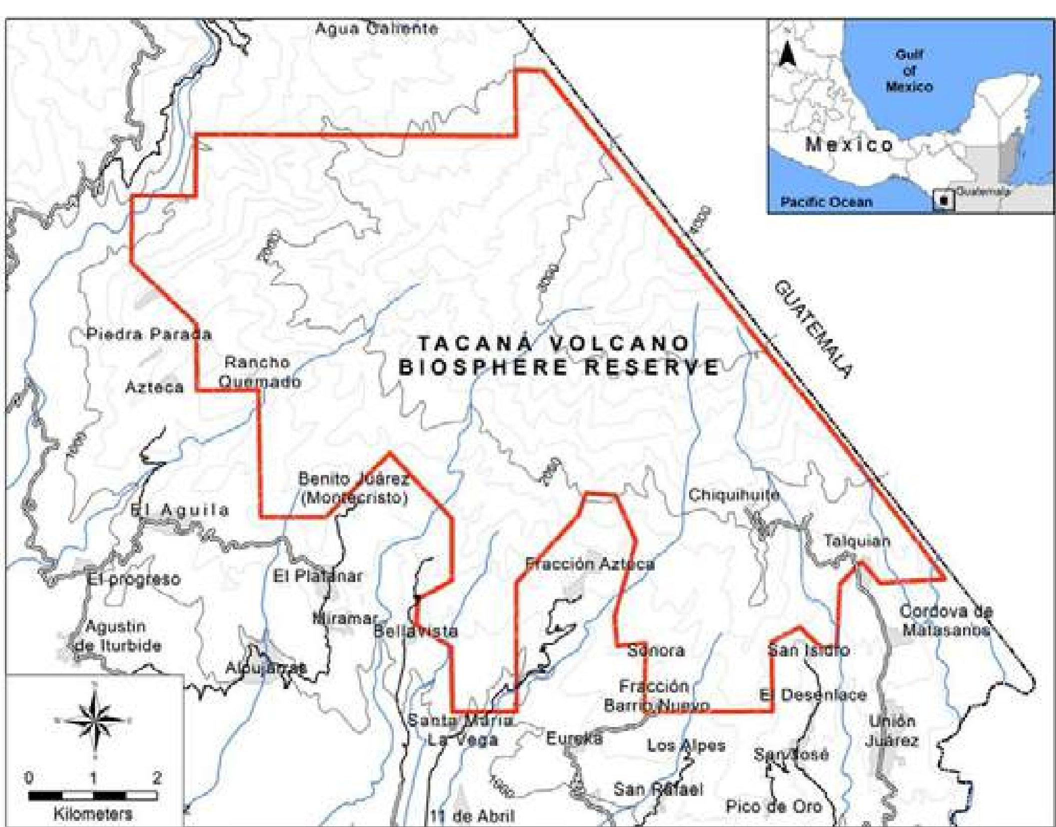


**Figure 1.** Locationof Tacaná Volcano Biosphere Reserve
Source: Geographic Information Lab (LAIGE) ECOSUR-Tapachula

## MATERIALS AND METHODS

### Study Subjects and location

The study concentrated on people that lived within the reserve and that could speak Mam. 15 people with these characteristics were detected, of which 11 were selected for this study. Study subjects averaged 65 years of age, ranging from 41 to 80 years. At all times Spanish was spoken; a Mam teacher from the local community helped with translating and any problems with communication.

We searched for and collected mushrooms at various sites within theTVBR. Our transect followed an already established route,well known by the inhabitants of the community, that runs from the village of Chiquihuites to the volcano summit. Mushroom gathering took place during the rainy season, from May to October, on a monthly basis. The biological material collected was described according to its morphological and ecological characteristics, according to Diaz-Barriga [1]; mushrooms were immediately placed in paper bags with their corresponding record; and carried in a plastic basket to facilitate transport on the steep volcano slopes.

### Interviews

Open and informal interviews were applied (in the form of talking) on the importance and use given to mushrooms in this region [21] Fresh biological material was subsequently shown to interviewees. The topic of the interviews was not limited to mushrooms, but included some economic, social and historical aspects, among others, all to avoid the stress of a formal "interrogation"[22].

The questions focused on the concept native people have on mushrooms, nomenclature, use, phenology, the source (substrate), location; and criteria for the identification of poisonous and medicinal mushrooms, preparation use, abundance and quality [23].The interviews were conducted during the six months we spent collecting biological material.The questions did not change and were applied to the same informants and at the same site, as suggested by Martinez-Alfaro [24].

### Drying and identification of mushrooms

The collected material was transferred to the facilities at the Ecosur Research Centre –in the city of Tapachula. They were dried, labeled and preserved in the mycological collection for further studies. Mushroom identification was carried out according to the literature. The characterization and dehydration methodology used was as proposed by Cifuentes *et al.* [25]. For taxonomic determination, standard techniques (26), and various general taxonomic keys [20, 27- 37] were used.

### Analysis of results

Information obtained during the interviews, was recorded in a field notebook.The data obtained from the informants was organized to produce tables and graphs (38). The information collected was analyzed so that it could be categorized into a dynamic constant comparison based on literature; each category was designated with a descriptive name [39].

## RESULTS AND DISCUSSION

In this study, mushroom collecting concentrated on species that the respondents use or know. Most of these mushrooms are large, so little emphasis was put on the collection of smaller fruiting bodies. Eleven people with Mam origins were selected and interviewed and more than 100 specimens of mushrooms (of which 50 were shown to the interviewees) were obtained belonging to the Basidiomycota and Ascomycota phyla.

The eldest respondents did not visit the places where the collections were made. They mentioned that mushrooms are scarce and have been increasingly harder to find. They also point out that few children and young people in the community have any knowledge related to mushrooms, indicating that the tradition is not being transmitted to the new generations.

The number of specimens recognized by respondents was 16. Table 1 shows the scientific name and name in Mam for each mushroom. It is noteworthy that three of the16 were not found (white mushroom *Pleurotus* sp, cob *Morchella* sp. and

**Table 1.** Mushrooms known by the Mam ethnic group living in the Tacaná Volcano Biosphere Reserve, Chiapas, Mexico

| Common Name | Name in Mam | Scientific Name | Fruiting period | Nutrition* | Substrate | uses |
|---|---|---|---|---|---|---|
| Red mushroom | Tx'iag'taporJak'tap | *Lactarius* sp. | May to August | M | Chicharro tree (*Platymiscium dimorphandrum*) | Edible |
| White mushroom | Sak'itzajorXa'ig'zak' | *Pleurotus* sp. | April | S | Alis tree (*Alnus* sp.), canac (*Chirantodendron pentadactylon*), and elder (*Sambucus* sp.) | Edible |
| Cob mushroomorcob | B'aj lag'orB'aj lak' | *Morchella* sp. | May to August | M | Ground around pine (*Pinus* sp.), pine leaves | Edible |
| White mushroom (in closter) | Rechum,Twi tok or Kui tok | *Sparassis crispa* | June to July | M | Cajete trunks**, Check fire tree**, coffee tree (*Coffea* sp.) | Edible |
| ———— | Xch' kbi lak' | *Agaricus sylvaticus* *Macrolepiota procera* | May to June | S | soil, sheep manure | Edible |
| SquirrelClaw | X' itx' k' ku' ku | *Schizophyllum commune* | May to August | S | Guarumo tree (*Cecropia* sp.) | Edible |
| Little yellow mushroom | X' ul | *Cantharellus cibarius* | | M | Alis tree (*Alnus* sp.) | Edible |
| Horn or deer antler | Tkach tx' iej | *Ramaria botrytis* | | M | Pine leaves | Edible |
| Blue mushroom | X' ew | *Lactarius indigo* | March to April | M | soil, pine leaves | Edible |
| Cattle belly | Tg' u'j wax | *Suillus tomentosus* | August | M | Chicharro tree (*Platymiscium dimorphandrum*) | Edible |
| ———— | Tzü | *Amanita muscaria* | May to Agust | M | Cow manure | Poisonous |
| Black mushroom | X' ewj, Tx' yolorX' oll | *Helvella lacunosa* | August | M | Pine root, below pine trees | Edible |
| Coyote | X o'j | *Boletus edulis* | June | M | soil | Edible |
| Dead eye(mature) | Wutz anim | *Lycoperdum umbrinum* | June to September | S | In the plain | Edible and medicinal |
| Ear | Xan cuch' | *Auricularia delicata* | July to September | S | In the plain | Edible |
| ———— | Tx' elok' | *Coprinus* sp. | ———— | S | Alis trunk (*Alnus* sp.) and elder (*Sambucus* sp.) | Edible |

**Note:** **for substrates labeled with two asterisks, no scientific name was found.
* saprophytes (S) y Mycorrhizal (M)

blue stain *Lactarius indigo*), for reasons attributable to the detrimental changes, which have taken place in recent years in the Reserve; the most important being: the presence of humans and animal grazing, burning and indiscriminate felling of trees. *Coprinus* sp.was another species that was not observed, however the respondents describe it as: brown, umbrella shape with black lamella, and when broken up turn to black like chicken droppings, as typically described for the genus [20].

Among the commonest genera found stand out: *Amanita*, *Lycoperdon*, *Helvella, Boletus, Schizophyllum* and *Suillus*. Less common genera include: *Lactarius, Auricularia, Lepiota, Agaricus, Geastrum, Cantharellus, Ramaria*; sporadic: *Sparassis*. The peak month for collection was June, when there is a vast diversity and abundance of mushrooms, especially in the higher parts of the Reserve. Some of the fungi identified in this study have previously been reported by Perez-Ibarra [19]; furthermore, their names in other languages and use by other ethnic groups have been reported by Guzman [40].

## Morphology

The Mam respondents associate the term “mushroom” with fruiting bodies of the agaricoid type (e.g. *Amanita*). When other fungi such as the boletoid type are mentioned it is common to designate them as “coyote” and the coraloid type as “deer horn,” even though they all belong to the Fungi Kingdom. This is also the case regarding **Xch’ kbi lak’** (*Agaricus sylvaticus*), a fungus without translation but considered as one of the most important and most represented fungi by this ethnic group.

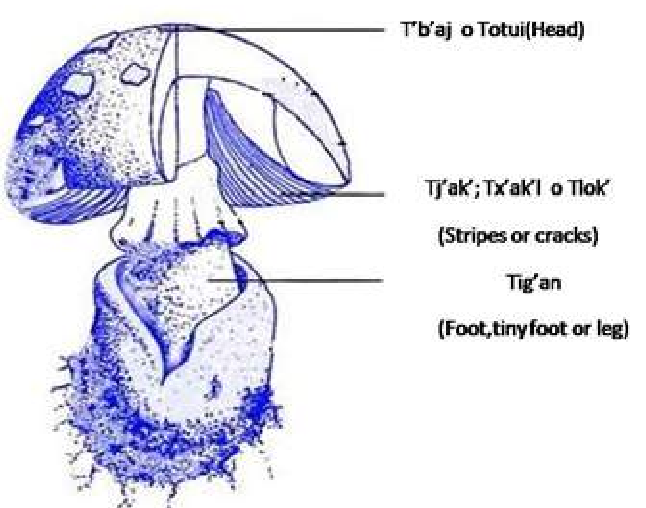


**Figure 2.** .Name of each part of a mushroom recognized by speakers of the Mam language

Respondents were able to recognize and name the different structures of a typical basidiocarp, such as the pileus, the hymenium and stipe; with the exception of ascomycota; unlike the Tzotzil ethnic group who only have knowledge based on the rhizomorphic mycelium or fruiting body, which they called “kaalzamte” or “root” [41].The names used for each structure are shown in Figure 2.

The names used to refer to each of the structures comprising a basidiocarp is evidence that the people of this ethnic group have acquired a substantial amount of knowledge relating to the following fungi genera: *Lactarius*, *Pleurotus, Agaricus, Cantharellus, Coprinus* and *Amanita*; unlike other species and genera, where these structures are not recognized. Moreover, the meanings of the fungi parts in English (pileus =head; hymenium=scratches or cracks and stipe=foot) which are given to the same structures of this phylum (Basidiomycota) are consistent with the names given to these parts in the Mam language [1, 22, 42].

## Phenology

Fruiting occurs from March to November (according to respondents). During the last two months of this period (October-November), the diversity and abundance of these organisms is considerable lower. The specimens were initially collected from May to September. Based on their mode of existence,11 species are mycorrhizal and 5 saprophytic (Table 1).

Some fungi show specificity towards a host plant (for example *Suillus* with *Pinus*), others are associated according to the age and successional stage of the plant or wood, such as *Laccaria* with young plants; *Boletus* and *Lactarius* with adult plants (Table1: substrate) [43].

However, most of the mushrooms, *Lactarius* sp., *Morchella* sp., *Sparassis crispa, Agaricus sylvaticus, Macrolepiota procera, Cantharellus cibarius, Ramaria botrytis, Suillus tomentosus, Helvella lacunosa, Boletus edulis*(representative

for the community mam), appear to be restricted to the volcano summit. The distribution of other fungi species is not completely defined, and they may be associated with more than one types of climate. Examples of this include the species *Amanitamuscaria*, *Cantharellus cibarius*, *Lactarius indigo* among others. Other fungi known to the ethnic group vary in their distribution and were found in areas ranging from temperate to warm where it is common to find saprophytic species such as *Pleurotus* sp., *Schizophyllum commune* and *Auricularia delicata* as reported by Bran *et al.*[44].

**Ecology**

The Mam indigenous inhabitants are able to differentiate several species of mushrooms.16 were identified in this study, the majority of them edible. The identification of these fungi is based on color, size, shape, texture, time of development and growth type of fruiting body, features that traditional taxonomy also take into account when discerning species.

Trying to identify particular mushrooms can be challenging and confusing; however, the Mam identify them through their ecology; examples being the red mushroom=Tx'iag'taporJak'tap (*Lactarius*sp.) usually foundon tree"chicharro" and other species that are found at specific sites or substrates. Most respondents commented that the fungi they recognize are found on the upper parts of the volcano. Each of these species may be found in different substrates (Table 1). Some studies provide ecological data for each of the mushrooms (from the time of year they can be found to the type of substrateon which they grow) matched with data provided by the studied community [1, 15, 20, 34, 44].

**Criteria used by the Mam ethnic group for traditional classification of mushrooms in the Volcano Tacaná Biosphere Reserve**

The Mam respondents identified mushrooms by their morphological characteristics; specifically consistency, with all the above mentioned species categorized as fleshy with the exception of *Auricularia delicata* included within the gelatinous category. Therefore, they do not recognize the ordering criteria mentioned by Mapes *et al.* [42] where by the classification of fungi, has varied according to morphology, phenology and even ecology.

The Mam respondents also classify fungi, according to whether they grow on soil or on "trunks" thus referring to the type of substrate on which they are found. This type of classification is consistent with that observed by several authors [39, 45, 46] who separate diverse groups of fungi populations according to the type of substrate (terrestrial fungi and fungi growing on wood), since the substrates present properties that result in important differences between both groups.

**Brief grammatical comparison of vernacular names used by the Mam ethnic group in theTacaná VolcanoBiosphere Reserve and in Guatemala**

The work of Arzu-Flores *et al.* [43] describes names in Mam of the mushrooms used for food in some parts of the Sierra of Guatemala and their translation into Spanish. The grammar is very similar to that presented by this ethnic group in Chiapas; but it is interesting to appreciate the meanings given to known mushrooms in Guatemala because most of them do not match those provided by members of the community under study, except one: *Ramaria* sp., known by both communities belonging to the same ethnic group, as "deer horn", ***Xew***is a Mamname provided by both communities, but which refers to different species on both the Mexican and Guatemalan side; X'ulmay indicate the same species, but the meaning may vary.

We tried to understand the meaning of each mushroom name obtained by comparing the names in the existing Carreño's Mam vocabulary[7]; for example for the term ***Tkach/tx'iej***, no similarities were found between the words provided by the group in our study and in this vocabulary. Probably the described term does not belong to the dialect type existing in the study area. However, data is consistent with the prevailing criteria used by the Mam ethnic group for the nomenclature of fungi, principally animal forms, associated plants or habitats, or mushroom colour.

Hosting *et al.* [47] do not specify the names of various fungi found in communities of Guatemala; however, the presence of *Boletus edulis* (Tpanku'k squirrel's bread), *Amanita caesarea* (Q'antzu), *Amanita muscaria* (Xq 'antzu), *Ramaria* sp. (Tuk'a Misat) and *Agrocybe aegerita* (TTx'yolil B'aqman) is confirmed and the authors note that the edible mushrooms *Lactarius indigo* and *L. deliciosus* do not present any names in Mam.

Many of the names provided by the Mam community, when translated into Spanish, are related with the morphological characteristics of mushrooms, such as "red mushroom" ***Tx'iag'tap*** named after its orange color and medium size, as well as appearing fleshier than individuals of the same or other species, but less developed. The "cob mushroom" ***B'aj lag'*** because the shape is similar to a corncob. Another example is ***Xch'kbilak'*** (*Agaricus sylvaticus*), that does not describe any particular features of the mushroom so the use of the name has gradually declined over the generations (in the Mamethnic group) and has no translation. In the case of ***Tzü*** (*Amanita muscaria*), the essential feature is its striking red color, but it does not have connection with the name in Mam.

The Mam names of some mushrooms are connected to their meaning in Spanish, and some others present similar phonetics, ***Tkach Tx'iej*** = horn or deer horn, projecting the sound "tkach' related to Spanish word "cacho" (horn). Chacón and Guzman [48] mention that several species of *Auricularia* (including *Auricularia delicata*) are known to the inhabitants of the Lacandon community as "chole", "ears" or "gummy ear" but may also have other common names such as "coolosh", "tzaan"inTzeltal [40,41].

Regarding nomenclature, the data provided by respondents is in keeping with Berlin's statement (49), that ethnobiological nomenclature usually refers to morphological features associated with its biological referent.

**Uses of mushrooms**

The Mam community uses most of their known mushrooms for food, although some areused in traditional medicine. Meso-American Indians also used mushrooms for their medicinal properties, an example being moldy tortill as containing various species of *Penicillium* (50) used to heal skin wounds.

There are mushrooms that are used for food and medicinal purposes, such as *Lycoperdon umbrinum* (in Mam= ***Wutz anim***) "dead's eye" with similar applications as those reported for *Geastrum saccatum* ("soil star" in central Mexico, or ichlo-um, lol-lum, pupulik, pupush-lu-um, sorta-ojo, xpupul-ikil, in the Yucatan peninsula) (40, 41, 51). Besides being used against the evil eye, boiled alone or with other plants and infusions, it is also used as a powder combined with other plants against asthma and to dry the navel of newborn babies.

In Chiapas, in addition to *Lycoperdon umbrinum,* there are other species such as *Bovista fusca*, *Calvatia cyathiformis, C. gigantea, Lycoperdon candidum, L. perlatum, L. pyriforme, Vascellumin termedium* and *V. pratense*, that are considered as edible and have medicinal uses when young ("when they are white"), such as coagulating blood and healing wounds, by directly applying the spores ("the powder"). These mushrooms are known by the name "bujtél", "bursting ball", "balls", "baby calves" and "little eggs" in other regions [15, 41, 43, 50].

The mushroom ***X'ew*** (*Lactarius indigo*) is used as a food both among the Mam and in the region of Acambay, Mexico. It has also been recorded as medicinal, as a purgative; however, its use in folk medicine is restricted [51, 52].

The majority of these mushrooms have been reported in coniferous forest, followed in order of importance by: oak forest, cloud forest, tropical forests, and agricultural and urban areas [53,54]; although Guzman [55] and Moreno-Fuentes *et al.* [56] indicate that most of the fungal biodiversity is found in tropical regions. Since many ethnomycological studies have been conducted in temperate are as, there is a lack of available data in tropical areas.

*Boletus edulis* (in Mam= ***X o'j***; English = coyote) is one of the fleshy fungi consumed by the Mam community, but it is also scarce and hard to find. There are records indicating that this fungus is edible and common in the Maya region with common names such as "zek um tulle " inTzotzil [50,51].

*Amanita muscaria* (in Mam= ***Tzü***, without translation to English) is known to be poisonous and is used as livestock feed, although there is no record of the latter use. It is a sacred mushroom in Latin America, as well as having important ethnomycological roots in Meso-america and possible ceremonial use among Mayans who identify *A. muscaria* as the "mushroom of Thunder", "mushroom Ray" or "weed Thunder "[40, 50, 51, 57]. In other parts of the world this species is also known as poisonous, medicinal or as an insecticide [58, 59].

*Schizophyllum commune* (in Mam= ***X' itx' k' ku' ku***) also known inother regions of Chiapasas "sulte" (wooden peel), is very popular and sold in the markets of Guatemala and is identified by the name of "asam". The studied community considered *S. commune* as edible and was one of the most frequently observed fungi in the study. It is one of the most consumed and is known invarious parts of the country and the world [15, 34,39]. This species is also reported in traditional Chinese medicine, used against leucorrhoea [60].

*Auricularia delicata* (Mam = ***xancuch'***) recognized as edible in both the Mam and Lacandon communities [15]. Ying*et al.* [60] refer to several species of the genus *Auricularia* in the Chinese pharmacopoeia as medicine for the stomach and body, improving blood circulation and preventing bleeding.

The Mam informants are not aware of the existence of a fungus with recreational use (as a toy); however, in the town of Teapa (Tabasco) people recognized the genus *Auricularia* as having this use [39]. The Mam informants considered Tx'elok' (*Coprinus*sp.) edible, although Guzman [61] reported no practical properties for this mushroom.

The use of species found in the Mam community during this study was distributed as follows:14 edible, one medicinal and one without use but recognized as poisonous.

These results concerning Mam autochthonous knowledge on species of fungi are similar to those found by Hosting*et al.* [48] in several Mam municipalities in Quetzaltenango, Guatemala. In a study conducted between 1994 and 1998; the authors mention that most of the farmers in the study areas recognized about six mushrooms by name at most and twelve as edible, inedible or poisonous.

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# SPAWN CRYOPRESERVATION OF *AGARICUS BISPORUS* AND *A. SUBRUFESCENS* STRAINS

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## ABSTRACT

One of the main problems for conservation of mushroom germplasm is that the traditional method of subculturing facilitates aging and contamination of cultures. The conservation of strains at ultra-low temperature in liquid nitrogen (-196 °C) is a widely used method, however, this method become expensive in collections with large number of strains and requires highly specialized personnel. The aim of this study was to evaluate the viability of spawn of *A. bisporus* and *A. subrufescens* strains frozen at different temperatures (-20 and -80 ºC). Two strains of *A. subrufescens* and one strain of *A. bisporus* were studied using spawn prepared in sorghum seeds. Fully-incubated sorghum seeds were placed in polycarbonate vials with a sterile glycerol solution (10% v/v) (G) or without glycerol (WG) and frozen at -20 or -80 ºC. After 3, 6 and 12 months the samples were thawed (30 °C, 10 min) and the percentage of sample recovery was evaluated. Mycelial growth was also evaluated in potato dextrose agar (PDA) or a medium with glucose and compost extract (C) recording mycelial diameter after 7 days of incubation at 25 °C. The results showed that in the samples frozen at -20 °C, the recovery was minimal (2 to 4%). However, the samples frozen at -80 °C showed a recovery of 100% from 1 to 6 days after thawing. The recovered samples had a significantly higher growth in the medium C. In all cases the recovered samples produced normal basidiomata when cultivated on compost substrate.

**Keywords**: white button mushroom, *Agaricus subrufescens*, ultra-low temperatures, edible mushrooms, germplasm conservation

## INTRODUCTION

Edible mushrooms of the genus *Agaricus* are currently valued not only for their nutritional properties but also for their medicinal and organoleptic qualities.The white button mushroom, *A. bisporus* (JE Lange) Imbach, is the most cultivated edible species in the world, with an estimated production of over 4 million tonnes annually [1]. Its nutritional and culinary properties have given it an important place in the world market preferences. Moreover, *A. subrufescens* Peck (named also in some publications *A. blazei* Murrill or *A. brasiliensis*), popularly known as cogumelodo sol, almond mushroom, or Himematsutakeis a well recognized species for its medicinal properties and for its delicate flavor [2]. One of main features of this latter species is its ability to grow at relatively high temperatures which makes it ideal candidate for growing in tropical and subtropical areas [3].

Growers face difficulties for maintaining spawn under refrigeration for long time periods without loss of mycelium viability and breeders and biologists have to find new preservation methods of the genetic resources other than conventional storage of mycelium at low temperature. Most of Basidiomycetes are usually preserved by successive mycelial subculturing but this method occasionally causes genetic degenerations, lost of commercial characteristics and contamination by other microorganisms (mainly moulds and bacteria) [4]. Furthermore this method become expensive to maintain collections with many strains and requires highly specialized staff for managing them. On another hand, cryopreservation with liquid nitrogen at -196 ºC has been successfully used for preservation of fungal species [5]. Other methods have been also tested for mycelial preservation [6], however one of the cheapest method is to use spawn of the strains as an alternative support for preservation [3, 6-9]. The use of spawn as an alternative support for preservation of *Agaricus* genetic resources has been proposed by several authors after San Antonio and Hwang [10] and Mata and Pérez-Merlo [8]. Among *Agaricus* species, *A. subrufescens* is the only species of the genus known to suffer damage when exposed for prolonged periods at temperatures of 4 °C or lower [11]. It diminishes its ability to grow in relatively short periods of time [3]. With the objective to find

options of spawn preservation, the aim of this work was to evaluate two different temperatures (-20 and -80 ºC) to preserve spawn of *A. bisporus* and *A. subrufescens*.

## MATERIALS AND METHODS

### Studied strains and spawn preparation

Three strains of *Agaricus* genus from different origins were studied: IE 623 strain of *A. bisporus* native from Mexico and strains of *A. subrufescens* IE 829 (= INRA Ca 454) originating from Brazil and IE 830 (= INRA Ca 487) originating from France, see Llarena *et al*. [12] for strain description. The strains were maintained in culture medium of potato dextrose agar (PDA). The strains were cultured for 7 days in Petri dishes with PDA. Spawn was prepared according to the method of Guzman *et al.* [13] in sorghum seeds (*Sorghum vulgare* Pers.), 65% hydrated and sterilized at 121°C for 1 h. The seeds, placed in Petri dishes were inoculated with a disc (± 0.5 cm diameter) of PDA with mycelium pre-cultures of each strain and incubated in the dark for 3 weeks at 25 °C to allow the grains to get completely covered by mycelium.

### Freezing samples (-20 and -80ºC)

For freezing samples the method proposed by Mata and Perez Merlo [8] was used. Fully incubated sorghum seeds were placed in sterile polycarbonate (Nalgene) vials (25 seeds per vial) each vial containing 1.5 ml of sterile cryoprotectant solution prepared with 10% glycerol (v/v). The seeds remained in contact with the cryoprotective solution for 1 h (G) and then samples were placed in polycarbonate boxes and transferred directly into the freezer at -20 or -80 ºC. Moreover vials containing 25 seeds without glycerol were also prepared (WG) and immediately placed into the freezers. All samples were kept frozen at -20 or -80 ºC for 3, 6 and 12 months (3M, 6M, 12M). After that time samples were thawed in polycarbonate boxes by dipping in water at 30 °C for 10 min [7]. Once thawed, the vials were cleaned for 1 min in an alcohol solution (70% v/v) and then seeds were removed from vials and placed in Petri dishes with PDA and incubated at 25 °C.

### Viability and vitality of the samples

After treatments the percentage of sample recovery was evaluated through daily observations of the seeds. A sample was considered recovered when mycelial growth was noted by observing the seeds with a stereoscopic microscope. The delay for recovering was also recorded (maximum 15 days). For each treatment (G, WG) and time of freezing (3M, 6M, 12M) recovery was evaluated with 50 spawn seeds.

Moreover, mycelia recovered from freezing treatments were used to prepare new spawn in order to evaluate mycelial growth in two culture media: PDA and other culture medium prepared with agar, glucose (100 g/l) diluted in compost extract (C). Mycelial diameter was recorded by placing a spawn seed in a Petri dish with PDA and C on 2 perpendicular axes after 7 days of incubation at 25 ºC [14]. Ten samples were prepared per treatment and strain.

### Basidiomata production test

To asses basidiomata formation, samples of 4 kg of commercial compost were inoculated with spawn (5%) prepared as above from each of the strains recovered after freezing at – 80 ºC. After 15 days of incubation at 25 ºC a casing layer of 5 cm was added to the samples and incubation continued for another 7 days. To favor appearance of primordia, samples were placed in a room with relative humidity of 90 – 95%, ventilation for 10 min 4 times a day, 12 h light and 12 h dark at 18 ºC for IE 623 strain and at 25 ºC for IE 829 and IE 830 strains was provided. Formation of basidiomata was registered.

### Statistical analysis of data

Data recorded for mycelial diameter were analyzed using ANOVA followed by Tukey's multiple-range test ($p = 95\%$) to identify statistical differences in the average diameter of the mycelia obtained with different treatments.

## RESULTS AND DISCUSSION

The results showed that in the samples frozen at -20 °C the recovery was minimal. In the 3M frozen samples, only one strain of *A. subrufescens* (IE 829) exhibits a recover percentage between 2 and 4%. No strain was recovered at 6M or 12M in samples frozen at -20 ºC. However, the samples frozen at -80 °C with or without cryoprotectant (G, WG) during the freezing times tested (3M, 6M, 12M) showed a recovery of 100% from 1 to 6 days after thawing (Table 1). In all strains and conditions tested at -80 ºC, the recovered mycelia showed normal appearance in color and texture, with no apparent alteration (Fig. 1).

**Table 1.** Percentage of recovered samples after frozen at -20 or -80 ºC with (G) and without glycerol (WG)

| | | -20 ºC | -80 ºC | | |
|---|---|---|---|---|---|
| | | 3M | 3M | 6M | 12M |
| G | IE 623 | 0 | * (5) | * (5) | * (3) |
| | IE 829 | 2 (14) | * (2) | * (5) | * (3) |
| | IE 830 | 0 | * (2) | * (6) | * (2) |
| WG | IE 623 | 0 | * (2) | * (5) | * (3) |
| | IE 829 | 4 (14) | * (2) | * (5) | * (5) |
| | IE 830 | 0 | * (1) | * (5) | * (1) |

* Means total recovery of 50 samples = 100 % recovery.
Numbers in parentheses are days for recovery.

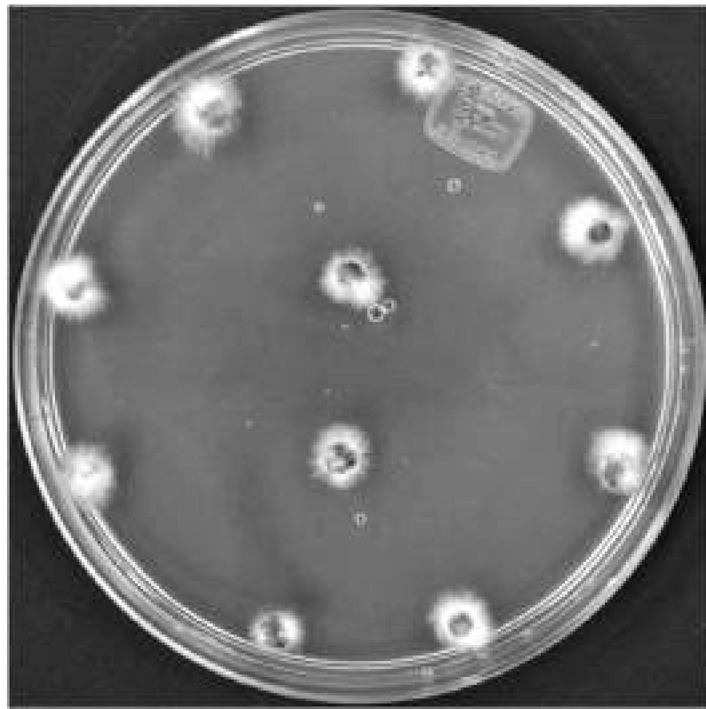

**Figure 1.** Mycelia recovered after 12 months of freezing at -80 ºC with glycerol (G) as a cryoprotectant

In Table 2 the results of the mycelial growth of the strains frozen at -80 °C are shown. In general, all strains showed higher growth in the culture medium C with significant differences from the average obtained in PDA,the medium of compost extract and glucose seems to favor mycelial growth after freezing. The average growth of the samples recovered from the WG treatment was 46.1mm while in treatment G was 47.0 mm, so it was not possible to establish significant differences between treatments. These results confirm those obtained in other studies on the recovery of mycelia frozen without cryo protectant using seeds as vectors [3, 8, 14].

The strain IE 623 of *A. bisporus* showed no difference in their growth both 3M and 12M samples in treatments G and WG in both culture media (PDA and C). Further more the strains IE 829 and IE830 of *A. subrufescens* showed significantly lower growth after 12 months. Under similar conditions, using vectors of sorghum seed, but frozen in liquid nitrogen, these *A. subrufescens* shown greater recovery than strain IE 623 of *A.bisporus* [3], however, the results obtained in this work confirm that the strains of *A.subrufescens* are sensitive to low temperatures [3, 6], and therefore, protocols for preservation must be carefully selected and tested. The average mycelial growth in samples recovered from the 3M (49.9 mm) and 12M (43.2 mm) showed significant differences, suggesting as lightly negative effect due to freezing.

Spawn prepared from mycelia recovered after freezing at -80 °C (3M and12 M) showed a normal growth in compost, completely covering the substrate at 15 days and having

**Table 2.** Micelial diameter in mm after 7 days of incubation in samples recovered from -80 ºC

| | IE 623 | | | | IE 829 | | | | IE 830 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PDA | | C | | PDA | | C | | PDA | | C | |
| | G | WG | G | WG | G | WG | G | WG | G | WG | G | WG |
| 3M | 20.6a | 23.7a | 50.8b | 52.8b | 44.6b | 48.6b | 72.5d | 68.7cd | 39.9b | 33.0ab | 75.3d | 66.5c |
| 12 M | 22.1a | 24.3a | 54.1b | 55.3b | 24.6a | 31.7a | 61.8c | 61.2c | 30.1a | 28.9a | 66.7c | 58.3c |

Different in the columns by species indicate significant differences in mycelialdiametres using Tukey's multiple range test ($p$ = 0.05 5).

primordial from day 21, which means 6 days after adding the casing. The basidiomes obtained in all cases showed normal morphological (color, size, shape) and organoleptic (odor) characteristics (Fig. 2).

**Figure 2.** Basidiomata of *Agaricus subrufescens* (strain IE 830) obtained after 12 months of freezing at -80 ºC

## CONCLUSION

Samples frozen at -20 °C showed a minimal recovery (2 to 4%), however, samples frozen at -80 °C showed a recovery of 100% with or without cryoprotectant (G, WG) in all freezing times tested (3M, 6M, 12M). The recovered samples had a significantly higher growth in the medium prepared with compost extract and glucose (C), so this culture medium could be recommended to favor recovery samples. The results showed a slight decrease in growth in the samples of *A. subrufescens* strains recovered if 12 M samples are compared with 3 M samples. In spite of good results obtained in the recovery of samples, strains of *A. subrufescens* appear to be more sensitive than *A. bisporus* to freezing at -80 ºC. Although in all recovered strains normal basidiomata were obtained when they were grown in compost, it would be advisable to test freezing during longer periods of time.

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# PHYLOGENETIC RELATIONSHIPS OF *GANODERMA* SPECIES BASED ON MITOCHONDRIAL AND NUCLEAR DNA SEQUENCES FROM TAMIL NADU, INDIA

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## ABSTRACT

Phylogenetic relationship of *Ganoderma* species confining to Tamil Nadu was assessed by three molecular markers viz., internal transcribed spacer region (ITS) 1 and 2, β-tubulin and ATP synthase subunit 6 (*atp*6). Forty-three isolates representing 11 species of *Ganoderma* were identified from ITS rDNA sequence data. The nucleotide variations found in the ITS region were suitable for discriminating the eleven species viz., *G. australe*, *G. cupreum*, *G. lucidum*, *G. resinaceum*, *G. tropicum*, *G. weberianum*, *Ganoderma* sp. 1, *Ganoderma* sp. 2, *Ganoderma* sp. 3, *Ganoderma* sp. 4 and *Ganoderma* sp. 5. The terminal clade represent that species and/or species complex were consistent with geographical origin of the isolates and cultural characters. The β-tubulin gene phylogeny provided more robust phylogenetic information for separating ten *Ganoderma* spp. than the ITS region and found to be suitable in identifying the *Ganoderma* species and/or species complex. The *atp*6 gene sequence data acquired highest phylogenetic informative character than nuclear DNA genes; however, due to the very low levels of nucleotide divergence, the gene is suitable at genus or lower level. *Ganoderma* spp. inferred from these three un-linked loci resulted in a robust phylogeny. The terminal clades of nuclear DNA genes represented the species or species complex and consistent with cultural characteristic, host relationship and biogeographical distribution rather than *atp*6. This study implies that the higher variability in the nucleotide sequences of the nuclear DNA genes have the potential to be the robust markers for molecular identification than *atp*6. Chlamydospores in culture provided valuable information in identifying *Ganoderma* spp.

**Keywords:** *Ganoderma*, ITS rDNA, β-tubulin gene, *atp*6, chlamydospores, multigene phylogeny, biogeography

## INTRODUCTION

The genus *Ganoderma* is a member of the Ganodermataceae, a family distinguished by unique double-walled basidiospores. *Ganoderma* has a world wide distribution growing on numerous perennial, coniferous and palmaceous hosts. Species of *Ganoderma* are the causal agents of root and stem rots [1] however, they are considered as the 'herb of longevity' [2]. The genus *Ganoderma* for the laccate and stipitate *Polyporus lucidus* W.Curt was established in 1881 [3] and later erected to the family Ganodermataceae based on the unique double-walled basidiospores [4]. Mycologists from different parts of the world used various criteria for the identification of *Ganoderma* sp. Approximately, 219 species have been described so far and 30% of name in *Ganoderma* have been proposed as synonyms [5]. The macroscopic (pileus, stipe, shape, size, color, context, tube) and microscopic (hyphal system, basidiospores and pilocystidia) characters have been used to distinguish the species [6]. However, the macro-morphological characters are influenced by environmental factors [7], the species identification and circumscription are often difficult and controversial [8] and there are many synonyms and several species complexes have been recognized [6, 7, 9]. Besides basidiomata characters, cultural studies were also investigated [6, 10-13] to discriminate the *Ganoderma* sp. The cultural characters are less polymorphic than the morphological characters [14]. The presences of chlamydospores in the cultures are considered to be useful character in distinguishing between the taxa [15]. Besides, isozyme analysis was carried out to investigate the species [16]. The molecular data have great potential in elucidation of the relationships between taxa and together with recent methodological advances, have instigated a resurgence of interest in phylogeny reconstruction. DNA taxonomy system provides a new scaffold for the accumulated taxonomic knowledge and convenient tool for species identification and description where traditional micro-morphological approaches have failed. Fungal systematists have begun to explore various protein-coding genes, including β-tubulin [17] and mitochondrial *ATP*6 [18]. The number of loci that are used in fungal molecular systematics will continue to grow, especially as more complete fungal genome sequences are produced. For the past one decade, *Ganoderma*

have been explored in different parts of the world using molecular techniques. Phylogenetic studies in *Ganoderma* using rDNA region and confirmed that variation found in the ITS rDNA could be appropriate for the species level identification [14]. Their study implied that many names reported earlier were synonyms. Similarly, ITS marker was used to distinguish the *Ganoderma* species in Australia and South America respectively [16, 19]. In India, all studies for identification was confined to macro-morphological, microscopical characters and cultural studies of the basidiomata alone. Therefore, many names used could be synonym, especially *G. lucidum*, *G. australe* and *G. applanatum* are more frequently described, which led to contradictory view of *Ganoderma* spp. in India and this also emphasized the importance of molecular tool in taxonomic studies. The aim of the present investigation is to identify the *Ganoderma* isolates collected from Tamil Nadu, India through ITS rDNA locus and to infer the phylogenetic relationship of *Ganoderma* spp. Furthermore it is to investigate the utility of partial β-tubulin (nuclear) and *atp*6 (mitochondrial) genes in molecular systematics of *Ganoderma*. Systematics of *Ganoderma* isolates are also ascertained from the combined dataset of nuclear (ITS and partial β-tubulin) and a mitochondrial gene (*atp*6).

## MATERIALS AND METHODS

### Organisms

Table 1 lists the isolates from Tamil Nadu examined in this study, which includes both basidiocarps and culture collections. Cultures were raised from context tissue of the fresh basidiomata and grown on PDA medium (Difco) at 25 °C.

### Cultural characters

The chlamydospore characters were studied only in twelve isolates that could be cultured *in vitro*. The occurrence, abundance, shape, size, and reaction to Melzer's reagent were recorded. The results were later compared with molecular data.

### Molecular techniques

DNA was isolated using 3% SDS extraction buffer according to the method described elsewhere [14].The ITS-rDNA region was amplified using primers ITS1 and ITS4 [20] in a reaction volume of 25 ml, as follows: 1.25 mM of each dNTP, 3.5 ml of dd$H_2O$, 2.5 ml of 10x PCR buffer containing 1.5 mM $MgCl_2$, 2.5 ml of BSA (10 mg / ml), 10 mM of each primer, 0.5 U of *Taq* polymerase and 10 ml of genomic DNA (5-10 ng). Thermal cycling was as follows: one cycle of 95 °C for 2 min; 36 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1.30 min; and one final cycle of 72 °C for 5 min. Partial β-tubulin gene was amplified by a modified method [21]. The PCR amplification of β-tubulin gene was performed with a primer pair B36/B12 (5'-CACCCACTCCCTCGGTGGTG-3') and (5'-CATGAAGAAGTGAAGACGCGGGAA-3') targeting exon 5 and 6 respectively. Mitochondrial gene, *atp*6was amplified by the modified method [18]. The ligonucleotide primers, ATP6-3 (5'-TCTCCTTTAGAACAATTTGA-3') and ATP6-2 (5'-AATTCTANWGCATCTTTAATRTA-3') were used to amplify the partial *atp*6 gene region. PCR products were purified with QIA quick PCR purification Kit (QIAGEN) and sequenced by the Big Dye v. 3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according the manufacturer's instruction, except that 2 ml of Big Dye was used and the reaction volume was 10 ml. PCR templates were sequenced in both directions using the respective forward and reverse primers. Sequencing reactions were run in an ABI PRISM® 3100 Genetic Analyzer (AppliedBiosystems). Bidirectional reads were assembled and edited in Sequencer™ version 4.2.2. (Gene Codes Corporation, Ann Arbor, MI, USA).

### Phylogenetic analyses

First analysis was conducted using only the Tamil Nadu sequences listed in Table 1 with the addition of a sequence of *Amauroderma rude* from Taiwan as outgroup taxon [14]. Sequence alignment was done manually and exported to PAUP* [22] for maximum-parsimony (MP) analyses. MP analyses in PAUP* was conducted as follows: alignment gaps treated as missing data, characters weighted equally, 100 heuristic searches with random addition sequence keeping no more than 100 trees in each replicates, TBR branch swapping, and branches collapsed when minimum length was zero. Bootstrap analyses used 1,000 replicates of random addition sequence with TBR branch-swapping, keeping no more than

10 trees per replicate, and retaining groups with frequency > 70%. The method of assessing the combinability of data sets, and the one adopted in this study, is by simply comparing highly supported clades among trees generated from different data sets to detect conflict [23]. If no conflict exists between the highly supported clades in trees generated from individual data sets, it suggests that the genes share similar phylogenetic histories and combining the data sets could ultimately increase phylogenetic resolution and support. BLAST searches in the NCBI database (Genbank) were used to retrieved highly similar sequences to the Tamil Nadu sequences produced in this study.

## RESULTS AND DISCUSSION

Seventeen isolates of *Ganoderma* were collected from different parts of Tamil Nadu were listed in Table 1.

### Cultural studies

*Ganoderma* sp. 1, 3, 4, 5 and *G. australe* do not produce chlamydospores in culture. *Ganoderma* sp. 2 produced inamyloid and amyloid chlamydospores were observed in cultures. The size ranges from 8 µm × 9-11 µm; globose or elliptical; intercalary or terminal in position. In *G. resinaceum,* they were ellipsoidal, hyaline, smooth, dexrinoid, 8.5-14 × 11.5-14 µm; intercalary in position and rarely terminal.*G. tropicum* produce inamyloid cylindrical chlamydospores with the size ranges from 4.5-6.0 µm × 12-6 µm. *G. weberianum* produced abundant chlamydospore with the size ranging from 4.5-7 µm × 8-12 µm; globose, subglobose and elliptical shape but they were not striated; intercalary in position; negative in Melzer's reagent. In addition, gastrospores were observed after 5 weeks.

### Phylogenetic analysis of ITS rDNA region

PCR amplification of ITS region of *Ganoderma* was ca. 560 bp. All the positions were generally able to be aligned within the collections of the same species; whereas alignment across the species required gaps. The ITS region varies in length from 546 to 562; the length of ITS1 ranges from 196 to 208 and sequences were aligned in 227 positions; ITS2 ranges from 184 to 199 and aligned in 212 positions. There is negligible amount of difference in AT% (50.3) and GC% (49.7) in ITS region. Seventeen different sequences were identified among the native collections examined (Table 1). Of the 429 characters included in the maximum parsimony (MP) analysis, 240 were constant; 63 variable characters were parsimony uninformative; 126 were parsimony informative. Tree statistics were; tree length=403, consistency index (CI)=0.6402, retension index (RI)=0.8294, rescaled consistency index (RC)=0.5310, homoplasy index (HI)=0.3598. Parsimony analysis based on an alignment of these sequences indicated that the isolates were grouped into 10 well supported clades as measured by bootstrapping (Fig 1). Phylogenetic analysis of ITS rDNA was useful in the identification and classification of 17 isolates of *Ganoderma* into 11 species viz., *G. australe* (5), *G. cupreum* (1), *G. lucidum* (11)*, G. resinaceum* (2), *G. tropicum* (2)*,G. weberianum* (1)*, Ganoderma* sp. 1 (1), *Ganoderm*sp. 2 (1), *Ganoderma* sp. 3 (7), *Ganoderma* sp. 4 (2) and *Ganoderma* sp. 5 (10) (Table 1). The five *Ganoderma* spp. 1, 2, 3, 4 and 5 remained unidentified since they occupied isolated position in ITS phylogenetic tree (Fig. 1).

### Non-laccate species (*Ganoderma* subg. *Elfvingia*)

Two strains viz., YER03 and K39 clustered with intersterile groups 1 and 2 of Taiwan isolates TAI-01 (94% Bootstrapping (BS)) and TAI-05 (100% BS), respectively. Although *G. australe* Group 1 (Yercaud) and Group 2 (Kodaikanal) were collected from different localities but they fall on different altitude i.e. below 1500 mts and above 2133 mts respectively. The result obtained in the present study is consistent with the earlier reports [24, 25]. *Ganoderma* sp. 5 was placed in isolated position with 100% BS, and hence considered as a distinct species. More samples were required from other parts of world to study the phylogenetic relationship.

### Laccate species (*Ganoderma* subg. *Ganoderma*)

*G. cupreum* MYC1 tended to group together with collections from Australia with 100% BS. *Ganoderma cupreum* has been referred to as *G. chalceum* in previous literatures [7] however, *G. cupreum* has nomenclatural priority [5, 16]. *G. cupreum* seems to be distributed in Australia, Pacific, Northern Africa [26] and Asia (present study). Additional molecular

**Table 1.** ***Ganoderma*** **isolates sequenced in this study and sequenced by Dr. Moncalvo**

| Determination | Source & Collection Number[a] | Geographical origin, Host | Gene Bank Accession |
|---|---|---|---|
| *G. adspersum* | CBS351.74 | Europe, *Salix* sp. | X78742/X78763 |
| *G. ahmadii* | FWP 14329 | Pakistan, *Dalbergiasissoo* | Z37047/Z37098 |
| ***G. australe*** | **YER03** | **Yercaud, India, *Bougainvillaea* sp.** | **AY993914/AY993915** |
| ***G. australe*** | **K39** | **Kodaikanal, India, Hardwood,** | **AY993916/AY993917** |
| *. australe* | RSH.0705 | Taiwan, N.d. | X78750/X78771 |
| *G. boninense* | LKM254 | Sabah, Malaysia, Palm | |
| *G. cupreum* | DFP3896 | Australia, *Casuarina* sp. | AJ27586/AJ627587 |
| *G. cupreum* | QFRI4336 | Australia, Dead wood | AJ627588/ AJ627589 |
| ***G. cuperum*** | **MYC1** | **Coutrallum, India, Hard wood** | **DQ051906/DQ015907** |
| *G. capense* | ACCC5.71 | China, N.d. | Z37070/Z37104 |
| *G. curtisii* | JM96/80 | North Carolina, USA, | Check |
| *G. lobatum* | BAFC651 | Chile, Dead stump | AF169983/AF169984 |
| ***G. lucidum*** | **I5** | **Chennai, India, Hard wood** | **DQ015904/DQ015905** |
| ***G. lucidum*** | **G14** | **Chennai, India, *Borassusflabillifer*** | **DQ011079/DQ011080** |
| "*G. lucidum*" | ATCC32472 | India, N.d. | X87351/ X87361 |
| "*G. lucidum*" | RSH.G001 | Taiwan, | X87345/ X87355 |
| *G. lucidum* | CCRC37053 | Taiwan | - |
| *G. lucidum* | RYV33217 | Norway, Hardwood | Z37096/Z37073 |
| *G. microsporum* | RSH.0821 | Taiwan, *Salix babylonica* | X78751/X78772 |
| *G. praelongum* | ATCC52410 | Argentina, *Platanusacerifolia* (?) | - |
| *G. resinaceum* | CBS152.27 | United Kingdom, N.d. | Z37062/Z37085 |
| ***G. resinaceum*** | **PTK3** | **Chennai, India, *Tamarindusindica*** | **DQ011095/DQ011096** |
| *G. subamboinense* | ATCC52419 | Argentina, *P. acerifolia* | X78736/X78757 |
| ***G. trophicum*** | **MYC6** | **Coutrallum, India, *Albizzialebbeck*** | **DQ011093/DQ011094** |
| *G. cf. tropicum* | RSH.1111 | Taiwan, N.d. | Z37068/Z37088 |
| *G. valesiacum* | CBS 282.33 | United Kingdom, *Larix*sp. | Z37056/Z37081 |
| *G. weberianum* | CBS219.36 | Philippines, *Mangifera* sp. | X78734/X78755 |
| ***G. weberianum*** | **19** | **Vandalur, India, *Polyalthia* sp.** | **DQ001761/DQ001762** |
| *G. weberianum* | DFP8405 | NSW, Australia, *Mangifera*sp. | - |
| *G. zonatum* | ME-GAN-33 | Florida, Oil palm | - |
| ***Ganoderma* sp.** | **CNB1 TN** | **Coonoor, Hard wood** | **DQ011635/DQ011636** |
| *Ganoderma* sp. | MW1 | Ontario, Canada, Chestnut. | - |
| ***Ganoderma* sp.** | **CN2** | **Coonoor, India, hardwood** | **DQ011633/DQ011634** |
| *Ganoderma* sp. | TNAU-CRS-1 | Veppankulam, India, *Cocosnucifera* | AY508882 |
| *Ganoderma* sp. | QFRI8647.1 | QLD, Australia, Dead wood | - |
| *Ganoderma* sp. | JMHK93.9 | Honk Kong, Hardwood | Z37069/Z37089? |
| *Ganoderma* sp. | JMCR.132 | Costo Rica, N.d. | AF255138 |
| *Ganoderma* sp. | JM97/3 | North Carolina, N.d. | AF255094 |
| *Ganoderma* sp | TAI-01 | Taiwan, N.d. | AF255110/AF255111 |
| *Ganoderma* sp. | ME-GAN-14 | Florida, N.d. | AF255130 |
| *Ganoderma* sp | TAI-05 | Taiwan, N.d. | AF255193/AF255194 |
| ***Ganoderma* sp.** | **MC3** | **Tambaram, India, Hard wood** | **DQ011623/DQ011624** |
| ***Ganoderma* sp.** | **G09** | **Chennai, India,*Tamarindusindica*** | **DQ015908/DQ015909** |
| ***Ganoderma* sp.** | **MC5** | **Tambaram, India, *Pterolobium* sp.** | **DQ011625/DQ011626** |
| ***Ganoderma* sp.** | **G05** | **Chennai, India, *B. flabellifer*** | **DQ056318 /DQ056319** |
| ***Ganoderma* sp.** | **ANN3** | **Chennai, India, *Cassia roxburgii*** | **DQ011097/DQ011098** |
| ***Ganoderma* sp.** | **CPT** | **Chengalpet, India, *Mangiferaindica*** | **DQ011103/DQ011104** |
| ***Ganoderma* sp.** | **IFE** | **Chennai, India, *Feroniaelephantum*** | **DQ011109/DQ011110** |
| *Ganoderma* sp. | BJ-1 | Indonesia, *Elaeisguineensis* | AY220539 |
| *Ganoderma* sp. | MUCL27886 | India, N.d. | AF255190 |
| *Ganoderma* sp. | MUCL38595 | Zimbabwe, N.d. | - |
| *Amaruoderma rude* | JM/ASP.1 | Taiwan, on buried roots | X78753/X78744 |

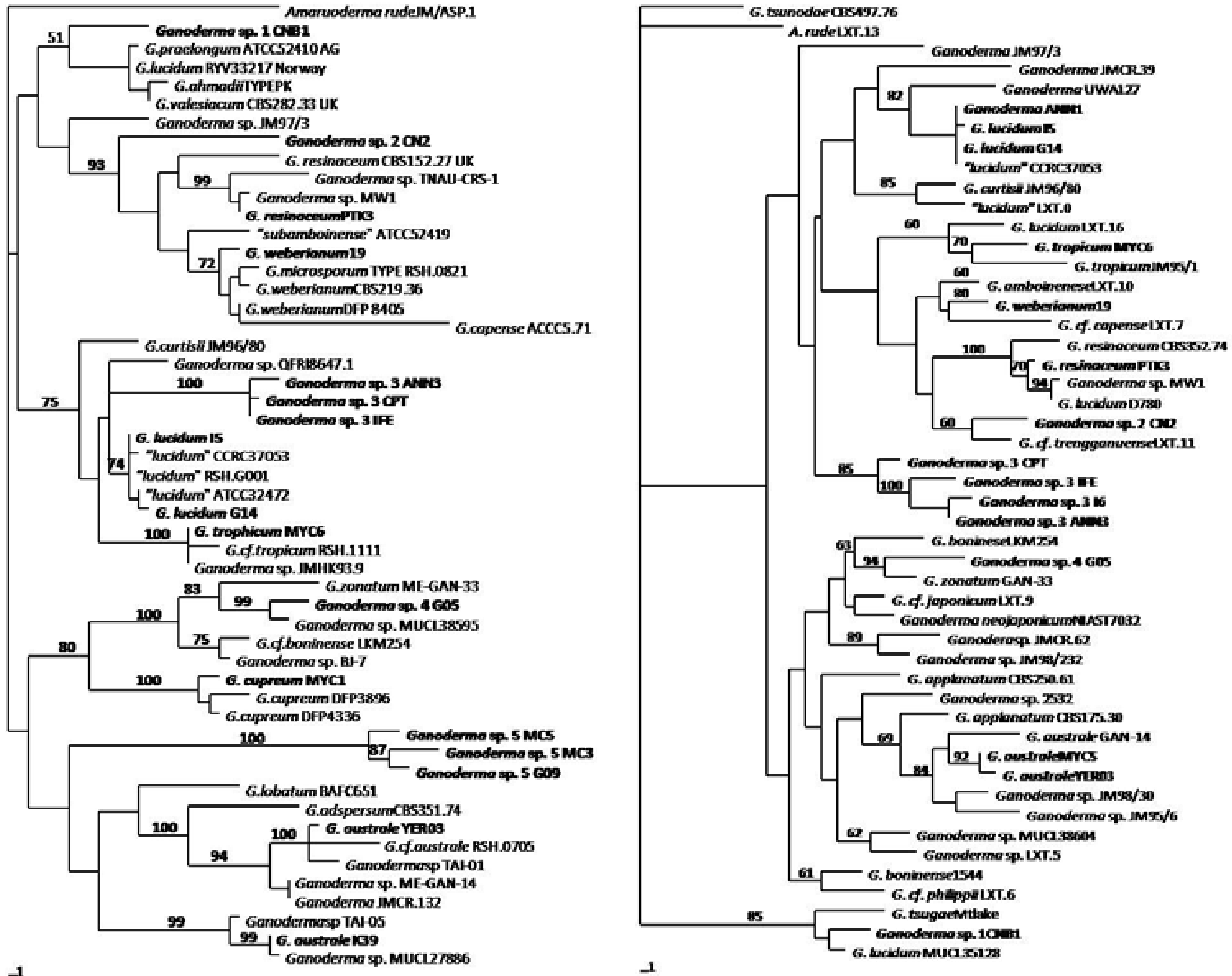


**Figure 1.** One of the most parsimonious trees inferred from ITS rDNA gene rooted with *Amauroderma rude* JM/ASP.1 sequence. The bold names indicate the eleven *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

**Figure 2.** Maximium parsimonious trees inferred from partial β-tubulin gene sequences of *Ganoderma* spp. rooted with *Amauroderma rude* JM/ASP.1. The bold names indicate the *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

evidences from Eastern Asia or South Africa are needed for more studies on taxonomy and distribution pattern of *G. cupreum*, *G. lucidum* (G14 and I5) closely clustered with Asian isolates with strong BS (74%). This clade comprises of *G. lucidum* from Asian counties. The Asian *G. lucidum* were similar with a lower level of nucleotide divergence [14]; they were intercompatible in di-monokaryotic mating studies [12]. But reports are not available whether this species are conspecific or not. Asian *G. lucidum* collected from hardwoods produced chlamydospores, which agrees with earlier reports [14]. *G. tropicum* (MYC6) was closely clustered with Asian collections with 100% BS. This group produces abundant, inamyloid, long, cylindrical chlamydospores which was consistent with earlier reports [27]. These isolates can be distinguished by morphological and cultural evidences [12]. *G. resinaceum* (PTK3) was closely clustered with Indian (TNAU-CRS-1) and North American collection (MW1) with 99 % BS and there is no nucleotide variation. They were completely conspecific irrespective of their geographical distribution or macro-morphological differences [6, 14]. The ITS sequence analysis clearly demarcated the populations of Europe, North America and South America and they were genetically isolated from each other and hence these populations needs more justification at the species level [27]. However, additional sampling

from South East Asia and Australia is required to confirm the taxonomic conclusion. This group produced abundant chlamydospores which were smooth, ellipsoid or subglobose; dextrinoid or inamyloid; intercalary or terminal in position [6, 13]. This species was more or less cosmopolitan in its distribution. *G. tropicum* is distributed throughout the tropical and subtropical regions. Only few collections were reported from Asian countries and additional molecular evidence from Africa would provide the firm taxonomic conclusion of this species. The monophyletic lineage of *Ganoderma* sp. 3, *G. lucidum* and *G. tropicum* are was well resolved (75 % BS). *G. weberianum* was considered to be the counter part of *G. resinaceum* in tropical countries and can be distinguished by cultural and molecular data. The former species closely clustered with Asia-Australian isolates with 72% BS. The robustness of the *G. resinaceum* and *G. weberianum* clade was highly supported (93% BS) and comparable to literature [14]. There is strong support for separating Asian and Australian collections as reported earlier [16]. The chlamydospores of *G. weberianum* were of two types viz., ellipsoid, striated and ellipsoid, non-striated [13]. The latter type was found in *G. weberainum* and was also reported earlier in a Taiwanian collection CCRC37081. In addition, globose, elliptical with echinulate and fissured gastrospores were also produced in less numbers as reported earlier in Australian [16] and Indonesian collections [7]. This result shows that chlamydospore and gastrospore producing groups were clearly demarcated in ITS phylogeny tree of the present study. This species seems to be distributed in temperate and tropical Asia, Australia and Pacific regions [16]. *Ganoderma* sp. 1 (CNB1) was isolated in its position but remain clustered with collections from temperate regions representing *G. lucidum* sensustricto and stands alone in phylogenetic analysis with no clearly supported relationships. *Ganoderma* sp. 1 is phylogenetically distinct from other members by >2.75% nucleotide substitution in ITS region without bootstrapping, therefore, we consider this taxa to be a distinct species. Members of this group lack chlamydospores and seem to be distributed in temperate region of both northern and southern hemisphere [14]. *Ganoderma* sp. 2 differed with *G. resinaceum* by >3.5% nucleotide variation in the ITS region. The distinction in cultural and molecular data was consider, *Ganoderma* sp. 2 could be a distinct species which might be closely related to *G. resinaceum* rather than *G. weberainum*. It produced abundant chlamydospores in culture which is comparatively smaller than that of *G. resinaceum* group but larger than *G. weberianum*. It produces both amyloid and inamyloid chlamydospore. ITS phylogeny shows that *Ganoderma* sp. 2 might be distributed in tropical and subtropical regions; still additional sampling from southern hemisphere is needed to confirm the distribution pattern. Three strains of *Ganoderma* sp. 3 represents only Indian isolates and stands alone in the ITS phylogenetic tree with high BS (100 %) and named as distinct ITS species. Additional data are required to confirm its phylogenetic relationship. *Ganoderma* sp. 4 closely clustered with pathogenic strains isolated from palm with 99% BS. ITS phylogeny clearly shows that the *Ganoderma* sp. 4 might be restricted to southern hemisphere (Zimbabwe and India) (unpublished data). However additional molecular evidence would provide more information concerning the distribution. The *Ganoderma* species 1, 2, 3, 4, and 5 are represented by only a few collections from Asia and South America thus the correct naming of species remains problematic and additional sampling would provide more information.

**Phylogenetic analysis of β-tubulin gene**

Partial β-tubulin, nuclear DNA gene was amplified with B36 / B12 primer pair which yielded ca. 450 bps. The present investigation revealed that the partial β-tubulin sequences proved to be an excellent tool for the identification of *Ganoderma* species/species complex and also resolved the relationship of *Ganoderma* species. The nucleotide composition was varied among the nuclear DNA genes; in β-tubulin, GC content was 55.5% and AT was 44.6% whereas in ITS rDNA, the GC (49.73%) content comparatively lesser than AT (50.3%). The transition and transversion ratio was comparatively higher (2.84) than ITS rDNA (2.299) gene. 56.1% of GC content of β-tubulin gene was reported in "*G. lucidum*" WC721 [21]. Heuristic searches performed under parsimony criterion (as described in Materials and Methods) produced 20 most parsimonious trees with tree length = 547, consistency index = 0.459, retention index = 0.631. Phylogenetic analysis of β-tubulin sequences was concurrent with ITS dataset and provided greater resolution as assessed by bootstrapping. However *Ganoderma* sp. 5 (MC3, MC5 and G09) and *G. australe* (K39) has been eliminated due to template or PCR product contamination. The remaining nine species remain clustered with respective clades and were consistent with ITS rDNA gene (Fig. 2). The GC content was higher (55.5 %) than that of AT content (44.6 %) and the transition and transversion ratio was comparatively higher (2.84) than ITS rDNA (2.299) gene.

## Phylogenetic analysis of *atp*6 gene

The mitochondrial protein coding gene *atp*6 was amplified with ATP3/ATP2 primers which yielded a single PCR of approximately 664 bps. Phylogenetic analysis of *atp*6 gene showed that there was less nucleotide divergence when compared to the nuclear DNA genes (ITS rDNA and β-tubulin). The percentage of the AT (73.5 %) content was comparatively higher than GC (26.5 %) content. The abundance of A/T transversion caused lowest transition / transversion ratio (0.730) than the other two nuclear DNA genes. *G. australe, G. lucidum*, *G. resinaceum*, *G. tropicum*, *G. weberianum*, *Ganoderma* sp. 2, *Ganoderma* sp. 3, *Ganoderma* sp. 4 and *Ganoderma* sp. 5 were well resolved as assessed by bootstrapping (Fig. 3). One of the strains of *Ganoderma* sp. 3 (CPT) conflicts with nuclear DNA gene and remain clustered with *G. lucidum*. When compared to ITS rDNA and β-tubulin, *atp*6 provided very little informative character towards the phylogenetics study. However it contributed important information for the resolution of the *Ganoderma* spp.

## Multigene phylogenetic analysis

*Ganoderma* phylogeny inferred from three unlinked loci resulted in a robust phylogeny that is largely in congruence with individual sequence dataset. The terminal and deep branches were well resolved when comparing the ITS, β-tubulin and *atp*6. Combined dataset clearly separated all the 10 species viz., *G. resinaceum, G. weberianum, G. tropicum, G. australe*, *G. lucidum* and unidentified species *Ganoderma* sp. 1, *Ganoderma* sp. 2, *Ganoderma* sp. 3, *Ganoderma* sp. 4 and *Ganoderma* sp. 5. However, *Ganoderma* sp. 3 (CPT) alone conflicted and closely clustered with *G. lucidum* clade with moderate support (70 %) (Fig. 4).

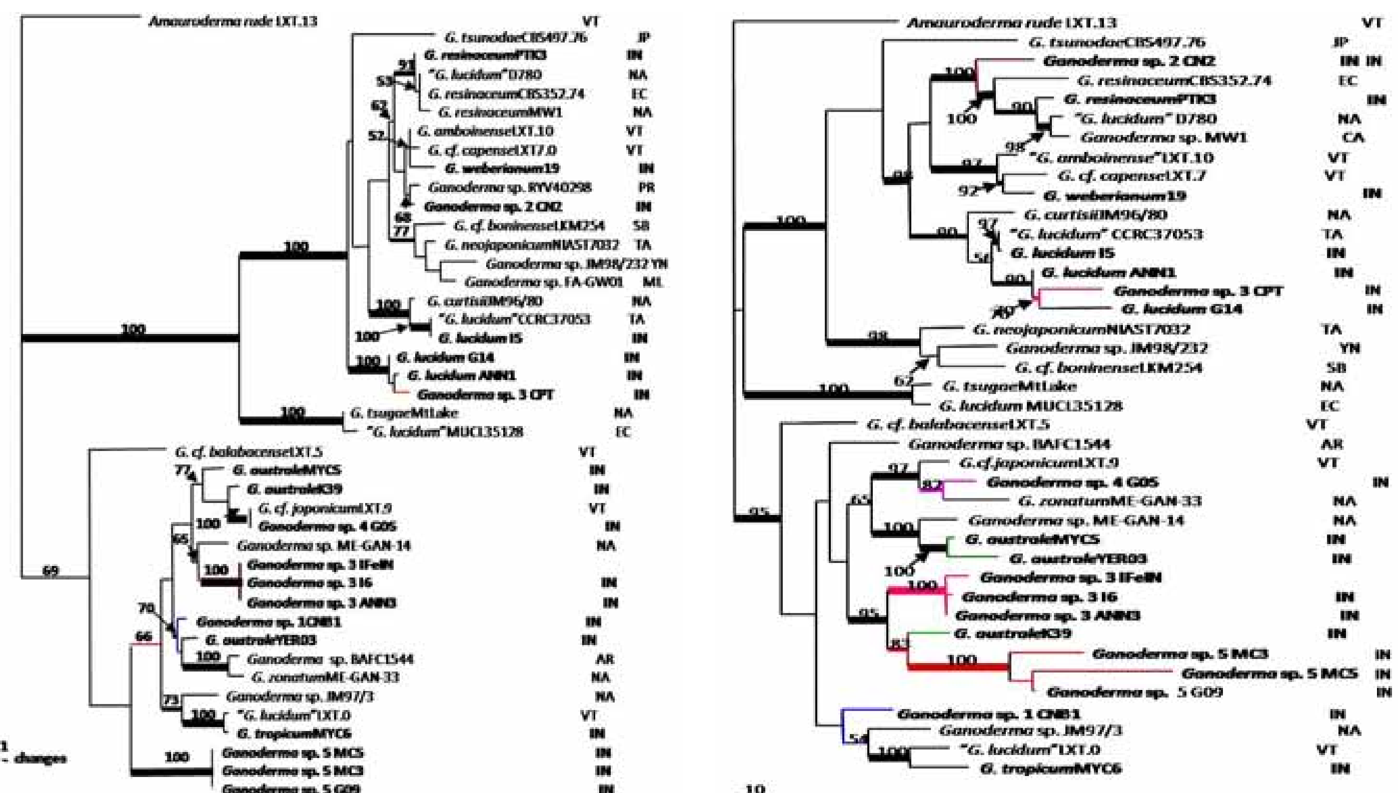

**Figure 3.** Maximium parsimonious trees inferred from partial *atp6*gene sequences of *Ganoderma* spp. rooted with *Amauroderma rude* JM/ASP.1. The bold names indicate the *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

**Figure 4.** Maximium parsimonious trees inferred from ITS r DNA region, β-tubulin and partial *atp6* gene sequences of *Ganoderma* spp. rooted with *Amauroderma rude* JM/ASP.1. The bold names indicate the *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

## CONCLUSION

This study has contributed important details to the understanding of the natural relationships within the *Ganoderma* species. Now it is clear that the nuclear ITS rDNA and β-tubulin genes do contain useful information to resolve the relationship of species, species complex and closely related species of *Ganoderma*. On the other hand, though *atp*6 contained low nucleotide divergence it provided significant information towards the *Ganoderma* phylogeny. However, *atp*6 based tree topology was in line with the multiple gene phylogeny. The results were consistent with cultural and geographical distribution of species. Since this is the first report of protein coding gene in *Ganoderma* phylogenetic study, no literature is available for the comparative analyses.

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# DNA MARKERS REVEAL GENOME-WIDE VARIATIONS IN THE STRAINS OF BUTTON MUSHROOM (*AGARICUS BISPORUS*)

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## ABSTRACT

The wild strains of a cultivated species constitute an important breeding material for the genetic enhancement of commercial lines. Cultivated strains of button mushroom, *Agaricus bisporus* have narrow genetic base due to their origin from few high-yielding hybrids and single spore progenies through tissue culture propagation. The genetic identities of wild and cultivated strains of *A. bisporus* were established by DNA sequencing of internal transcribed spacer (ITS) regions of 5.8S rRNA gene. Molecular variation among the different strains was assessed using RAPD and AFLP markers. ITS1, 5.8S rRNA gene and ITS2 were 290, 154 and 208 bases, respectively in length in all the strains of *A. bisporus* studied. SNPs in ITS2 region were identified, which distinguished different strains within the species. RAPD and AFLP markers differentiated all the germplasm strains. White pileus cultivated strains were separated from the wild brown strains by a phylogenetic branch. The wild strains possessed a broad range of genetic variation (53.4%) and exhibited 53.7% genetic distance from the cultivated strains and hybrids, indicating a high level of DNA polymorphism in the germplasm. However, the cultivated strains showed less DNA polymorphism (16.8% genetic variation) as compared to the hybrid cultivars (26.8% variation). The cultivated strains showed a genetic distance of 33.6% from the hybrids and were clearly separated in the UPGMA dendrogram and PCO plot. These results have practical implications for the future breeding programmes as these molecularly diverse germplasm strains could be selected for genetic improvement of this commercially important button mushroom in India.

**Keywords:** *Agaricus bisporus,* AFLP, ITS sequencing, RAPD, wild strains

## INTRODUCTION

The button mushroom, *Agaricus bisporus* (Lange) Imbach is a commercially important vegetable crop and significantly contributes to the economies of many countries [1]. This species has a secondarily homothallic life cycle [2] with low levels of crossing over and normal segregation [3-4]. Now, with the acquisition of a large collection of wild types through the *Agaricus* Resource Program (ARP) [5-8], the *A. bisporus* species encompass three different life cycles *viz.*, primary homothallism in *A. bisporus* var. *eurotetrasporus* Callac et Guinberteau [9], pseudohomothallism (= secondary homothallism) in traditional variety, *A. bisporus* var. *bisporus*, and heterothallism in *A. bisporus* var. *burnettii* Kerrigan & Callac [10-11].The wild relatives of a cultivated species represent a valuable genetic resource and provide the foundation for the genetic improvement. Thus, the reliable assessment of genetic variation and identity of the wild strains and their relationship with the cultivated germplasm becomes the prerequisite for the planning of a new breeding programme. These wild genetic resources can also provide useful information on the evolution of the species. The use of DNA markers is considered best for the above purpose as the phenotypic traits are stage specific and their expressions are considerably influenced by ecological factors.

DNA sequencing provides the ultimate fine scale measurement of genetic polymorphism directly at the nucleotide level. Nuclear ribosomal RNA genes (18S, 5.8S and 28S) evolve relatively slowly and are useful for studying distantly related organisms. The internal transcribed spacer (ITS) regions evolve faster and may vary among species within a genus or among strains of the same species [12]. The ITS region is an area of particular importance to fungal diagnostics. ITS regions show more sequence divergence than flanking regions [13] and are often used to distinguish related mushroom species and to infer phylogenetic relationships [9, 14-16]. Molecular characterization based on random amplified polymorphic DNA (RAPD) markers has been used for the assessment of genetic variation and strain identification in a wide variety of

fungal species [17-20]. DNA polymorphisms are easier to identify with RAPD markers than with restriction fragment length polymorphism (RFLP) markers [21]. In button mushroom *A. bisporus*, RFLPs [22-23] and RAPDs [24-28] were used for the assessment of genetic diversity in wild and cultivated strains.

The more robust and highly polymorphic amplified fragment length polymorphism (AFLP) markers have been utilized to study the genetic relationship and DNA polymorphism in plants [29-31] and fungi [32-33] including mushrooms [34-36]. Thus, the objectives of present study were i) to DNA fingerprint and assess molecular variation in *A. bisporus* germplasm that includes quondom cultivars, currently cultivated strains and hybrids, and the wild strains from Russia, France, USA and India using RAPD and AFLP markers, and ii) to determine the extent of intra-specific molecular variation among the wild strains and their genetic relationship with the commercially cultivated white strains and hybrids. In this paper we report the genetic identities of ARP wild strains of *A. bisporus* using ITS sequence data and the presence of a high degree of DNA polymorphism in ARP strains using reproducible RAPD and AFLP markers.

## MATERIALS AND METHODS

### Mushroom strains

The mushroom strains included 42 germplasm accessions of *A. bisporus* that comprised of wild collections from geographically isolated regions of the world, quondom cultivars, currently cultivated strains and hybrids in India, and a strain of closely related species, *A. subfloccosus* (Table 1).

### DNA extraction

Genomic DNA from the somatic tissues of young fruitbodies of individual strains was isolated using the CTAB method [37] with modifications as previously described [38]. DNA samples were purified with RNase and purified DNA was run on a 0.7% agarose gel with diluted uncut lambda DNA (25 ng/ml) as standard to assay its concentration and integrity. DNA was also quantified with UV/VIS spectrophotometer of Hitachi model U-1500 by measuring $OD_{260}$ and $OD_{280}$. The DNA samples showing $OD_{260}/OD_{280}$ ratio of 1.8 to 1.9 were used for RAPD analysis. The quantified DNA samples were diluted in TE buffer to make a final concentration of 50 ng/µl for PCR reactions.

### Molecular analysis

The ITS regions, comprising ITS1+5.8S rRNA gene + ITS2, were amplified using ITS1 ext B and ITS4 ext A forward and reverse primers, respectively [16]. The PCR amplifications were carried out in 50 µl reaction volume following the protocol detailed in Yadav *et al.* [28]. ITS amplified products were purified using Qiagen columns following the QIAquickPCR purification kit protocol. Purified ITS DNA was eluted in 1 mMTris-HCl pH 8.0. Cycle sequencing reactions were performed using ABIPRISM™BigDye Terminator Sequencing ready reaction kits (Applied Biosystems, UK) for sequencing the ITS products. The cycle sequencing reaction consisted of the following components (10 µl): 2 µl Big Dye reaction mix, 1.6 µl Primer (1pmole/ml), 1ml PCR product DNA (15-20 ng/reaction) and 5.4 ml deionized water. The sequencing primers ITS1 and ITS4 [12] were used for cycle sequencing reactions. The PCR conditions for sequencing were: 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. The contents of the sequencing reaction were spin down and processed as per the protocol of ABI Prism 3100 Genetic Analyser from Applied Biosystems. The samples were electrophoresed at sequencing facilities of Delhi University, South Campus, India.

RAPD reactions were performed in 22 strains of *A. bisporus* as per Williams *et al*. [39] protocol with minor modifications to enhance reproducibility and consistency of RAPD profiles [38]. PCR amplicons were separated on a 1.5% agarose gel pre-stained with ethidium bromide solution using 1X TAE buffer. The gels were run for 4 hours at 45 V and the RAPD amplicon profiles were recorded using Syngene Gel Documentation System using GeneSnap software. The size of the amplified fragments was determined using 100 bp plus ladder (MBI Fermentas, Lithuania) and Gene Tools software. All RAPD reactions were performed twice to test the reproducibility of the amplicon profiles.

**Table 1.** List of commercial strains, hybrids and wild strains of *Agaricus bisporus* and a wild strain of *A. subfloccosus* used in the molecular analysis

| Sl. No. | Original code | Gene bank code[1] | Source/origin | Germplasm description[2] |
|---|---|---|---|---|
| 1. | S-11 | A-1 | NRCM, Solan, India | White pileus, commercial strain |
| 2. | U-3 | A-13 | NRCM, Solan, India | White pileus, commercial hybrid |
| 3. | U-3 | A-97 | Wang Nan, EFI, China | White pileus, commercial hybrid |
| 4. | MS-39 | A-51 | NRCM, Solan, India | White pileus, commercial strain |
| 5. | S-44 | A-2 | NRCM, Solan, India | White pileus, commercial strain |
| 6. | S-56 | A-4 | NRCM, Solan, India | White pileus, commercial strain |
| 7. | S-791 | A-6 | NRCM, Solan, India | White pileus, commercial strain |
| 8. | RRL-89 | A-7 | NRCM, Solan, India | White pileus, commercial strain |
| 9. | S-310 | A-10 | NRCM, Solan, India | White pileus, commercial strain |
| 10. | P-1 | A-11 | NRCM, Solan, India | White pileus, commercial strain |
| 11. | ITCC-3708 | A-44 | ITCC, New Delhi, India | White pileus, commercial hybrid |
| 12. | ITCC-3710 | A-45 | ITCC, New Delhi, India | White pileus, commercial hybrid |
| 13. | ITCC-3709 | A-46 | ITCC, New Delhi, India | White pileus, commercial hybrid |
| 14. | ITCC-1924 | A-47 | ITCC, New Delhi, India | White pileus, commercial strain |
| 15. | ITCC-1933 | A-48 | ITCC, New Delhi, India | White pileus, commercial strain |
| 16. | ITCC-3554 | A-49 | ITCC, New Delhi, India | White pileus, commercial strain |
| 17. | ITCC-3609 | A-50 | ITCC, New Delhi, India | White pileus, commercial strain |
| 18. | NCS-100 | A-68 | NRCM, Solan, India | White pileus, commercial strain |
| 19. | NCS-101 | A-69 | NRCM, Solan, India | White pileus, SSP from hybrid |
| 20. | NCH-102 | A-70 | NRCM, Solan, India | White pileus, commercial hybrid |
| 21. | ARP-247 | A-73 | ARP collection, USA | White pileus, strain |
| 22. | Hybrid-1 | A-98 | NRCM, Solan, India | White pileus, New hybrid |
| 23. | WI-1 | - | Wild collection, India | White pileus, wild strain |
| 24. | Chail-1 | A-80 | NRCM, Solan, India | White pileus, commercial hybrid |
| 25. | SM-110 | A-82 | SMF, New Delhi, India | White pileus, commercial hybrid |
| 26. | SM-170 | A-84 | SMF, New Delhi, India | White pileus, commercial hybrid |
| 27. | SM-210 | A-85 | SMF, New Delhi, India | White pileus, commercial hybrid |
| 28. | SM-270 | A-86 | SMF, New Delhi, India | White pileus, commercial strain |
| 29. | ARP-209 | A-15 | ARP collection, USA | Brown pileus, wild strain |
| 30. | ARP-210 | A-16 | ARP collection, USA | Brown pileus, wild strain |
| 31. | ARP-214 | A-18 | ARP collection, Russia | Brown pileus, wild strain |
| 32. | ARP-216 | A-20 | ARP collection, Russia | Light brown pileus, wild strain |
| 33. | ARP-217 | A-21 | ARP collection, Russia | Brown pileus, wild strain |
| 34. | ARP-224 | A-28 | ARP collection, Russia | Brown pileus, wild strain |
| 35. | ARP-243 | A-67 | ARP collection, USA | Brown pileus, commercial strain |
| 36. | ARP-246 | A-72 | ARP collection, France | Brown pileus, wild strain |
| 37. | ARP-249 | A-75 | ARP collection, USA | Brown pileus, wild strain |
| 38. | ARP-250 | A-76 | ARP collection, USA | Brown pileus, wild strain |
| 39. | ARP-251 | A-77 | ARP collection, USA | *A. subfloccosus* |
| 40. | ARP-256 | A-91 | ARP collection, USA | Brown pileus, wild strain |
| 41. | ARP-257 | A-92 | ARP collection, USA | Light brown pileus, wild strain |
| 42. | ARP-259 | A-94 | ARP collection, USA | Brown pileus, wild strain |
| 43. | ARP-261 | A-96 | ARP collection, USA | Brown pileus, wild strain |

[1]Mushroom Gene Bank, DMR, Solan-173 213, India; EFI = Edible Fungi Institute, China; SSP = single spore progeny; ITCC = Indian Type Culture Collection, Mycology and Plant Pathology Division, IARI, New Delhi 110 012, India; SMF = Swadeshi Mushroom Farm, New Delhi, India; *Agaricus* Resource Programme, USA; [2]Grow out tests of these genotypes were conducted at DMR during 2000-2005.

AFLP fingerprints were generated based on the protocol of Zabeau and Vos [40] with slight modifications enumerated in Yadav *et al*. [36] using PCR reagents from AFLP analysis system kits of Life Technologies (Invitrogen), California, USA. AFLP fingerprints were obtained using six AFLP primer-pairs; five with three selective nucleotides in each of *EcoR*I and *Mse*I primers (AFLP Analysis System I) and one with only two selective nucleotides in *EcoR*I and three selective nucleotides in *Mse*I (AFLP small genome kit). All the PCR reactions were carried out in a PE-9600 thermocycler (Perkin Elmer Corp., Norwalk Conn., USA). AFLP reaction products were mixed with an equal volume of Formamide dye (98% Formamide, 10 mM EDTA, 0.005% each of Bromophenol blue and Xylene Cyanol). Three µl of each sample were loaded on a 5% denaturing polyacrylamide gel, run in 1X TBE electrophoresis buffer. The gel was dried for 2 h before subjecting it to autoradiography for 1-3 days at –70 °C depending on the signal intensity. The size of the fragments was estimated using 20 bp size standards (BioWhittakar Molecular Applications, USA).

## Statistical analysis

The ITS nucleotide sequences were analyzed after generating the complementary and reversed sequences of ITS4 primer and then comparing with the ITS1 sequences using GeneDoc software [41]. Multiple sequence alignments of consensus sequences were made via ClustalX (1.83) algorithm [42]. The multiple aligned sequences were then utilized to generate a bootstrap ($\eta$ = 1000) N-J phylogenetic tree [43]. The tree was viewed with the help of TREEVIEW software (http://taxonomy.gla.ac.uk/rod/treeview.html). Sequence divergence (d) and base substitutions were calculated based on Kimura's two-parameter model [44] and are presented as per cent value ($d \times 100$).

The RAPD and AFLP amplification products (amplicons) were scored as present (1) or absent (0) for each primer-genotype combinations. Molecular data were entered into a binomial matrix and were used to determine Jacquard's similarity coefficients [45] using NTSYS-PC software version 2.02h [46]. The dendrograms depicting the genetic relationship were constructed employing the Unweighted Paired Group Method of Arithmetic Averages (UPGMA) algorithm and Sequential, Agglomerative, Hierarchial and Nested (SAHN) clustering. The principal coordinate analysis (PCO) was carried out with NTSYS-pc using DCENTER and EIGEN procedures. The Jaccard's similarity matrix was also used to generate a 3-D plot of principal coordinates to resolve the patterns of variation among the strains. The bootstrap analysis was carried out using the WINBOOT program [47] with 1000 replications. Most informative primers were selected based on high polymorphism information content (PIC) values of individual primers. The PIC was calculated using the formula [48]:

$$PIC = \sum_{i=1}^{n} \{2 \times F(1-F)\}$$, where, F is the frequency of presence of a maker band, i.

The per cent genetic distance (GD) was measured using the Jaccard's similarity coefficients as: % GD = (1- average similarity coefficient) $\times$ 100. Similarity matrices from high PIC primers were compared with that of all the primers using MXCOMP sub-program of NTSYSpc. Discrimination power (DP) was estimated to test the efficacy of individual primers in distinguishing the strains, employing the formula DP = number of pairs of strains differentiated /total number of pairs of strains $\times$ 100.

# RESULTS

## Genetic identity and ITS sequence polymorphism

Genetic identities of ARP wild strains were established by amplifying and sequencing the 5.8S rRNA gene along with flanking ITS1 and ITS2 spacer regions. The ITS regions comprising ITS1 + 5.8S rRNA gene + ITS2 were amplified as a single unit from 21 strains of *A. bisporus* along with a strain of related species *A. subfloccosus*. In all cases, the PCR yielded a single product without any visible length variations in the ITS profiles. The length of the PCR product was approximately 755 bases on gel. Multiple nucleotide sequences of the ITS region were generated for a subset of 14 strains. The full length ITS region was 756 bp in all the *A. bisporus* strains analyzed. The 5.8S rRNA gene sequences were found to be conserved

within the species as well as among the species *A. bisporus*, *A. bitorquis* and *A. subfloccosus*. The ITS1 region, 5.8S rRNA gene and ITS2 region were 290, 154 and 208 nucleotides long, respectively in all the genotypes of *A. bisporus* studied. Within the species *A. bisporus*, nucleotide sequence divergence ranged from 0 to 0.43% with the average value of 0.21%. The genotypes of *A. bisporus* exhibited 1.6 and 3.8% sequence divergence from *A. subfloccosus* and *A. bitorquis*, respectively. The ITS2 region had single nucleotide polymorphisms (SNPs) at four base pair positions of 509, 515, 550 and 590. The transition mutations (T→C) occurred at 509, 550 and 590 SNP positions, whereas the SNP at 515 bp exhibited a transversion mutation (C→A) in the wild strains ARP-224 (from Russia) and ARP-261 (from USA). The presence of informative indels in ITS1 (a triple base TGT deletion in *A. bisporus* and *A. subfloccosus*) and in ITS2 region (a double base TG deletion in *A. bisporus*) were used to delimit the wild strains into the species *A. bisporus*.

### *RAPD fingerprinting*

Twenty-eight decamer primers amplified identifiable and consistent PCRamplicons that ranged from 275 bp to 3500 bp in length, and 283 RAPD marker bands, out of which 256 (90.5%) were polymorphic. A total of 2401 amplicons were produced in 22 genotypes with an average of 3.9 amplicons per genotype per primer. The wild strain ARP-224 from Russia exhibited the maximum number of amplicons (120), whereas the *A. subfloccosus* strain ARP-251 produced the minimum (93). The highest number of amplicons was produced by the primer OPN-04 (166), while the least amplicons were obtained with primer OPO-18 (31). Twelve most informative primers showing high PIC values were identified as OPN-02, 05, 06, 08, 09; OPO-01, 02, 03, 12, 13, 14 and OPO-20 (Fig. 1a-d).

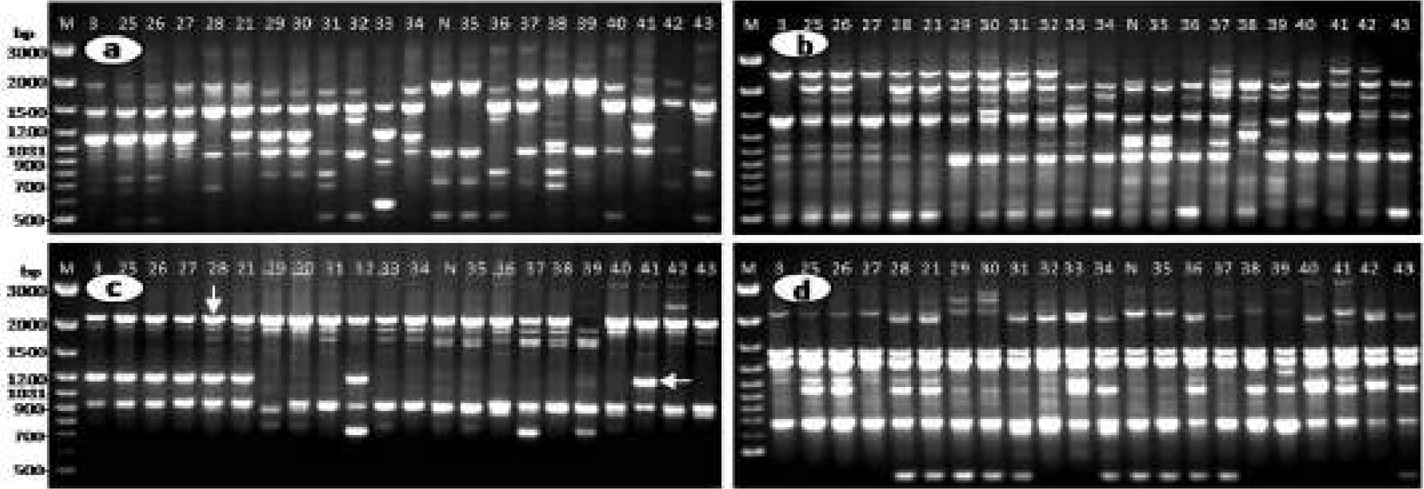


**Figure 1.** RAPD profiles of 21 germplasm strains of *A. bisporus* and one strain of *A. subfloccosus*. M: DNA ladder, lanes 2-23: correspond to germplasm strains listed under serial No. 3, 25-28, 21, 25-43 in Table 1 and N = ARP-234. DNA polymorphism obtained with primer OPO-12 (a), primer OPN-05 (b), primer OPN-02: Horizontal arrow indicates an OPN-$02_{1200}$ band which is present in white and off-white pileus strains and hybrids; Vertical arrow indicates an OPN-$02_{2200}$ band which is present only in *A. bisporus* strains (c),and primer OPN-08 (d)

These primers amplified a total of 168 bands (59.4% of the bands amplified by all the 28 primers) of which 160 (95.2%) were polymorphic. The PIC value for RAPD primers ranged from 0.25 to 4.64. The maximum number of polymorphic markers (18) was obtained with primer OPO-14. The DP values were more useful in the discrimination of strains and were varied from 25.5 – 98.7% with the average being 86.4 (Table 2). Primer OPN-02 proved useful for distinguishing the pileuscolor and for species identification. An RAPD band of approximately 1200 bp (OPN-$02_{1200}$) was present in all the white and off-white (light brown) pileus strains, while OPN-$02_{2200}$ band which was present only in *A. bisporus* strains (Fig. 2c). Similarity indices estimated on the basis of all the 28 RAPD primers in a subset of 22 strains ranged from 0.30 to 0.94. The white pileus strains and hybrids exhibited relatively more genetic similarity (0.710) in comparison to brown and off-white pileus wild strains (0.488). The brown and off-white wild strains showed a high degree of genetic variation (51.2%) amongst them. The highest genetic variation (69.7%) was recorded between brown strains ARP-251 (*A. subfloccosus*) and ARP-246 (*A. bisporus*); while, the white pileus hybrids SM-110 and SM-170 exhibited the least (6.2% genetic variation).

**Table 2.** Efficacy of individual primers for detecting DNA polymorphism and discrimination between 21 strains of button mushrooms *A. bisporus* and one strain of *A. subfloccosus*

| Primer | Sequence (5'-3') | No. of RAPD bands | Size range (approx. bp) | PIC* value | Discrimination power (DP) (%) | Number of pairs of strains not distinguished (Total No. of pairs = 231) |
|---|---|---|---|---|---|---|
| OPN-01 | CTCACGTTGG | 7 | 400-2850 | 1.96 | 91.3 | 20 |
| OPN-02 | ACCAGGGGCA | 10 | 775-3500 | 2.94 | 92.6 | 17 |
| OPN-04 | GACCGACCCA | 13 | 450-2600 | 2.84 | 96.1 | 9 |
| OPN-05 | ACTGAACGCC | 16 | 500-2500 | 4.19 | 95.7 | 10 |
| OPN-06 | GAGACGCACA | 14 | 600-2700 | 4.42 | 98.3 | 4 |
| OPN-07 | CAGCCCAGAG | 8 | 350-3200 | 1.63 | 90.5 | 22 |
| OPN-08 | ACCTCAGCTC | 14 | 375-3100 | 3.46 | 98.3 | 4 |
| OPN-09 | TGCCGGCTTG | 17 | 750-2300 | 4.36 | 97.8 | 5 |
| OPN-10 | ACAACTGGGG | 10 | 750-3000 | 2.73 | 93.5 | 15 |
| OPO-01 | GGCACGTAAG | 11 | 490-3200 | 3.76 | 98.7 | 3 |
| OPO-02 | ACGTAGCGTC | 15 | 400-1900 | 3.14 | 96.1 | 9 |
| OPO-03 | CTGTTGCTAC | 15 | 400-2900 | 4.64 | 95.7 | 10 |
| OPO-04 | AAGTCCGCTC | 6 | 475-2100 | 1.23 | 77.9 | 51 |
| OPO-05 | CCCAGTCACT | 7 | 650-2900 | 1.74 | 78.8 | 49 |
| OPO-06 | CCACGGGAAG | 6 | 350-1350 | 1.47 | 87.9 | 28 |
| OPO-07 | CAGCACTGAC | 8 | 275-2700 | 1.54 | 87 | 30 |
| OPO-08 | CCTCCAGTGT | 7 | 500-3000 | 1.34 | 86.6 | 31 |
| OPO-09 | TCCCACGCAA | 2 | 650-875 | 0.25 | 25.5 | 172 |
| OPO-10 | TCAGAGCGCC | 11 | 650-3000 | 2.34 | 90 | 23 |
| OPO-11 | GACAGGAGGT | 10 | 700-3200 | 2.22 | 87.4 | 29 |
| OPO-12 | CAGTGCTGTG | 14 | 475-1900 | 4.39 | 97.8 | 5 |
| OPO-13 | GTCAGAGTCC | 12 | 550-2400 | 3.43 | 96.1 | 9 |
| OPO-14 | AGCATGGCTC | 18 | 425-2700 | 4.42 | 96.1 | 9 |
| OPO-15 | TGGCGTCCTT | 10 | 550-2800 | 2.41 | 88.3 | 27 |
| OPO-16 | TCGGCGGTTC | 4 | 550-1150 | 1.6 | 71 | 67 |
| OPO-18 | CTCGCTATCC | 2 | 700-850 | 0.48 | 50.6 | 114 |
| OPO-19 | GGTGCACGTT | 4 | 625-1800 | 0.66 | 59.3 | 94 |
| OPO-20 | ACACACGCTG | 12 | 325-2700 | 3.34 | 95.2 | 11 |

*Polymorphism information content

## AFLP profiling

Six AFLP primer-pairs amplified a total of 467 marker bands in 42 strains of *A. bisporus* and a strain of *A. subfloccosus* with an average of 77.8 bands per primer. Out of 467 AFLP markers, 431 (92.3%) were polymorphic and the rest were monomorphic (7.7%). Out of six primer-pairs tested, two (E-ACT + M-CAC and E-ACT + M-CAG) were able to differentiate all the genotypes studied and had the discrimination power of 1.00 (Table 3).

Wild brown strains were differentiated by all the primer-pairs studied. Pair-wise Jaccard's similarity values varied from 0.282 to 0.936 with average being 0.567. The highest genetically similar strains were S-44 and S-56 (0.936 similarity), while strains S-791 *(A. bisporus*) and ARP-251 (*A. subfloccosus*) were found to be the least similar ones (0.28.2 similarity). The average genetic distance between white pileus cultivars (strains and hybrids) and brown pileus wild collections was found to be 53.7%, indicating a high level of DNA polymorphism amongst the germplasm of *A. bisporus.* However, the white pileus strains had less DNA polymorphism (av. similarity 0.832). The white pileus hybrids exhibited relatively

**Table 3.** Primer-pairs used for selective amplification in AFLP analysis and their utility in molecular differentiation of white pileus cultivars and wild brown strains of *A. bisporus*

| Primer- pair | No. of AFLP markers | Polymorphic AFLP markers (%) | Discrimination power | Number of pairs of strains not distinguished (total number of strain pairs = 903) |
|---|---|---|---|---|
| E-AC + M-CAT | 68 | 89.71 | 0.97 | **24** (pedigree related white pileus strains and hybrids were not differentiated) |
| E-AAG + M-CAT | 38 | 86.84 | 0.95 | **43** (pedigree related white strains and hybrids were not discriminated) |
| E-ACA + M-CTT | 56 | 91.07 | 0.97 | **25** (pedigree related white strains and hybrids were not differentiated) |
| E-ACG + M-CAA | 70 | 94.29 | 0.99 | **1** (pedigree related white strains S-44 and RRL-89 were not discriminated) |
| E-ACT + M-CAC | 117 | 92.31 | 1.00 | **0** |
| E-ACT + M-CAG | 118 | 94.92 | 1.00 | **0** |

more molecular variation between them (av. similarity 0.732) and had an average genetic distance of 33.6% from the white strains.

## Genetic relationship and DNA polymorphism

The genetic relationships inferred from the nucleotide sequence polymorphism in the ITS region of different genotypes of *A. bisporus* are depicted in Fig. 2. Three sub-clusters were formed within the major cluster of *A. bisporus*.

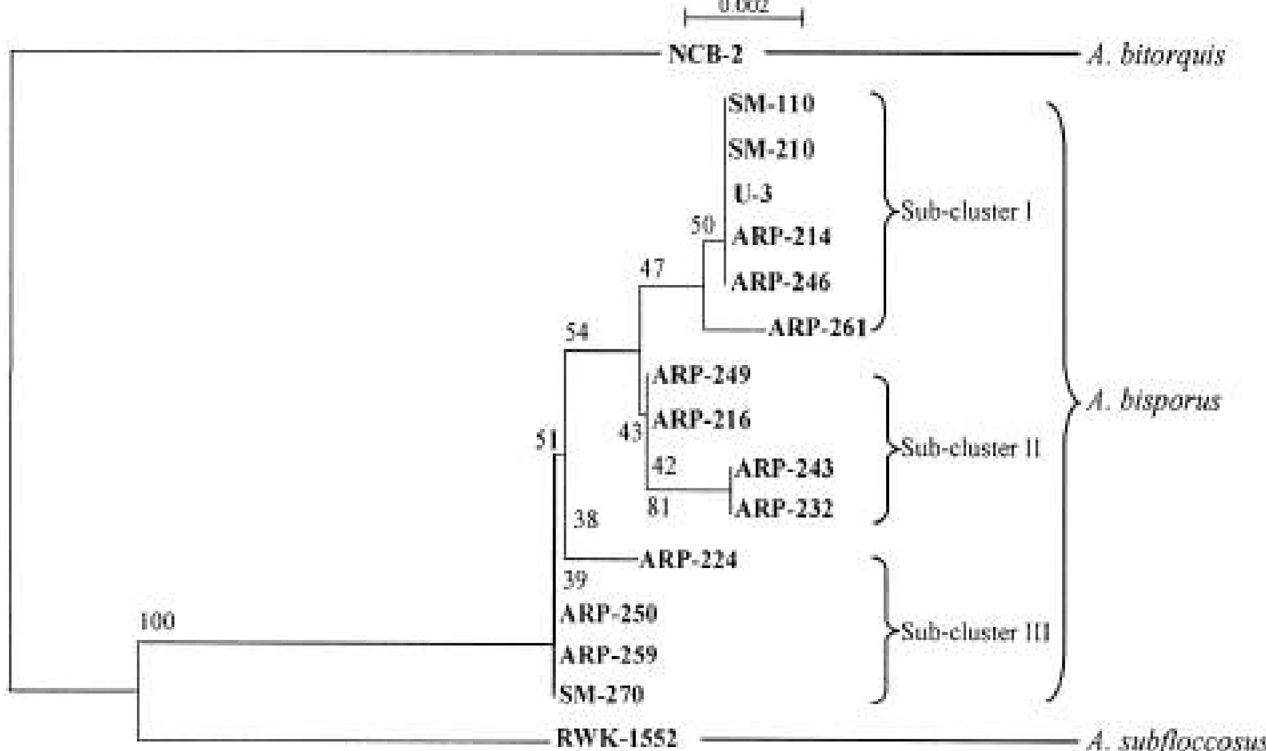


**Figure 2.** Neighbour-joining tree inferred from sequence polymorphisms in the ITS region of wild and cultivated strains of *Agaricus bisporus*. Numbers on the branches denote per cent bootstrap support to each node. The tree was rooted using two related species *A. bitorquis* (NCB-2) and *A. subfloccosus* (RWK-1552)

The wild strains from Russia showed high sequence polymorphism and were placed in all the three sub-clusters. The wild strain ARP-232 (from Canada) was clustered with ARP-243 (a brown strain from USA) within the sub-cluster II that also included the wild strains ARP-216 (from Russia) and ARP-249 (from USA).ARP-224, a wild collection from Russia along with wild strains ARP-250, ARP-259 (both from USA) and SM-270 (white strain) constituted the third sub-cluster. Cluster analysis based on RAPD markers grouped all the white pileus strains and hybrids into one cluster with 86% bootstrap value, whereas the wild brown ARP strains formed several sub-clusters exhibiting a high level of genetic polymorphism (Fig. 3).

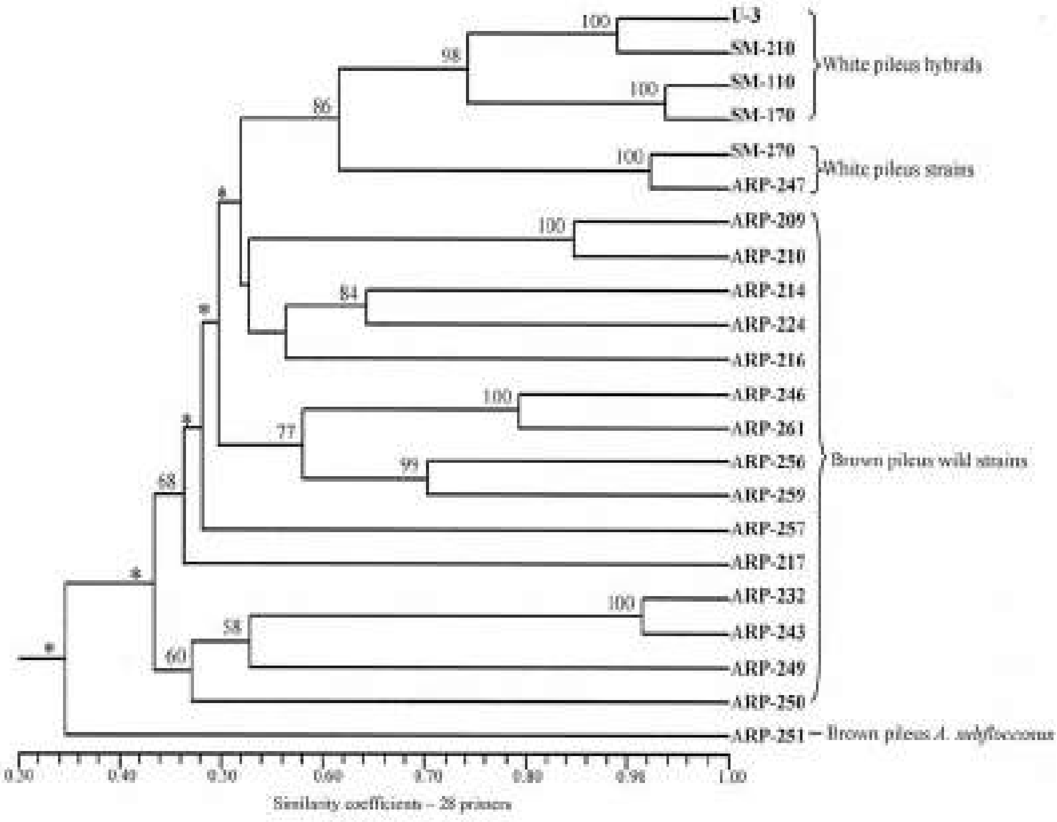


**Figure 3.** UPGMAdendrogram constructed from the RAPD profiles of 21 germplasm strains of *A. bisporus* and one strain of *A. subfloccosus*. The numbers on the forks denote per cent bootstrap support to each node (branches showing less than 50% bootstrap values are indicated by asterisks). Major clusters are indicated at the right margin

All the strains were distinguished using aggregated primer data. Individual primers were also informative in providing specific RAPD polymorphisms for strain differentiation (Table 2). However, none of the primers was able to differentiate all the 22 strains. Primer OPO-01 with 11 RAPD markers differentiated all the genotypes except three pairs namely U-3 and SM-110, SM-170 and ARP-210, and ARP-246 and ARP-261. Wild brown pileus strains exhibited a high degree of genetic variation and were clearly separated from cultivated white pileus strains and hybrids. Genetic relationships among 42 strains based on AFLP profiles are depicted in Fig. 4. Dendrogram based on UPGMA algorithm and SAHN clustering clearly distinguished the strains from the hybrids and the wild strains. *Agaricus subfloccosus* strain ARP-251 showed average genetic distance of 68.3% from *A. bisporus* strains and clustered with wild strains from USA namely ARP-249 (42.2% similarity) and ARP-250 (40.7% similarity) in the dendrogram constructed based on all the six AFLP primer-pairs (Fig. 4).

Within the *A. bisporus* accessions, all the white pileus strains and hybrids were separated from the brown pileus wild collections by a phylogenetic branch with 70% bootstrap value. The wild brown strains exhibited a wide range of genetic variation (av. genetic variation 53.4%). Within the wild brown strains, collections from Penza region of Russia were clearly

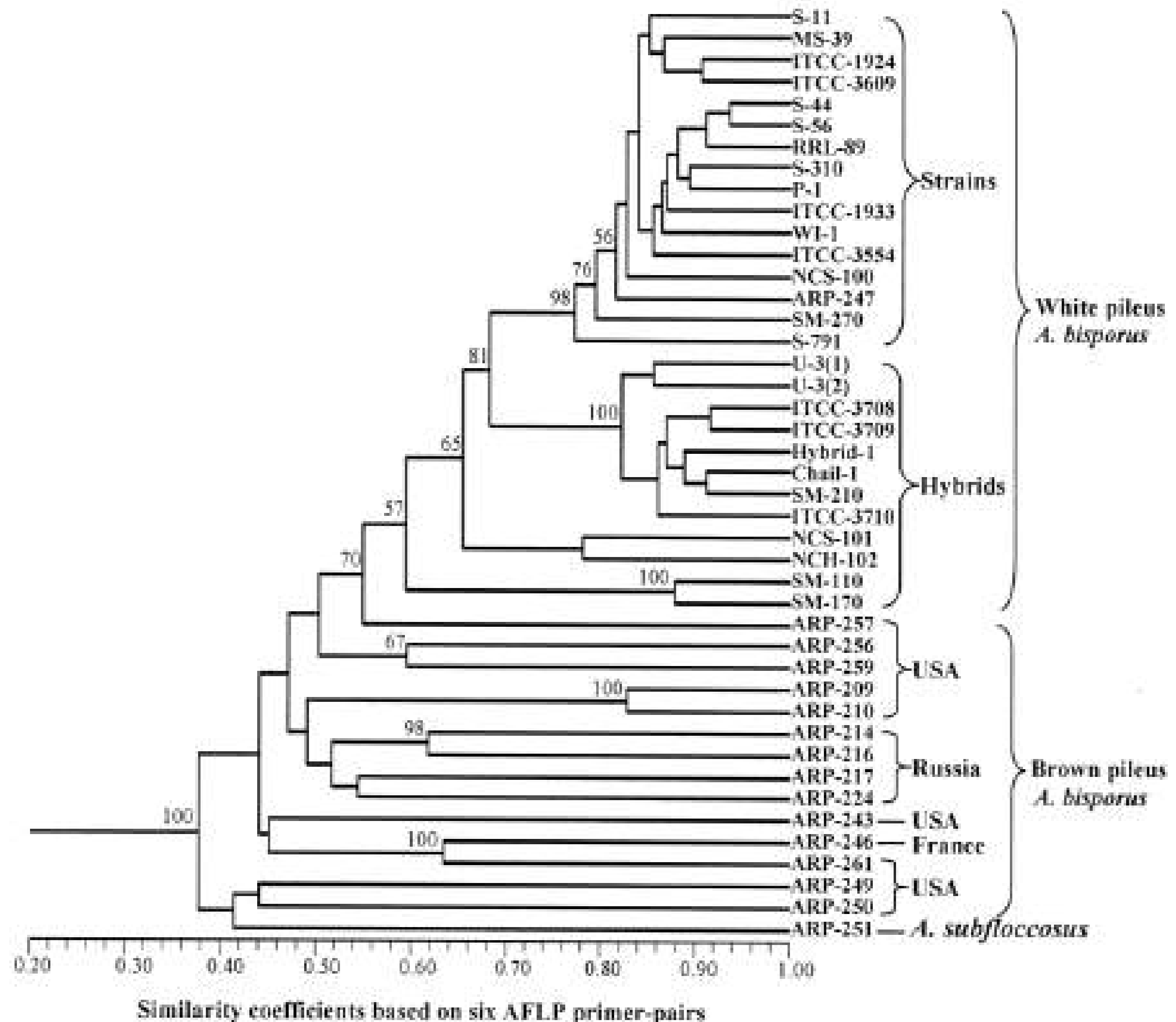


**Figure 4.** UPGMAdendrogram constructed from 467 AFPL markers showing genetic relationships amongst 42 strains of *Agaricus bisporus* and a strain of *A. subfloccosus*. The scale at the bottom represents Jaccard's coefficients of similarity. The numbers on the forks denote per cent bootstrap support to each node. Major clusters are indicated at the right margin

separated, whereas, the collections from different parts of USA exhibited wide genetic variation and formed divergent sub-clades. The wild strain ARP-246 from France clustered with wild strains ARP-261 and ARP-243 of USA. White pileus cultivated strains and hybrids exhibited a narrow genetic base and collapsed in the 3-D PCO plot (Fig.5). The Jaccard's similarity matrices obtained with the individual AFLP primer-pairs were compared with the matrix of all the six AFLP primer-pairs. The matrix correlation (r) values ranged from 0.80 - 0.96 indicating good to very good fit of the two matrices compared. The dendrogram based on AFLP data from primer-pair E-ACT + M-CAC was the most similar to the dendrogram based on all the six primer-pairs with matrix correlation of $r = 0.96$ ($p = 1.00$).

## DISCUSSION

Ribosomal RNA genes evolve cohesively within a single species [49] and exhibit only limited sequence polymorphism in the ITS regions between individuals of a species [50]. Nucleotide sequences polymorphisms of the ITS region have been utilized in establishing the phylogenetic relationships among species of the same or related genera of mushrooms [14-15, 51]. Four SNPs were identified at 509, 515, 550 and 590 base pair positions in ITS2 region which could distinguish the wild strains of *A. bisporus* collected from geographically isolated regions. The presence of informative indels in the ITS1 region (a triple base – TGT – deletion in ITS1 of both *A. bisporus* and *A. subfloccosus*) and in ITS2 region (a double base – TG – deletion in *A. bisporus* only) unequivocally demonstrates that the genetically diverse strains studied in this work belong to

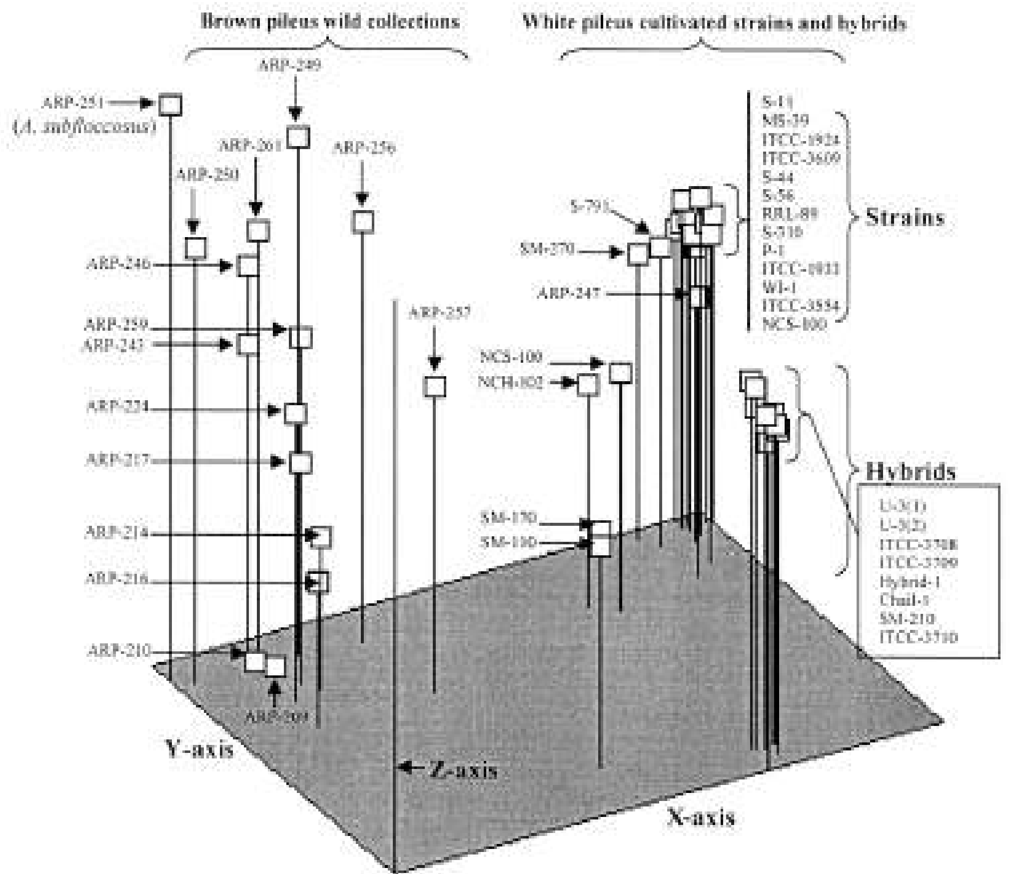


**Figure 5.** A 3-D plot of the Principal Coordinates Analysis of AFLP data showing genetic relationships among brown pileus wild collections from geographically different regions of the world and white pileus cultivated strains and hybrids of *A. bisporus*. White pileus cultivated strains and hybrids exhibited a narrow genetic base and coalesced in the 3-D plot

the species *A. bisporus*. In the previous finding [16] a tetrasporic *A. bisporus* var. *burnettii* [10] was distinguished by a single transition mutation (G→A) at 261 bp in ITS1, while the homothallic *A. bisporus* var. *eurotetrasporus* had the characteristic deletion (a single base –T– at 118 bp). Similarly, ITS sequence polymorphisms at five base pair positions, two in ITS1 region and three transition mutations in ITS2 region at 509 (T→C), 550 (T→C) and 628 (G→A) bp positions were reported in the sexually diverse wild specimens of *A. bisporus* [9]. In the present work, we have compared the ITS sequences from 14 strains (differentiated based on RAPD markers) of *A. bisporus* and found that the ITS1 region is conserved (does not have base substitutions or deletion) perhapsdue to the absence of both the *A. bisporus* var. *burnettii* and *A. bisporus* var. *eurotetrasporus* cultures in the germplasmanalyzed. In our study, we have found a transversion mutation (C→A) at 515 bp position in the wild strains ARP-224 (from Russia) and ARP-261 (from USA) and an additional transition mutation (T→C) at 590 bp position in the ITS2 region. The ITS sequence data of this work clearly validate the presence of European strains in USA as observed by Kerrigan *et al*. [10] using nuclear and mitochondrial markers.

RAPD fingerprinting was found efficient to reveal usable levels of DNA polymorphism among white pileus strains and a very high amount of molecular variation among wild strains of *A. bisporus*. A high degree of genetic homogeneity among hybrids has been reported using RAPD markers [24, 26]. Wild strains analyzed in this study are from the geographically isolated regions (Russia, France, Canada and USA) and thus exhibited a high degree of DNA polymorphism with RAPD. The wild strains ARP-217 (from Russia) and ARP-257 (from USA) exhibited unique bands in some RAPD profiles and consequently formed a solitary cluster in the dendrogram. AFLP markers revealed the overall similarity index of 0.57, which indicates the broad genetic base of germplasm. The similarity value of 0.83 between white pileus strains suggests that the level of genetic variation (16.8%) is low among the cultivated lines; while the white pileus hybrids exhibited relatively more genetic variation (av. similarity 73.2%) and had an average genetic distance of 33.6% from the white pileus strains. However, AFLP markers have differentiated pedigree related and genetically close white pileus strains and grouped them

into a phylogenetic cluster with 98% bootstrap value and form the strong point of the present investigation. The wild brown strains analysed with AFLP markers showed a high degree of DNA polymorphism with genetic variation of 53.4 per cent.

In earlier studies, the wild collections of *A. bisporus* exhibited a high level of genetic variation using allozymes [53] and nuclear DNA RFLPs [22, 23]. The wild strains of the *Agaricus* Resource Program were characterized using nuclear and mitochondrial DNA RFLP probes [5-8, 52-54] and repetitive DNA sequences [55] and were found to be genetically highly variable. However, allozyme and RFLP analyses scan only a limited number of loci in the genome, while the utility of RAPD and AFLP markers for the germplasm characterization and assessment of genetic variation has been well documented in various fungal species [17-20, 32,33] including mushrooms [24-28, 34-36].

## CONCLUSION

The availability of genetically highly variable germplasm from ARP has opened new vistas for research in molecular genetics and breeding of *A. bisporus*. This research represents one of the most comprehensive analyses of molecular variation among the wild and cultivated strains of *A. bisporus*, and demonstrates the effective use of RAPD and AFLP markers for multilocus genotyping and assessment of genetic variation in the germplasm and for establishing the genetic relationships between wild and commercial strains of *A. bisporus*. The ITS sequence analysis unequivocally delimited these genetically highly variable wild strains into a single taxon of *A. bisporus*. These findings have an immediate practical application by the breeders to involve the genetically diverse strains in hybrid breeding programmes.

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# GENETIC VARIABILITY IN STRAINS OF *VOLVARIELLA VOLVACEA* COLLECTED FROM THE STATE OF ODISHA, INDIA

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## ABSTRACT

*Volvariella volvacea*, the straw mushroom is an edible mushroom of tropics and sub-tropics, valued for its flavor, texture and nutraceutical properties. However, it is among the least studied mushrooms in India with respect to genetic variability and strain improvement programme. The present study aimed at studying the genetic variability in wild *Volvariella* germplasm collected from different regions of the state of Odisha, India. The mycelial cultures raised from *V. volvacea* fruit bodies, collected from nine different locations spread in seven districts of Odisha were used in the study. Out of total ten strains, seven were fast growing (>90 mm radial growth), while rest three were slow growing. The strains also exhibited variability in other mycelial growth characteristics and extracellular lignocellulolytic enzymes activity profiles. Fastest growing strain OSM-1 exhibited highest activity of exo-glucanase, low of endo-glucanase and superior levels of β-glucosidase and xylanase. Laccase activity was comparatively low in slow growing strains compared to fast growing strains. In grow out trials, four strains including three slow growing (OSM-5, OSM-8 and OSM-10) and one fast growing (OSM-2) did not colonize the spawn substrate. Highest fruit body yield was recorded in strain OSM-9 (23.60 kg/100 kg dry substrate), followed by strain OSM-3 (17.47 kg/100 kg dry substrate). Rest four strains gave negligible yield. The fruit bodies of strain OSM-9 were very light in weight (7.80 g) compared to strain OSM-3 (15.85 g). The strains formed two separate groups with respect to length of their 5.8S rRNA gene amplicons and the neighbor joining tree deduced from the 5.8S rRNA gene sequences. Group-I comprised of all fast growing strains (OSM-01 to OSM-04 and OSM-07), while group-II with three slow growing (OSM-5, OSM-8 and OSM-10) along with two fast growing strains (OSM-6 and OSM-9). The ClustalW2 analysis exhibited deletions at two base pairs, one each in ITS-1 and ITS-2 regions, and substitution at four different places in ITS-1 region in strains of group-II. The present study paves the way for further breeding programmes in this mushroom.

**Keywords**: *V. volvacea*, variability, morphological characteristics, enzymes activity, yield

## INTRODUCTION

The genus *Volvariella* comprises about 50 species world over [1], including the cultivated *V. volvacea* (paddy straw mushroom). Using the 'strips' recognized by Singer [2], several morphological/ecological groups have been differentiated within this genus.The straw mushroom (*V. volvacea*) ranks sixth among the world's most important species in terms of production [3]. It has significant pharmacological properties, including anti-tumor polysaccharides, immunopromotive proteins and immune modulatory lectins [4-6]. It is a popular mushroom of tropics/subtropics, grows well between 30-35 °C and completes its cropping cycle very fast, within three weeks time. Compared to many cultivated species of mushrooms like *Agaricus bisporus*, *Lentinula edodes* and *Pleurotus* spp., the productivity of *V. volvacea* is low [7]. However, the productivity of a mushroom species is attributed to many factors, important among that are the hydrolytic enzyme production potential of the mushroom species [7], quality of substrate used, method of substrate preparation and the growing conditions. The hydrolytic enzyme production potential of a mushroom species has direct bearing on its mushroom production potential; the cellulases play role during substrate colonization stage, while laccase during sporophore development stage [8, 9]. This mushroom has also been found to produce an array of extracellular hydrolytic and oxidases enzymes - i.e., endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21) and laccase (EC 1.10.3.2) [10-15]. The enzyme copper binding regions and the N-terminal amino acid sequences have also been used to generate complete sequence of six number of laccase from this mushroom and out of these lac1 and lac4 have been suggested to play role in morphogenesis in this mushroom [16, 17].

Among morphological characteristics, the mycelial growth rate, mycelial growth intensity, and formation of aerial hyphae and chlamydospores are considered as important attributes for selecting a potential high yielding strain both for commercial mushroom growing as well as strain improvement programme [18]. However, selection of a strain based only upon morphological characteristics gives misleading results, as these characteristics vary under different cultural and growing conditions [18]. Recently, genetic characterization by using molecular markers has helped in phylogenetic characterization of the strains of different mushroom species and other related fungi without any effect of extraneous factors [19-23]. The present study was aimed at studying the variability in *V. volvacea* specimens collected from different locations of the state of Odisha, India and to use that variability for strainal improvement programme and for understanding the molecular mechanisms of substrate utilization and fructification in *V. volvacea*.

## MATERIALS AND METHODS

### *V. volvacea* strains

The mycelial cultures of different strains were raised from the fruit bodies collected from nine different locations falling under seven districts of Odisha. The nine different locations belonged to five different agro-climatic regions. Maximum five strains were collected from East and South eastern coastal plain, two from mid-central Table land and one each from Northeastern ghat, North central plateau and West-central Table land (Table 1). The specimens were brought to pure culture by tissue culture method and coded as OSM-1 to OSM-10.

### Morphological characteristics of strains

Strains were characterized for radial mycelial growth (dia. in mm) and colony morphology on malt extract agar (MEA) Petridishes and for downward mycelial growth as well as mycelial growth density and the extent of mycelial growth on pounded paddy straw filled in wide mouth test tubes. For each strain, the 6 mm (dia.) uniformly grown mycelial bit was placed in the center of the MEA Petridish and these were incubated at 34±2 °C for 7 days. Three replications were kept for each strain. For downward mycelial growth the pounded paddy straw was wetted overnight and the substrate with 70-72% moisture was filled in wide mouth test tubes, plugged and sterilized at 20 psi for 1.30 h. The sterilized paddy straw was inoculated with one mycelial bit of 6 mm dia./tube. The inoculated tubes were incubated at 34±2 °C for 9 days. The downward mycelial growth was measured in mm along the extent and the density of the mycelial growth. Three replications were kept for each treatment.

### DNA extraction and 5.8S rRNA gene amplification

The mycelial cultures of all strains were grown separately on MEA Petridishes at 30±2 °C for 7 days. The mycelia from individual strain were scrapped and put in 1.5 ml micro-centrifuge tubes, kept at -85 °C for 2 h and freeze dried for 16-18 h. Genomic DNA was extracted from approximately 100 mg of freeze dried mycelia using DNeasy Plant Mini Kit (QIAGEN GmbH, D-40724 Hilden) following the protocol supplied by the manufacturer.

The polymerase chain reaction (PCR) primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') developed by White *et al.* [24] were used to amplify the ITS region along with 5.8S rDNA. PCR amplification was performed in a reaction mixture of 50 µl, containing 0.2 µl *Taq* DNA polymerase (5 U $\mu l^{-1}$), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2.0 mM each), 1 µl each of ITS-1 and ITS-4 primers (0.01 mM), 1 µl glycerol (5%), 2 µl $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). PCR reaction was performed in PCR Master Cycler Gradient in 36 cycles each of 95 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min 20 sec and final elongation at 72 °C for 10 min with lid heating option at 104 °C. The presence and yield of amplicons was ascertained on 2.0% agarose gel (w/v) prepared in 1% TBE. Gel electrophoresis was carried out at 90 V for 1.30 h in 0.5X TBE buffer. Staining was done with ethidium bromide and the gel was visualized and photographed using Bio Imaging System (Gene Genius, Syngene).

**PCR products cleaning, sequencing, annotation and blasting**

Amplicons of 5.8S rRNA of different strains were cleaned by using RCB kit (Banqiao City, Taipei County 220, Taiwan). Cleaned amplicons were got sequenced using 3730Xl (96 capillary) electrophoresis instruments from Bioserve Biotechnologies, Hyderabad, India. Received sequences in ab1/SCF format were cleaned to remove the misleading sequences and were improved upon using Chromas Lite 2.01 software (Copyright © 1998-2005 Technelysium Pty Ltd). The improved consensus sequences were blasted using BLASTn tool of NCBI [25] and the species against which highest similarity exhibited, was considered as the species identified.

**Sequence alignment, phylogeny and evolutionary relationships**

The improved consensus sequences of 5.8S rDNA of different strains were studied for variability in their nucleotide sequences by using ClustalW2 tool of European Bioinformatics Institute (EBI). Phylogenetic and molecular evolutionary analyses for strains were conducted using MEGA version 5.0 [26]. The evolutionary history was inferred using the neighbor-joining method [27].

**Enzyme assay**

The activity of different enzymes of the different strains was studied first by growing them on sterilized paddy straw substrate with 70% moisture in flasks. The enzymes were extracted from the mycelium-colonized substrate in 50 ml phosphate buffer (0.1 M), pH 7.0 by keeping the buffer mixed substrate at 40 °C for 30 min in an incubator shaker maintained at 100 rpm. The extract was filtered through glass microfibre filter (GF-C) and stored at 4 °C for further use. The enzyme assay was carried out in triplicate for all the enzymes and data were subjected to statistical analysis using AGRES software.

The cellulases were measured according to the method of Mandels *et al.* [28] as modified by Sandhu and Kalra [29]. The reaction mixture for exoglucanase (FPase, EC 3.2.1.91) comprised of eight filter paper (Whatman No. 1) discs of 0.6 cm dia in 0.5 ml acetate buffer of pH 5.0 and 0.25 ml of the enzyme source. The reaction mixture was incubated at 40 °C for 4 h and the reducing sugars released were measured by Nelson Somogyi method [30]. The endoglucanase (CMCase, EC 3.2.1.4) activity was measured following the above method, replacing filter paper discs with 0.5 ml of 5 mg $ml^{-1}$ carboxy methyl cellulose. Xylanase (EC 3.2.1.8) was assayed at 40 °C following a method described by Reese and Mandels[31].

Laccase (EC 1.10.3.2) was assayed by adding 0.3 ml enzyme source to 2.5 ml of 30 μM guaiacol in phosphate buffer (0.1 M) of pH 6.0 and Δ A was read at 470 nm after incubating the reaction mixture for 30 min at room temperature against zero time control. Polyphenol oxidase (EC 1.10.3.1) was assayed using catechol as substrate in place of guaiacol. One unit of laccase and polyphenol oxidase activity was calculated as change in absorbance of 0.001 $min^{-1}$ $ml^{-1}$ of enzyme source at 25 °C, while that of FPase, CMCase and xylanase as the μ mol glucose released $h^{-1}$ $ml^{-1}$ of enzyme source.

**Mushroom spawn preparation and crop raising**

The ready to mix spawn of different strains was prepared by using chopped paddy straw following the method of Ahlawat *et al*. [32].The strains were evaluated for fruit body yield using compost prepared with paddy straw + cotton ginning mill waste in 1:1 ratio (w/w). Composting was carried out by following the method of Ahlawat *et al*. [32]. Chicken manure and $CaCO_3$ were added @ 5.0% and 1.5% (dry wt. basis), respectively on day 0 and day 3 of stacking, while turnings were given on day 1, 2, 3 and 4 of outdoor composting. Beds of 180 cm x 70 cm x 12 cm (l x w x h) size were prepared with 35 kg wet substrate on shelves of iron racks in cropping room. Six replications were kept for each strain and the experiment was conducted in a randomized block design. The data recorded was subjected to statistical analysis using AGRES software. The fresh mushroom yield was recorded in g/20 kg of ready to use composted substrate. Data was also recorded for the time taken for first harvest in days post-spawning and mean fruiting body wt (g).

## RESULTS AND DISCUSSION

### Morphological characteristics

A total of 10 strains cultured from mushroom specimens collected from nine different locations falling in seven different districts and five agro-ecological regions were used for the study (Table 1). The strains were recorded for radial and downward mycelial growth, type of growth and formation of aerial hyphae as well as intensity of chlamydospores on MEA medium in Petridishes and pounded paddy straw in wide mouth glass test tubes (Table 2).

**Table 1**. Particulars of *Volvariella volvacea* isolates collected from different agro-climatic situations of Odisha (2010-11)

| Isolates | Place of collection | Date of collection | Agro-climatic zone |
|---|---|---|---|
| OSM-1 | Athgarh, Cuttack | 06.09.2010 | Mid-Central Table Land |
| OSM-2 | Bhubaneswar, Khurda | 09.08.2010 | East and South Eastern Coastal Plain |
| OSM-3 | Bhubaneswar, Khurda | 12.08.2010 | East and South Eastern Coastal Plain |
| OSM-4 | Salepur, Cuttack | 14.08.2010 | East and South Eastern Coastal Plain |
| OSM-5 | Lambodarpur, Dhenkanal | 13.09.2010 | Mid-Central Table Land |
| OSM-6 | Aska, Ganjam | 26.07.2010 | North Eastern Ghat |
| OSM-7 | Nalapari, Kendrapara | 23.08.2010 | East and South Eastern Coastal Plain |
| OSM-8 | Balakati, Khurda | 1.08.2010 | East and South Eastern Coastal Plain |
| OSM-9 | Betnoti, Mayurbhanj | 5.09.2010 | North Central Plateau |
| OSM-10 | Padiabahal, Sambalpur | 2.08.2010 | West-Central Table Land |

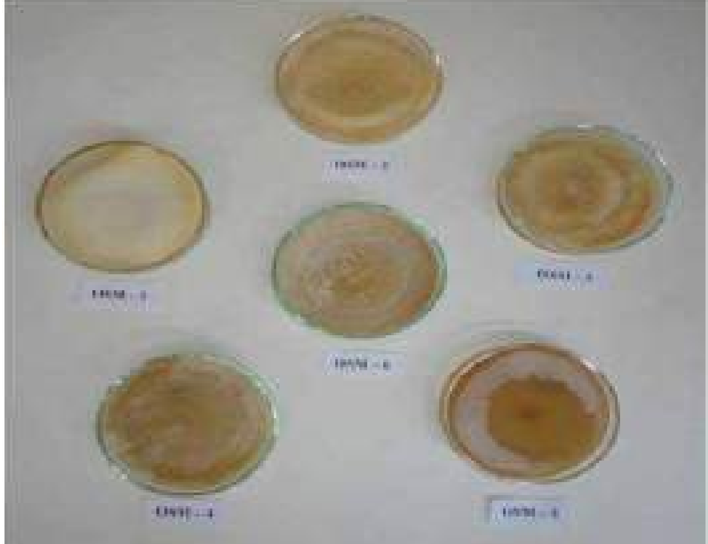

**Figure 1.** Mycelial growth of strains grown on Malt Extract Agar in Petridishes

The data presented in Table 2 reveal that strains OSM-1, OSM-2, OSM-3, OSM-4, OSM-6, OSM-7 and OSM-9 were fast growing as compared to strains like OSM-5, OSM-8 and OSM-10. The strains also varied in density and extent of aerial mycelial growth. The aerial mycelia were almost absent in slow growing strains. Strains, OSM-1 and OSM-7 exhibited highest mycelial growth density, followed by strains OSM-2, OSM-3 and OSM-9. The data for the downward mycelial growth on pounded paddy straw reveal that it was highest in strain OSM-1 (106 mm), followed by strains OSM-7 (85 mm), OSM-3 (78 mm), OSM-2 and OSM-9 (77 mm) and OSM-4 (68 mm). It was lowest in strains OSM-8 and OSM-10 (22 mm), followed by OSM-5 (44 mm). The majority of the fast growing strains formed creamy

white colonies, except of strains OSM-1 and OSM-6, which formed white coloured colonies. These low growing strains OSM-5, OSM-8 and OSM-10 formed white, creamy and yellowish colonies (Table 2).

**Table 2.** Morphological growth characteristic of different strains of *Volvariella volvacea* on malt extract agar medium.

| Strains | Mycelial growth characteristics | | | | |
|---|---|---|---|---|---|
| | Radial mycelial growth (Dia. mm) | Downward mycelial growth (mm) | Aerial mycelia | | Colony colour |
| | | | Mycelial density | Extent | |
| OSM - 1 | 90 | 106 | ++++ | +++++ | White |
| OSM - 2 | 90 | 77 | +++ | ++++ | Creamy White |
| OSM - 3 | 90 | 78 | ++ | ++++ | Creamy White |
| OSM - 4 | 90 | 68 | - | +++ | Creamy White |
| OSM - 5 | 44 | 44 | - | + | White |
| OSM - 6 | 90 | 61 | + | +++ | White |
| OSM - 7 | 90 | 85 | ++ | +++++ | Creamy White |
| OSM - 8 | 57 | 22 | - | ++ | Creamy |
| OSM - 9 | 90 | 77 | + | ++++ | Creamy White |
| OSM - 10 | 35 | 22 | - | + | Yellowish |

Least - +; highest - +++++; absent –

In present study majority of the strains exhibited the characteristics of a 'typical' *V. volvacea* strain (vigorously growing mycelia with abundant aerial and horizontal hyphae, mycelia usually thick at the margins of agar plate) [18], excepting 3 strains (OSM-5, OSM-8 and OSM-10), which showed quite 'atypical' characteristics matching characteristics of single spore isolates of *V. volvacea* [9, 18]. The variations in morphological characteristics in strains of *V. volvacea* and changes in their morphological growth characteristics at different stages of growth have also been reported earlier [15, 18]. So, broadly two different types of strains came in picture; one comparatively fast growing both on MEA and paddy straw and second quite slow growing on both types of media. Within the group, the strains also varied in their type of growth and colony characteristics.

## Phylogenetic analysis of different strains

**Nucleotide BLAST of Sequences:** As the BLAST of all 10 sequences done, the first hit strain or species of each query sequence from which the query sequence was maximum aligned is given here. All strains were found to belong to the species *V. volvacea*.

**Diversity analysis using CLUSTALW:** The diversity analysis exhibited 5.8S rRNA gene sequences of two different sizes. The first group of five strains (all fast growing) comprised of strains OSM-1, OSM-2, OSM-3, OSM-4 and OSM-7 exhibited amplicons of 638 bp long, while the second group of five strains (three slow and two fast growing) comprised of strains OSM-5, OSM-6, OSM-8, OSM-9 and OSM-10 exhibited ampliconsof 636 bp long (Fig. 2). There were deletions at two different places in second set of

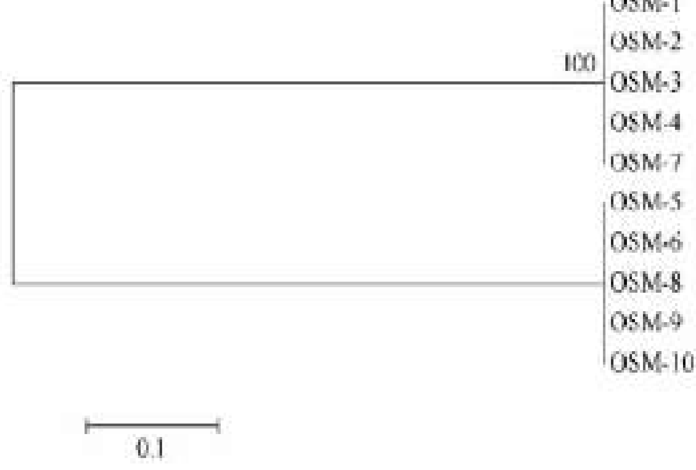


**Figure 2.** Neighbor-Joining T reededuced from 5.8S rRNA gene sequences of different strains

strains, one each in ITS-1 and ITS-2 regions. The sequences in two sets of strains also showed substitution at four different nucleotides in ITS-1 region (Fig. 3). In several earlier studies variations have been reported both in parent strains as well as in SSIs of the strains based upon their RAPD profiles [21, 22] and the laccase gene sequences [33]. However, only in a recent study, the variations in 5.8S rRNA gene sequences have been used for studying the variability in SSIs of *V. volvacea* [34].

```
OSM-1     CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-2     CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-3     CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-4     CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-7     CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-8     CAGAATCGAACGTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-6     CAGAATCGAACGTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-5     NNNNNNNNNNNNNTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-10    NNNNNNNNNNNNNTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
OSM-9     NNNNNNNNNNCGTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC     60
                       ***********************************************
OSM-1     GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC     120
OSM-2     GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC     120
OSM-3     GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC     120
OSM-4     GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC     120
OSM-7     GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC     120
OSM-8     GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC     120
OSM-6     GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC     120
OSM-5     GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC     120
OSM-10    GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC     120
OSM-9     GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC     120
          ******************* *************************** ************
OSM-1     GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     240
OSM-2     GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     240
OSM-3     GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     240
OSM-4     GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     240
OSM-7     GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     240
OSM-8     GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     239
OSM-6     GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     239
OSM-5     GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     239
OSM-10    GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     239
OSM-9     GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA     239
          ******* ******* ********************************************
OSM-1     GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     600
OSM-2     GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     600
OSM-3     GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     600
OSM-4     GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     600
OSM-7     GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     600
OSM-8     GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     598
OSM-6     GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     598
OSM-5     GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     598
OSM-10    GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     598
OSM-9     GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG     598
           ******* ****************************************************
```

**Figure 3.** ClustalW analysis of the 5.8S rRNA gene sequences of different strains

**Extracellular lignocellulolytic enzymes activity profile:** The crude enzyme extract extracted from fully mycelial colonized paddy straw was used as an enzyme source. Exoglucanase activity was highest in fast growing strain OSM-1, followed by slow growing strain OSM-5 and another fast growing strain OSM-2. It was least in fast growing strain OSM-9, followed by two slow growing strains OSM-9 and OSM-10. Endoglucanase activity was highest in fast growing strain OSM-6, followed by strain OSM-9 and two slow growing strains OSM-10 and OSM-8. It was least in strain OSM-2. β-glucosidase activity was highest in strain OSM-2, followed by strains OSM-3, OSM-4 and OSM-1. Its activity was lowest in strain OSM-9. Activity of xylanase was highest in strain OSM-6, followed by strains OSM-4 and OSM-1. Its activity was lowest in slow growing strain OSM-5. Laccase activity was highest in strain OSM-7, followed by OSM-2 and slow growing strain OSM-8. Its activity was lowest in strain OSM-10. Similarly, activity of PPO was highest in strain OSM-7, followed by OSM-2 and least in OSM-3 (Table 3).

**Table 3.** Extracellular lignocellulolytic enzymes activity of strains of *Volvariella volvacea*

| Strains | Enzyme activity | | | | | |
|---|---|---|---|---|---|---|
| | Exo-glucanase | Endo-glucanase | β-glucosidase | Xylanase | Laccase | Polyphenol oxidase |
| OSM- 1 | 0.0066 | 0.0045 | 0.0093 | 0.0066 | 164.911 | 54.588 |
| OSM- 2 | 0.0054 | 0.0038 | 0.0114 | 0.0049 | 371.005 | 100.773 |
| OSM- 3 | 0.0048 | 0.0052 | 0.0107 | 0.0050 | 90.916 | 47.910 |
| OSM- 4 | 0.0049 | 0.0064 | 0.0107 | 0.0070 | 84.815 | 51.071 |
| OSM- 5 | 0.0056 | 0.0053 | 0.0074 | 0.0046 | 43.938 | 57.610 |
| OSM- 6 | 0.0050 | 0.0121 | 0.0072 | 0.0075 | 74.572 | 58.110 |
| OSM- 7 | 0.0053 | 0.0064 | 0.0095 | 0.0060 | 388.694 | 122.316 |
| OSM- 8 | 0.0041 | 0.0087 | 0.0094 | 0.0064 | 263.70 | 64.638 |
| OSM- 9 | 0.0036 | 0.0110 | 0.0068 | 0.0048 | 47.527 | 57.932 |
| OSM-10 | 0.0037 | 0.0105 | 0.0070 | 0.0050 | 1.549 | 56.094 |

**Units of measurement:** Exo and Endo-glucanase/Xylanase - μ mole glucose released/min/ml of filtrate; PPO/Laccase - change in absorbance by 0.001/min/ml of filtrate; β–glucosidase - μ mole p-nitrophenol released/min/ml of filtrate.

In extracellular lignocellulolytic enzymes activity profiles, the strains did not exactly behaved as per extant of their mycelial growth on MEA and paddy straw. In case of β–glucosidase and xylanase, the highest activities were exhibited by fast growing strains, however, mixed responses were recorded in laccase, polyphenol oxidase and endo-glucanase activities. Here again the superior activity of an individual enzyme was not found sufficient to support the fast mycelial growth on MEA and paddy straw as in case of an earlier study [21]. Like a few earlier studies with monosporous isolates [9] and with parent strains of *V. volvacea*. [8], several fast growing strains were recorded to exhibit quite low level of activities of laccase and polyphenol oxidase. Here, again it is proved that mycelial growth in *V. volvacea* strains is not regulated by any one specific enzymes and laccase in particular. The variability in activities of extracellular enzymes in *V. volvacea* strains along with role of cellulases in mycelial colonization and laccase in sporophore formation have also been reported earlier by other workers [5, 7].

**Mushroom yield parameters and yield potential:** Out of ten strains selected in the beginning and used in other *in vitro* studies, only six strains, which showed good mycelial growth on master spawn and commercial spawn substrates, were selected for yield evaluation trials. Out of six strains selected, superior spawn run was recorded in strain OSM-1, followed by strains OSM-3 and OSM-9 (Table 4). In rest three strains it was almost of same level. Pinning intensity was highest in strain OSM-9, followed by OSM-3, while pinning was not recorded in strain OSM-6. Highest mushroom yield was recorded from the beds of strain OSM-9 (1416 g/bed). It was followed by yield in beds of strain OSM-3 (1048.12 g). Strains OSM-1, OSM-6 and OSM-7 gave only few scattered mushrooms.

**Table 4.** Yield potential of different strains on composted substrate of paddy straw + cotton ginning mill waste (1:1, w/w).

| Strains | Mycelial colonization of substrate | Pinning | Time taken for 1st harvest (days post spawning) | Yield (1st week) (g/number/bed of 20 kg substrate) | Average wt. of fruit bodies (g) |
|---|---|---|---|---|---|
| OSM- 1 | 5.0 + | 0.166 + | 15.66 | 13.16/0.5 | 26.32 |
| OSM- 2 | — | — | — | — | — |
| OSM- 3 | 3.87 + | 0.333 + | 15.48 | 1048.12/66.12 | 15.85 |
| OSM- 4 | 2.0 + | 1.0 + | 15.00 | 19.6/1.66 | 11.84 |
| OSM- 5 | — | — | — | — | — |
| OSM- 6 | 2.33 + | — | 17.33 | 55.5/4.16 | 13.34 |
| OSM- 7 | 2.50 + | 0.5 + | 14.00 | 26.66/1.66 | 16.06 |
| OSM- 8 | — | — | — | — | — |
| OSM- 9 | 3.83 + | 1.0 + | 14.41 | 1416/181.5 | 7.80 |
| OSM- 10 | — | — | — | — | — |

+: visible, —: not visible

The strains were also evaluated for time taken for first harvest (day post spawning) and the mean fruiting body wt. Lowest time for first harvest was recorded in strain OSM-7 (14.0 days), closely followed by strain OSM-9. In rest strains, it was almost same. The fruit body weight was highest in strain OSM-1 (26.32 g), followed by strain OSM-7 (Table 4). Amongst good yielding strains, the fruit body wt. was highest in strain OSM-3 (15.85 g) and just almost half in strain OSM-9 (7.80 g). Yield evaluation trials for selecting a better performing strain or single spore isolate have also been performed earlier by several workers [9, 15, 32, 35, 36] but only few of them have correlated the morphological and biochemical characteristics of a strain with its yield potential and have helped in selecting several high yielding strains [15, 32].

The present study reveals a relationship between the origin of the strains and its phylogenetic belongingness and it can be attributed to the origin of strains from a common parent in that area. There are only very few reports on using the lignocellulolytic enzymes activity or the protein profiles of the strains in studying polymorphism in different mushrooms and that too are restricted to use of laccase polymorphism in *Agaricus bitorquis* [37] and protein profiles in *A. bisporus* and *A. bitorquis* [38]. The relationship between origin of strains and the variability existed in them have also been studied earlier [21] and reported variability in strains originated from Odisha and Kerala states of India. However, in present case variability in the strains obtained from different regions of same state Odisha has been highlighted, which will strengthen the hypothesis of existence of variability in the strains of *V. volvacea*, which otherwise is considered as primary homothallic leaving very less scope of variability in this species. The fast growing strains like OSM-1 and OSM-7 did not gave fruiting even after exhibiting quite fast and dense mycelial growth on two media, contrary to medium level growing strains like OSM-3 and OSM-9 forming not very dense mycelial mat on media but gave fruiting in fruiting trials. These two strains even did not exhibit very high activities of any specific enzymes except of endo-glucanase in OSM-9 and β–glucosidase in strain OSM-3. The two strains were also isolated from far distant places and falling in two phylogenetically distinct groups. The present study nullifies the notion that fast growing attribute is the deciding factor for fruiting body formation or higher fruit body yield. Very few studies of this type have been conducted earlier, Chen *et al*. [8] and Ahlawat *et al*. [9], who had reported the role of cellulases in substrate colonization and laccase in sporophore formation in this mushroom. The present study has provided an insight into the subject, which will help in developing a solid base for existence of variability in *V. volvacea* growing under natural conditions.

## CONCLUSION

The specimens collected were found to vary both at mycelial growth characteristics and their 5.8S rRNA gene sequences levels, and broadly can be placed under two different groups. The strains exhibited varied levels of extra cellular lignocellulolytic

enzymes activities and not much correlation between the mycelial growth characteristics and the enzymatic activity profiles was recorded. Wide variations were also recorded at the levels of mycelial colonization of spawn and cultivation substrates and the fruiting pattern, as the slow growing strains along with one fast growing strain OSM-2 did not colonize the wheat grain based spawn substrate, while many fast growing strains did not gave fruiting. The strains have provided a good germplasm for understanding the biology of substrate colonization and fruiting, and their use in strain development programme.

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# GENETIC CHARACTERIZATION OF SINGLE SPORE ISOLATES OF *AGARICUS BISPORUS* (LANGE) IMBACH

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## ABSTRACT

The yield and quality of the mushroom produced is determined by three factors, the genetic makeup of the mushroom strain, the environmental conditions in which the mushroom is grown, interaction of genotype and environment and the quality of nutrition. In India, introduction of exotic strains of button mushroom and their selection on the basis of their evaluation is a continuing process. The present investigation was undertaken to develop improved strains of *Agaricus bisporus* through single spore isolates (SSIs) and genetic characterization of developed SSIs using SDS-PAGE protein profiling. On the basis of yield performance, four strains of *Agaricus bisporus*, namely, S-11, MS-39, NCS-100 and NCS-101 were selected (designated as A,B,C and D, respectively) and used for the isolation of single spores. A total of 410 SSIs were developed out of which 255 SSIs performed below or at par to their respective parent while 155 of SSIs out yielded the parent. Further, on the basis of higher yields, 5 SSIs of A (S-11), 2 SSIs of B (MS-39), 5 SSIs of C (NCS-100) and 6 SSIs of D (NCS-101) were selected and analyzed by protein profiling. On the basis of protein profiles, the above 18 isolates were classified into seven groups. The total number of bands ranged from 5 to 13. Within group, the isolates had very little difference in banding pattern both for position and intensities of bands, however, between group difference was observed.

Keywords: spore print, single spore isolates, protein profiling, *Agaricus bisporus*

## INTRODUCTION

Mushrooms are delicious, nutritionally rich, medicinally important and non-conventional source of human food. Mushroom production is regarded as the second most important commercial microbial technology, next only to yeast, for large scale profitable bioconversion of lignocellulosic wastes from agro-industry. Presently, mushrooms are being cultivated in about 100 countries. In India, *Agaricus bisporus* (button mushroom) contributes about 80-85 per cent of the total annual production of mushrooms. The yield and quality of the mushroom is determined by three factors, the genetic makeup of the mushroom strain, the environmental conditions in which the mushroom is grown and the quality of nutrition. In India, introduction of exotic strains of button mushroom and their selection on the basis of their evaluation is a continuing process. The present investigation was undertaken to develop improved strains of *A. bisporus* through single spore isolates (SSIs) and genetic characterization of developed SSIs using SDS – PAGE protein profiling.

## MATERIALS AND METHODS

On the basis of yield and quality performance, four strains of *Agaricus bisporus* viz., S-11, MS-39, NCS-100 and NCS-101 were selected as parent for the studies.

### Collection of spore print

A well formed and healthy sporophore with veil still intact but tightly stretched was selected for the spore print. Sporophore was surface sterilized and mounted on a sterilized wire stand and placed over the bottom plate of a sterilized petriplate and covered with a sterilized bell jar. After a thick deposit of spore mass, the lid was replaced and sealed with a parafilm strip, then stored in a refrigerator for further use.

### Preparation of spore suspension

A loopful of spores from spore print was transferred aseptically to 10 ml of sterilized distilled water in a test tube. The suspension was shaken thoroughly and 1 ml of this suspension was transferred to another tube containing 9 ml of sterilized distilled water. Likewise the serial dilution of spore suspension was made four or five times, till 1 ml of the suspension contain 20-25 spores.

### Plating for spore germination and isolation of SSIs

For spore's germination, 1 ml of spore suspension was added to 9 ml of sterilized and melted wheat agar media and poured into sterilized petriplate. Then the plates were rotated clockwise and anti-clockwise direction to mix and spread the spores in the media. The plates were then allowed to solidify. After that the plates were inverted and seeded with living mycelia in the lid plate. Then these plates were kept under incubation for spore germination. After a week of incubation, the lids of Petri plates containing living mycelia were substituted with another set of sterilized lids. The plates were then observed under high resolution Inverted Microscope. The individual germinating spores were marked and individual spore were transferred to the slants having malt extract agar media. The isolated spores were then incubated at 25±1 °C temperature. After 5-6 days of incubation, the SSIs start growing and mycelium was visible.

### Spawn preparation and yield evaluation

Selected SSIs were then taken to spawn preparation, for that, the wheat grain spawn was prepared using the standard methodology. The isolates were cultivated in the winter season from October to March and cultivation trials were conducted under natural climatic conditions of temperature 15-20 °C and relative humidity (75-90%) at Mushroom Research and Training Center, Pantnagar using standard cultivation technology on the compost prepared by short method of composting.

### Protein Profiling

Identification of different isolates of *A. bisporus* was carried out using protein profile, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). For doing the estimation, average sized fruit bodies were collected and washed with distilled water. After that the protein profiling was done using standard protocol applied for SDS-PAGE method and the results obtained were described.

## RESULTS AND DISCUSSION

### Development of Single Spore Isolates

120 SSIs of strain S-11, 80 SSIs of strain MS-39, 100 SSIs of strain NCS-100 and 110 SSIs of strain NCS-101 were developed and were designated as SSIs A, B, C & D, respectively, for convenience for further use (Table 1). All the isolates were then tested for their yielding ability and fruiting test. None of the isolates were found sterile for the use in hybrid development. All the SSIs developed (410) from 4 strains of *A. bisporus* were further divided into three categories on the basis of their yield performance as (i) below parent, (ii) at par with the parent and (iii) above parent. The number of SSIs grouped in these categories was 180, 75 and 155 respectively (Table 1). [1] tested yield performance of SSIs from 3 strains of *A. bisporus* and the evaluation of various SSIs indicated that maximum numbers of isolates were below or at par in yield with the parents, however, few number of isolates out yielded the parent strains.

**Table 1.** Performance of single spore isolates from four strains of *A. bisporus*

| Strain | Number of Isolates yielding | | | |
|---|---|---|---|---|
| | **Below parent** | **At par** | **Above parent** | **Total** |
| S-11 (A) | 43 | 16 | 61 | 120 |
| MS-39 (B) | 27 | 23 | 30 | 80 |
| NCS-100 (C) | 51 | 18 | 31 | 100 |
| NCS-101 (D) | 59 | 18 | 33 | 110 |
| **Total** | **180** | **75** | **155** | **410** |

### Screening of high yielding SSIs

The isolates selected on the basis of their yield from 6 weeks of harvesting period were tested for their performance in respect of yield first and then for quality parameters. Those, which yielded higher or at par with their parental strains were

selected for further tests and categorized as low, high and very high yielders with respect to their yield in a period of 6 weeks harvest. On the basis of higher yields 5 SSIs of A, 2 SSIs of B, 5 SSIs of C and 6 SSIs of D were selected (Table 2). Various workers have concluded that single spore isolates had variation in the cultures and in yield than that of parent strains and an increase in yield up to 21 percent was recorded in one of the single spore isolates of the parental strain S-11 by Kumar & Munjal [2, 4, 5]. The single spore isolates were found to show considerable difference in growth, morphological characters and yield [5-8]. Bhandal & Mehta [9] and Mehta *et al*. [10] isolated for promising single spore isolates with regards to mycelial characteristics, spawn run, yield and sporophore characters which resulted in release of two single spore isolates, NCS-100 and NCS-101 for commercial cultivation.

**Table 2.** Yield performance of different high yielding *A. bisporus* SSIs

| Isolates | Yield kg/q compost | | | |
|---|---|---|---|---|
| | Crop I | | Crop II | |
| | Numbers | Weight | Number | Weight |
| C-6 | 1725 | 16.08 | 1423 | 17.21 |
| C-13 | 1816 | 18.26 | 1460 | 16.77 |
| C-15 | 1630 | 18.75 | 1540 | 19.71 |
| A-27 | 2030 | 19.80 | 1799 | 21.48 |
| A-89 | 2482 | 23.37 | 1916 | 23.40 |
| A-61 | 2000 | 19.84 | 1655 | 20.22 |
| B-8 | 1798 | 17.66 | 1582 | 20.90 |
| B-49 | 1955 | 19.92 | 1760 | 22.49 |
| D-47 | 1975 | 20.03 | 1425 | 16.65 |
| D-54 | 2204 | 22.55 | 1410 | 19.38 |
| C-68 | 1945 | 20.31 | 1790 | 22.40 |
| D-20 | 2060 | 20.55 | 1827 | 19.97 |
| D-2 | 2720 | 23.58 | 1952 | 22.50 |
| A-46 | 2190 | 20.59 | 1985 | 20.80 |
| D-48 | 2240 | 23.09 | 1904 | 22.59 |
| C-47 | 2710 | 23.37 | 1806 | 17.82 |
| D-63 | 1230 | 10.61 | 2695 | 20.04 |
| A-67 | 1490 | 15.59 | 1895 | 20.55 |
| Check I(A) | 1635 | 14.97 | 1354 | 16.75 |
| Check II(B) | 1440 | 15.05 | 1520 | 16.68 |
| Check III(C) | 1116 | 13.39 | 1213 | 14.26 |
| Check IV(D) | 1380 | 15.68 | 1233 | 13.64 |
| CD at 5% | 40.5 | 0.62 | 35.44 | 0.75 |

## Protein profiling analysis

The total protein was analyzed by SDS-PAGE. Banding pattern obtained on gel was utilized for checking zymogram, representing the relative position and intensities of bands. The whole zymogram was divided into three zones (A,B and C) as three different band intensities were obtained viz., dark, medium dark and light bands (Table 3, Plate 1). Total number of protein bands ranged from five to thirteen spread over three zones. On the basis of total number of bands and their intensity, isolates were classified into seven groups. There were two isolates in five bands group, four in six bands group, three in nine bands group, one in ten bands group and one isolate was grouped in thirteen bands group (Plate 1). In five band groups, two isolates A-61 and D-63 were present. D-63 had bands spread in all the three zones while A-61 had no band in zone A. In A-61 most of the bands were present in zone C while in D-63 bands were uniformly distributed in all the

**Table 3.** Banding pattern of pileus of different *A. bisporus* SSIs

| Isolate | Total number of bands in different zones | | | | Intensity of bands | | |
|---|---|---|---|---|---|---|---|
| | Total no. | A | B | C | Dark | Medium | Light |
| A-61 | 5 | 0 | 1 | 4 | 1 | 4 | 0 |
| D-63 | 5 | 1 | 2 | 2 | 5 | 0 | 0 |
| C-15 | 6 | 3 | 1 | 2 | 2 | 4 | 0 |
| A-27 | 5 | 3 | 1 | 2 | 2 | 2 | 2 |
| D-20 | 6 | 1 | 1 | 4 | 0 | 2 | 4 |
| D-2 | 6 | 2 | 2 | 2 | 5 | 0 | 1 |
| C-13 | 7 | 2 | 2 | 3 | 0 | 2 | 5 |
| B-8 | 7 | 3 | 0 | 4 | 0 | 0 | 7 |
| C-68 | 7 | 3 | 1 | 3 | 0 | 3 | 4 |
| A-67 | 7 | 2 | 1 | 4 | 7 | 0 | 0 |
| D-18 | 8 | 2 | 1 | 5 | 6 | 0 | 2 |
| A-89 | 8 | 3 | 1 | 4 | 1 | 0 | 7 |
| D-47 | 8 | 2 | 2 | 4 | 1 | 6 | 1 |
| B-49 | 9 | 2 | 3 | 4 | 3 | 3 | 3 |
| D-54 | 9 | 2 | 3 | 4 | 0 | 4 | 5 |
| C-47 | 9 | 3 | 1 | 5 | 9 | 0 | 0 |
| A-47 | 10 | 3 | 2 | 5 | 8 | 2 | 0 |
| C-6 | 13 | 6 | 4 | 3 | 2 | 1 | 10 |

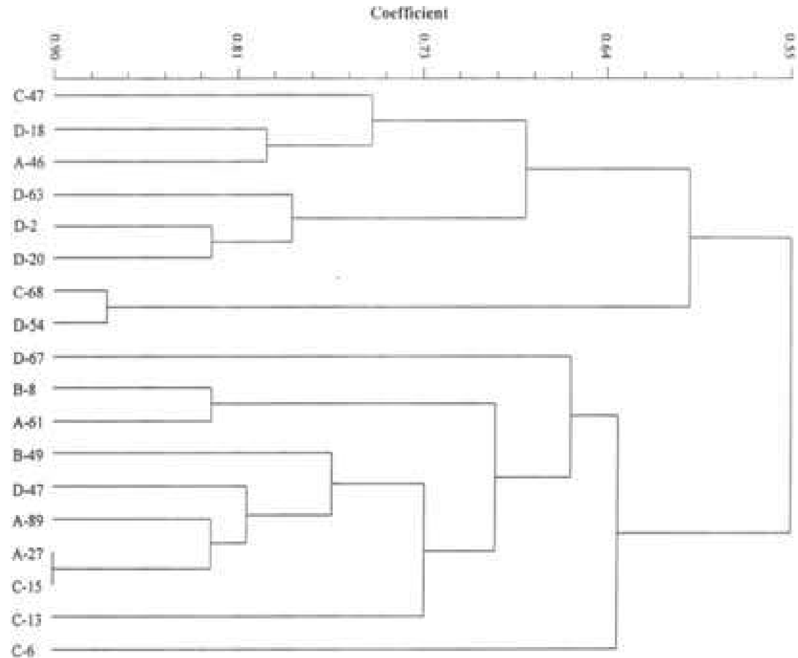


**Figure 1.** Combined Dendogram

three zones. Intensities wise both the isolates had mostly dark and medium intensity bands (Plate 1). In six band group, out of four isolates, two C-15 and A-27 had similar relative position of bands in zone A and zone C. Remaining two isolates had different banding pattern with D-20 having one band each in zone A and B and four bands in zone C while D-2 had two bands in each zone. C-15 had no light bands while D-20 had no dark band. D-2 had maximum number of five dark bands.

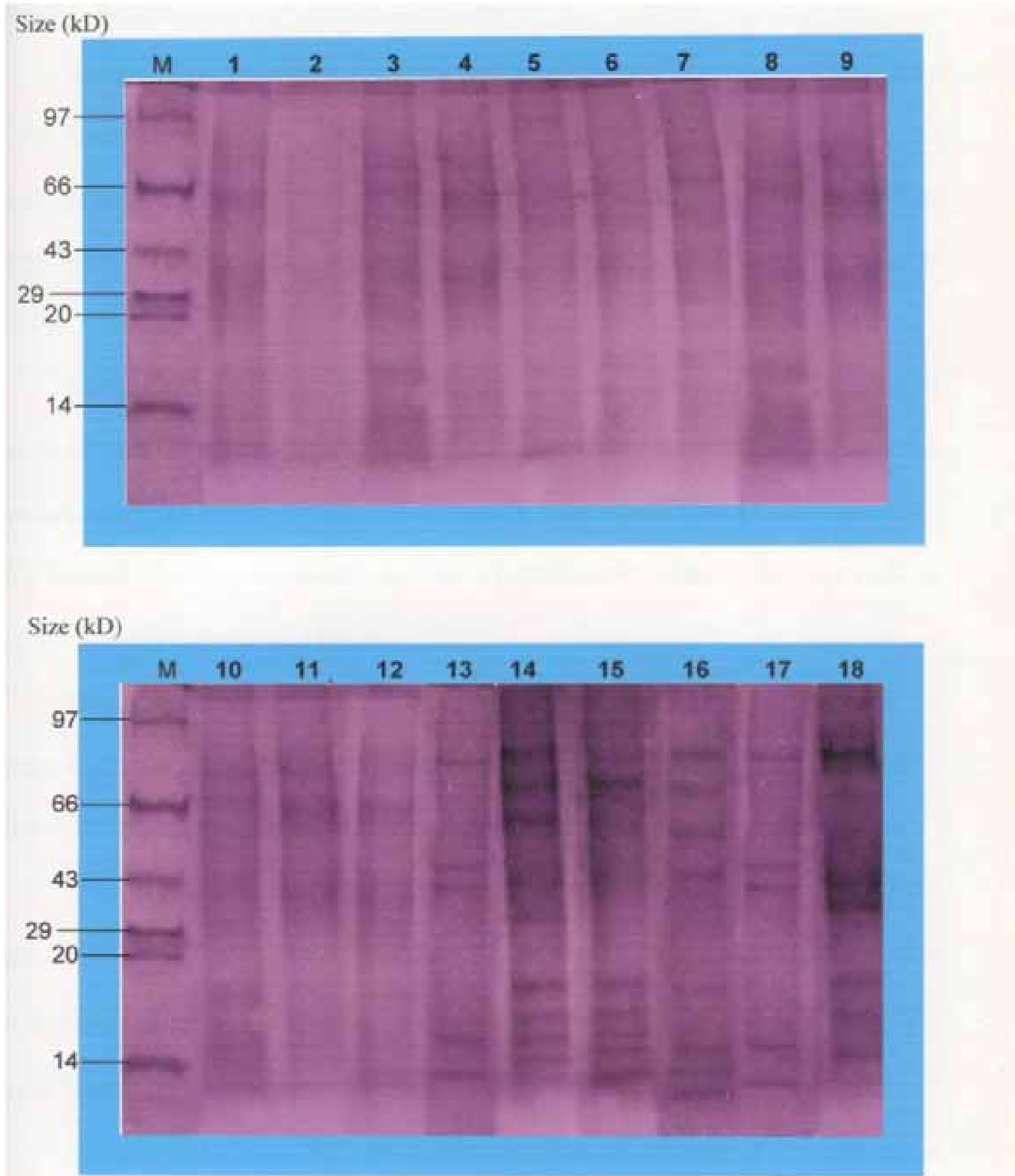


**Plate 1:** Protein profiling of fruit bodies of different isolates of *A.bisporus*
1=C-6, 2=C-13, 3=C-15, 4=A-27, 5=A-89, 6=A-61, 7=B-8, 8=B-49, 9=D-47,10=D-54, 11=C-68, 12=D-20, 13=D-2, 14=A-46, 15=D-18, 16=C-47, 17=D-63, 18=A-67 And M=Marker

In seven band group, there were four isolates out of which except B-8 all the isolates had bands in all the three zones with most of the bands being clusters in zone A and zone C. Intensity wise, except A-67 which had all dark bands, no isolates had dark band. B-8 had all light bands. In eight bands group, all the three isolates had band in each zone and most of the bands distributed in zone A and zone C. In terms of relative position of bands spread over different zones, it was seen that overall C zone was similar for all the member of this group, while variation in position of bands were observed in zone A and B. There were nine bands in three isolates (B-49, D-54 and A-47) but B-49 and D-54 had equal number of bands in all the three zones although the relative positioning of bands were different. Intensity wise, C-47 had all the bands with dark intensity (having maximum number of dark bands) while other two isolates had all the three intensity of bands. One isolate (A-47) had ten bands and isolates C-6 had a maximum of 13 bands in it. C-6 was the isolate which had maximum number of bands (six) in zone A. This isolate also had maximum number of light intensity bands (Ten). Overall it was observed that

in zone A, total number of bands varied from zero to six. A-61, being the only isolate having no bands in zone A, most of the isolates were having band of 2-3 which varied between and within groups. The bands lying in the zone were medium to light in intensities. The range of bands in zone B was found to be in the range of 1-4 in this zone. 50 percent isolates had one band while other had 2-3 bands. Only C-6 had four bands. All types of bands intensities were present in this zone. In zone C the range of bands were in the range of 2-5. As far as intensity was concerned, it was observed that almost half of the isolates had either zero or one dark band. Six isolates had five dark bands. Medium intensity bands were present in all the zones but most of them were accumulated in zone A and B. Light intensity bands were mostly present in zone A. A full range of zero to ten light bands was present. Maximum numbers of bands were present in C-6 (Plate 1).

## CONCLUSION

Isolation of single spores is one of the techniques widely used for the genetic improvement in various species of mushrooms. But on an average about 50 per cent of the isolates are not performing superior than the parents in terms of yield and quality. Amongst the superior isolates we can select the best isolate as one of the strain for cultivation purposes but there is need to check it further for the quality characters. These SSIs may vary genetically among themselves. Therefore, protein profiling is another way to know the genetic diversity. On the basis of protein profiles, all the 18 isolates were classified into seven groups. The total number of bands ranged from 5-13 and spread over three zones (A, B and C). One isolate was in 13 band group while two isolates were in 5 band group, four in 6 bands group, three in 9 bands group, four in 7 bands group, three in 9 bands group and one in 10 bands group. Within group, the isolates had very little difference in banding pattern both for position and intensities of bands, however between group difference was observed. Irrespective to group, isolates varied more for high molecular weight protein band as compared to low molecular weight proteins.

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# SEQUENCE ANALYSIS OF PARTIAL *TEF1α* AND *RPB2* GENES OF DIFFERENT *PLEUROTUS ERYNGII* ISOLATES BY MEANS OF PCR-RFLP

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## ABSTRACT

The *Pleurotus eryngii* species complex includes several varieties and certain groups with ambiguous taxonomic position. At the moment the following taxa are recognized: var. *eryngii*; var. *ferulae* /syn.: *P. fuscus* var. *ferulae*/; var. *elaeoselini*; var. *nebrodensis*; var. *tingitanus*; var. *tuoliensis*; *P. hadamardii*; *P. fossulatus*. In the course of our studies sequence analysis of the *tef1á* and *rpb2* genes was performed, in order to distinguish varieties and reveal variability between isolates.

Specific regions of the *tef1á* and *rpb2* genes were amplified in PCR experiments. The fragments were cleaned, sequenced, aligned then BLAST searched against the NCBI GenBank nucleotide database. Some point mutations were detected in the sequences, which were used for selection of differentiating restriction enzymes for subsequent PCR-RFLP experiments.

The *tef1α* gene sequences showed 100% identity in each strain. In contrast to that, point mutations were detected in the 21, 372 and 957 positions of the *rpb2* sequences. *In silico* digestion was performed on the sequences and two restriction endonucleases, the *BsmA*I and *TspDT*I were selected for PCR-RFLP experiments. As a result of the digestions, the isolates could be grouped into two groups with both enzymes. At the same time, BLAST search with both amplified sequences did not give reliable information neither on varietas, nor on species level. The *rpb2* locus, with its higher level of polymorphism, is a potential candidate for differentiation between varieties or identification on varietas level.

Based on the results we plan to investigate which loci and molecular methods may be suitable for differentiation of isolates.

**Keywords:** *Pleurotus eryngii*, *tef1á*, *rpb2*, PCR-RFLP

## INTRODUCTION

As it was described in former studies, the *Pleurotus eryngii* species complex consists of numerous varieties and taxa with uncertain taxonomic positions. Nevertheless, each species share a common feature: they are associated with certain species of the *Apiaceae* (*Umbelliferae*) and *Asteraceae* (*Compositae*) families, as facultative biotrophs or white rot fungi in their natural habitat [1–7].

The term 'species complex' is used in general for fungi species that are closely related, but partially or completely incompatible [8, [9, [4], [10.

Identification of the species might be very difficult, because the classical, mostly micro- and macro morphological characters might be masked by environmental effects. Hence, taxonomic research is often based on nucleic acids nowadays. The ITS (Internal Transcribed Spacer) region of the ribosomal gene cluster is one of the most frequently investigated DNA fragment in fungal taxonomy. In former studies, phylogenetic relations amongst members of the *P. eryngii* species complex were thought to be revealed on basis of the ITS region and partial â-tubulin gene sequences. Unfortunately, none of these regions showed such variability that could be used for differentiation of varieties [11–14].

Additional candidates for molecular phylogenetics and identification on varietas level are the genes coding certain regions of the translation elongation factor (EF1α) and the second largest subunit of the RNA polymerase II (*rpb2*) [11, 12, 14-19]. The EF1á is a binding protein used in ribosomal protein synthesis in eukaryotic cells. Marongiu *et al*. [20] found

nucleotide substitutions in the *tef1á* gene (coding EF1á) that might be useful in differentiation of *eryngii* and *ferulae* varieties.

The *rpb2* gene has a strongly conserved domain, so it is a good candidate for PCR primers. Formerly, the *rpb2* was used (with additional genomic regions) for identification of *Cortinarius* and *Inocybe* species [18, 21].

In addition to the beta-tubulin, *tef*1 and *rpb*2 genes, there are numerous other loci available for fungal taxonomists, including nuclear and mitochondrial ribosomal rDNA (18S, 28S, ITS, IGS, mtSSU, mtLSU) and protein coding genes, e.g. *rpb*1, gamma-actin or ATP synthase. In previous experiments, those protein coding genes that are present in the genome in a single copy, were successful candidates in phylogenetic experiments [22–25]. Nevertheless, those genes that have multiple copies in the genome, might be potential sources of misleading results [26]. The type of gene one would like to use for phylogenetic analysis depends on how long evolutionary history is intended to be revealed. Quickly evolving genes offer good support for detecting recent evolutionary events, whereas genes with lower mutation rate are more suitable for long term evolutionary research. Ribosomal genes, such as ITS, IGS and mtSSU rDNA are subjects of high and moderate rate of mutation; hence, these loci are good choice for discrimination of isolates at genus and species level. Unfortunately, non-coding regions of these genes tend to undergo length variation, which might cause serious problems during alignment. Nowadays sequencing is popular method for investigation of organisms, but sequencing of entire fungal genomes or even multiple loci is not an option for many taxa, because fungal material convenient for molecular experiments is often limited and culturing of numerous species is not possible [27].

## MATERIALS AND METHODS

### Strains

King oyster mushroom strains were isolated from grassy fields of Novaj, Eger Felnémet-Pásztorvölgy, Bogács, Tószeg, Kecskemét and Heves (Hungary) in autumns between 2006 and 2008. Furthermore, some strains were made available to us from strain collection of the Species Research Laboratory of Korona Mushroom Union. Codes and place of origin of the strains used in this study are the following: Hungarian strains: PEA – Tószeg; PEP – Eger, Pásztorvölgy; PEG – Tószeg; PEF-i – Demjén, Vas-tanya; PEF – Kecskemét; PEC – Eger, Pásztorvölgy; Ple1V – Novaj; Ple2V – Novaj; Ple3V – Bogács; Ple4V – Hevesi Füves Puszta; Ple5V – Novaj; Ple6V – Novaj. Strains from abroad: PES – the Netherlands; PE-SZM – Malaysia; PEL – North-Italy; PEK – China.

### DNA extraction and PCR conditions

DNA extraction was done according to the method of Szarvas et al. (2011) [28]. Amplification and sequence analysis was performed on the *tef1á* and *rpb2* regions, in order to differentiate *P. eryngii* strains and varieties.

Fig.1 shows the expected approx. 550 bp long fragment of the *tef1á* locus, amplified in the PCR reactions. Primers annealed to and amplified the 4-6 exon regions (green) that included the 4-5 introns (I.4 and I.5), as well. Sequences of the primers are the following: EF595F (5' CGT GAC TTC ATC AAG AAC ATG 3') and EF1160R (5' CCG ATC TTG TAG ACG TCC TG 3').

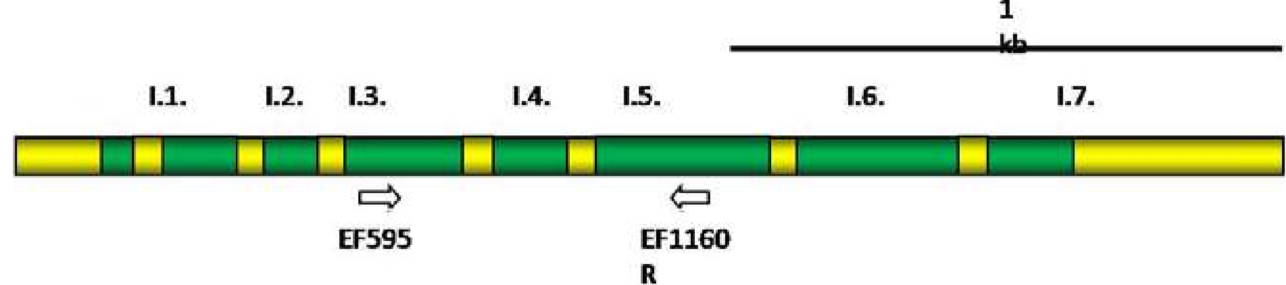


**Figure 1.** Annealing sites of primers (empty arrows) in the *tef1á* gene (coding translation elongation factor EF1á). Yellow: introns (I.1-I.7); green: exons (based on data of Wendland & Kothe 1997 [29]).

An almost 1100 bp long fragment of the *rpb2* (RNA-polymerase II) locus was amplified by PCR, too. The primers amplified the 4-5 exon (domain 7-11) region (Fig. 2). Primers used in this reaction were bRPB2-6.9F (=b6.9F) (5' TGG ACN CAY TGY GAR ATY CAY CC 3') and bRPB2-11R1 (=b11R1) (5' TGG ATY TTG TCR TCC ACC AT 3').

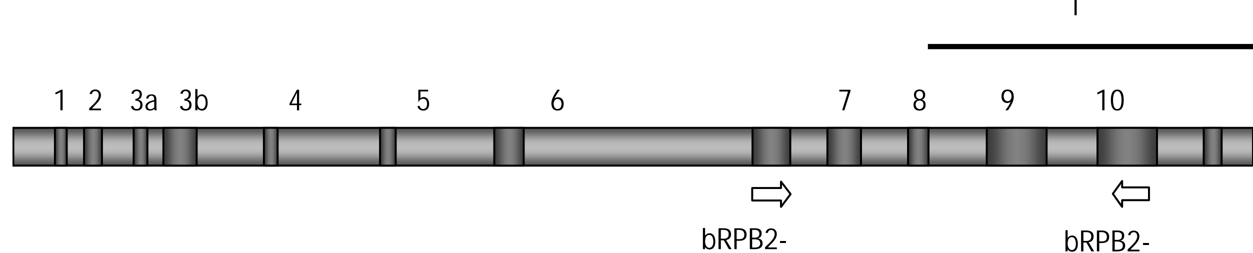


**Figure 2.** Structure of *rpb2* locus of Basidiomycetes [30]. Primer positions and direction of synthesis are shown by arrows. Eukaryotic conserved regions are shown in blue

In the first step conditions of the PCR reaction were optimized. Annealing temperature of primers was determined by temperature gradient PCR experiments, it was followed by the regular reactions. DNA of the 16 *P. eryngii* strains was used as template, after the concentration of each one was set to 10 ng/ìl, in order to avoid false results due to unequal amount. The PCR reactions were based on the DreamTaq Kit (Fermentas) and set up as follows (for one reaction): 2.5 ìl DreamTaq Buffer, 0.5 ìl 25 mM $MgCl_2$, 0.5 ìl 10 mM dNTP, 0.2 ìl DreamTaq Polymerase (5U/ìl), 1 ìl forward primer (5 pM/ìl), 1 ìl reverse primer (5 pM/ìl), 2 ìl template DNA (10 ng/ìl); 17.3 ìl distilled water. The PCR program was optimized for both primer pairs and was the following: 1. 95 °C 3 min; 2. 95 °C 5 s, 57 °C 10 s, 72 °C 30 s; 3. 72 °C 5 min. The second step was repeated 35 times. PCR reactions were performed in a Corbett Research Thermocycler (Corbett Life Science, Australia) instrument.

The amplicons were visualized by gel electrophoresis on 1% (w/v) agarose gel (SeaKem LE Agarose, Lonza, USA) in 1× TBE (Tris-Borate-EDTA) for 30 min at 130 V and made visible by GelRed (Biotium, USA) staining and UV transillumination in a BioDocAnalyze (Biometra, Germany) instrument. A 100 bp BenchTop DNA ladder (Promega, USA) was used as molecular size marker.

**Sequencing and alignment of sequences.**

The fragments were cleaned by EZ-10 Spin Column Kit PCR Purification Kit (Biobasic Inc., Canada), according to the manufacturer's instructions. Quality of the processed amplicons was checked again on 1% agarose gel as above and 10 ìl of each sample was sent to be sequenced with both primers in the sequencing laboratory (LGC Genomics, Germany).

In the next step the fragments sequenced from the reverse primer were used to generate their reverse complement version, in order to be used for alignment. Reverse complement sequences were made by the EditSeq software of DNAStar package. It was followed by the alignment of reverse complement sequences with fragments sequenced by the forward primer, and then the complete fragments of each strain were aligned with each other. Both steps were performed by ClustalX (http://www.clustal.org/clustal2/), and the final alignment was visualized by BoxShade http://www.ch.embnet.org/software/BOX_form.html). Due to the alignment point mutations, identities and differences between strains became visible, which might help in identification of varieties in future experiments.

Identification of strains was performed by blasting the aligned sequences against the GenBank database of NCBI by BLAST algorithm.

**Analysis of fragments by RFLP (Restriction Fragment Length Polymorphism).**

Alignment of sequences revealed the existence of point mutations in some strains in the same position. These minor differences offer the possibility for differentiation of strains by RFLP, if proper restriction enzymes are available. Restriction enzymes

were selected by using the RestrictionMapper online tool (http://www.restrictionmapper.org/); *in silico* digestion was performed on the sequences to find enzyme(s) capable of cleaving the DNA at the point mutations.

In total, two enzymes were found that showed ability to cut the DNA at the mutations. These enzymes were *Bsm*AI and *Tsp*DTI, with recognition sites and digestion profile showed on Fig. 3 and Fig. 4.

*BsmA*I

5′ GTCTC$(N)_1$↓ 3′

3′ CAGAG$(N)_5$↑ 5′

**Figure 3.** Cleavage site of *BsmA*I.

*TspDT*I

5′ ATGAANNNNNNNNNNN↓ 3′

3′ TACTTNNNNNNNNNN↑NN 5′

**Figure 4.** Cleavage site of *TspDT*I.

Reaction setup for one reaction with the *BsmA*I (New England Biolabs, USA) digestion was the following: 3 ìl NE buffer 4, 1 ìl *BsmA*I, 16 ìl distilled water and 10 ìl PCR mix, containing the amplified fragments. Digestion was performed at 55 °C for 60 min.

Reaction setup for one reaction with the *TspDT*I (EURx, Poland) digestion was the following: 3 ìl 1 × *TspDT*I buffer, 1 ìl *TspDT*I, 16 ìl distilled water, 10 10 ìl PCR mix, containing the amplified fragments. Digestion was performed at 70 °C for 3 hours.

The amplicons were visualized by gel electrophoresis on 2% (w/v) agarose gel (SeaKem LE Agarose, Lonza, USA) in 1× TBE (Tris-Borate-EDTA) for 180 min at 80 V and made visible by GelRed (Biotium, USA) staining and UV transillumination in a BioDocAnalyze (Biometra, Germany) instrument. A 100 bp BenchTop DNA ladder (Promega, USA) was used as molecular size marker.

## RESULTS AND DISCUSSION

### PCR experiments

Amplification of the *tef1α* gene resulted only one fragment, which size was the expected 550 bp and non-specific product was not found (Fig. 5). In contrast to that, non-specific fragments were visible below the main product (1000 bp) on agarose gel after the amplification of *rpb2* gene, probably due to the fact that primers used in this reaction were degenerated. Nevertheless, these by-products could be easily removed by cleaning and only the main product was present and used for further processing (Fig. 6).

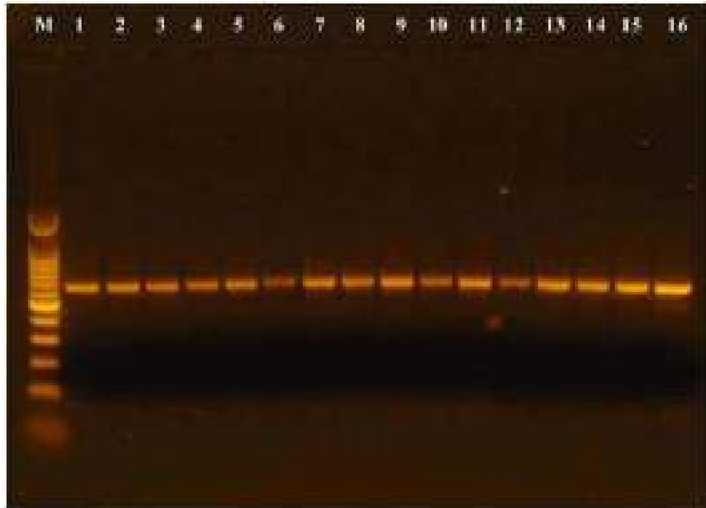


**Figure 5.** Amplification of the *tef1α* gene of 16 *P. eryngii* strains resulted only the expected fragment

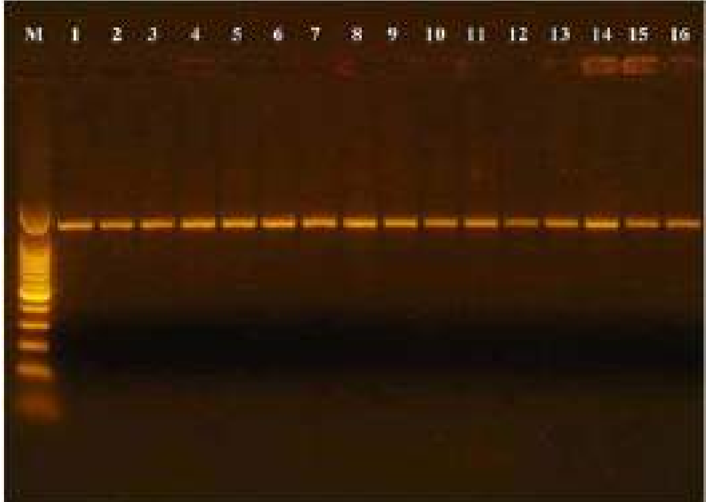


**Figure 6.** Main products of the *rpb2* PCR with DNA of 16 *P. eryngii* strains after removing non-specific products.

M: size marker, 1. PEP, 2. PEC, 3. PES, 4. PEL, 5. PEF, 6. PEA, 7. PE-SZM, 8. PEG, 9. PEF-i, 10. PLE-1V, 11. PLE-2V, 12. PLE-3V, 13. PLE-4V, 14. PLE-5V, 15. PLE-6V, 16. PEK*(M: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp).*

After sequencing and alignment of sequences, we had the data available for further analysis: 553 bp long fragment of the translation elongation factor *(tef1á)* and 1031 bp long fragment of the RNA polymerase II (*rpb2*).

## Results of sequencing and BLAST search

Sequence analysis of the *tef1α* fragments showed that no polymorphism was present amongst the strains; homology of sequences was 100%. Rodriguez Estrada *et al*. (2010) [14] found minor differences between the *tef1* sequences of var. *nebrodensis* and var. *eryngii* isolates which were used for discrimination of these varieties. Based on our results we can state that there was no var. *nebrodensis* amongst our isolates.

In the same study, Rodriguez Estrada *et al*. (2010) [14] found that differences in the *rpb2* gene are sufficient for discrimination of *eryngii*, *elaeoselini* and *ferulae* varieties. In our sequences we found only a few nucleotide substitutions (Table 1).

**Table 1.** Nucleotide substitutions in the *rpb2* sequences of 16 *P. eryngii* strains

| Position of nucleotide | Nucleotide | Isolate |
|---|---|---|
| 21 | C | Ple-1V and Ple-2V |
| | T | other strains |
| 372 | G | Ple-1V, Ple-2V, PEG, Ple-3V, Ple-4V |
| | A | other strains |
| 957 | G | Ple-1V, Ple-2V, Ple-3V, Ple-4V, PEG, PES |
| | A | other strains |

Aligned and BoxShade processed sequences are shown on Fig. 7. Since nucleotide substitutions might mean differences between varieties, we tried to check our sequences in the GenBank database.

Blasting the *tef1á* and *rpb2* sequences against the GenBank database resulted in interesting findings. Results of *tef1á* blasts showed that all of our isolates should be *P. fossulatus* with 100% identity and 0 E-value, and only 94% identity was found with *P. eryngii* sequences. Blasting with *rpb2* resulted that our isolates are *P. ostreatus* with 100% identity and 0 E-value. Similarity with *P. eryngii* var. *eryngii* sequences was only 70%, but the same value was found with var. *elaeoselini* and var. *ferulae* sequences, as well. Since these results were unexpected, we investigated a couple of sequences downloaded from the GenBank. Our analysis showed that there is uncertainty in certain extent in the data; hence, more sequences should be uploaded after thorough analysis in order to increase confidence of BLAST search results.

Concluding the results above, only the *rpb2* locus contained nucleotide substitutions in our isolates. Investigation of the sequences of both loci revealed nucleotide polymorphism only in a low degree. Our analysis showed that deposited sequences of *tef1á* and *rpb2* loci in the GenBank should be revised, because these sequences are not suitable for strain identification. At the same time it became clear that investigation of the ribosomal region, *tef1á* and *rpb2* genes is not sufficient for discrimination of strains and varieties of *P. eryngii*, more loci must be involved in phylogenetic experiments. It is clear that molecular identification of varieties still has some serious drawbacks, which need more time to eliminate.

```
Ple_5V        1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
Ple_6V        1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEFI          1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PE-SZM        1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEA           1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEFA          1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEL           1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEK_4         1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEC           1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEP           1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PES           1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
Ple_E_1V      1  TCACAATCAGGTATGTCGTTCATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
Ple_2V        1  TCACAATCAGGTATGTCGTTCATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
PEG           1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
Ple_3V        1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
Ple_4V        1  TCACAATCAGGTATGTCGTTTATCGTTAAGACGCATGCATGCTCGCCTCACGCAGTTTGT
consensus     1  ********************.***************************************

Ple_5V      361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
Ple_6V      361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEFI        361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PE-SZM      361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEA         361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEFA        361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEL         361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEK_4       361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEC         361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEP         361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PES         361  CTCGAAAAGAAATCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
Ple_E_1V    361  CTCGAAAAGAAGTCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
Ple_2V      361  CTCGAAAAGAAGTCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
PEG         361  CTCGAAAAGAAGTCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
Ple_3V      361  CTCGAAAAGAAGTCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
Ple_4V      361  CTCGAAAAGAAGTCTGGAATCCAGCAGTTGGAAGAATTCGAGAAGCCTTCTCGTGACAAC
consensus   361  ***********.************************************************

Ple_5V      901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
Ple_6V      901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PEFI        901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PE-SZM      901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PEA         901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PEFA        901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PEL         901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PEK_4       901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PEC         901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PEP         901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGACAG
PES         901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGGCAG
Ple_E_1V    901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGGCAG
Ple_2V      901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGGCAG
PEG         901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGGCAG
Ple_3V      901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGGCAG
Ple_4V      901  GGTGACGCTACTCCATTCACCGATCTTACGGTCGAGTCCGTTTCCGCCTTCTTGAGGCAG
consensus   901  ********************************************************.***
```

**Figure 7.** Aligned *rpb2* sequences of 16 *P. eryngii* isolates

**Results of RFLP analysis.**

*In silico* digestion showed that both restriction enzymes were able to divide the strains into two groups.

<u>*BsmA*I</u>

*Group 1:* 504 bp, 456 bp, 71 bp (Ple-5V, Ple-6V, PEF-i, PE-SZM, PEA, PEF, PEL, PEK, PEC, PEP, PES)

*Group 2:* 504 bp, 373 bp, 83 bp, 71 bp (Ple-1V, Ple-2V, Ple-3V, Ple-4V, PEG)

Results of the digestion by the real *BsmA*I enzyme were the same as the *in silico* digestion, but not each fragment was well visible on the agarose gel. In case of strains belonging to Group 2 (Ple-1V, Ple-2V, Ple-3V, Ple-4V and PEG), the 456 bp fragment is not present, though it is well visible at the other strains. So, this enzyme can be used for sorting the isolates into two groups (*Fig. 8*). It should be noted that fragment sizes in of *in silico* digestion are smaller than size of real fragments, because sequences were chopped prior to the theoretical digestion.

The *TspDT*I enzyme was not able to separate the strains (Fig. 9), though it was successfully performed during *in silico* digestion. During digestion a 9 bp long fragment is removed from the 313 bp fragment, but this difference is too small to visualize it agarose gel, so polyacrylamide electrophoresis or capillary electrophoresis is highly recommended in this case.

<u>*TspDT*I theoretical digestion</u>

*Group 1:* 586 bp, 304 bp, 132 bp, 9 bp (Ple-1V, Ple-2V)

*Group 2:* 586 bp, 313 bp, 132 bp (Ple-3V, Ple-4V, Ple-5V, Ple-6V, PEF-i, PE-SZM, PEA, PEF, PEL, PEK, PEC, PEP, PES, PEG)

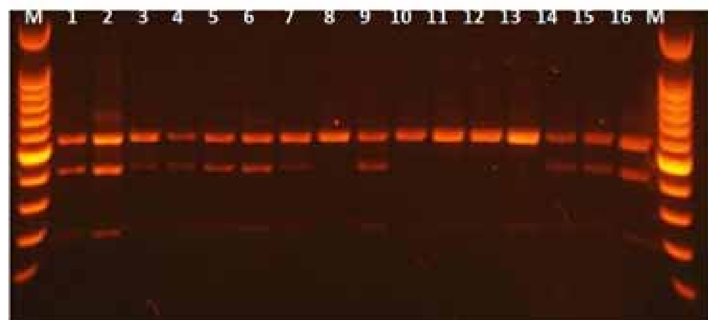


**Figure 8.** Digestion by *BsmA*I enzyme. (M: 100 bp size marker, 1. PEP; 2. PEC; 3. PES; 4. PEL; 5. PEF; 6. PEA; 7. PE-SZM; 8. PEG; 9. PEF-i; 10. PLE-1V; 11. PLE-2V; 12. PLE-3V; 13. Ple-4V; 14. PLE-5V; 15. PLE-6V; 16. PEK).
*(M: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp)*

**Figure 9.** Digestion by *TspDT*I restriction enzyme. (M: 100 bp size marker, 1. PEP; 2. PEC; 3. PES; 4. PEL; 5. PEF; 6. PEA; 7. PE-SZM; 8. PEG; 9. PEF-i; 10. PLE-1V; 11. PLE-2V; 12. PLE-3V; 13. Ple-4V; 14. PLE-5V; 15. PLE-6V; 16. PEK).
*(M: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp)*

## SUMMARY

In this study we presented the sequence analysis of *tef1á* and *rpb2* genes of 16 *P. eryngii* isolates (of mostly Hungarian origin) in order to reveal variability between isolates and varieties.

Fragments from of the *tef1á* and *rpb2* regions were amplified by specific primer pairs (*tef1á*: EF595F/ EF1160R; *rpb2*: bRPB2-6.9F/ bRPB2-11R1). The amplicons were cleaned, sequenced and blasted against the GenBank database. After sequence analysis, theoretical *in silico* digestion (RFLP) was performed on the sequences, which was followed by real digestion with restriction enzymes. The isolates showed 100% homology in the *tef1á* region, and a few nucleotide substitutions were found amongst the *rpb2* sequences of the various strains (in position 21, 372 and 957). The real digestion experiments were done with *BsmA*I and *TspDT*I restriction endonucleases, because these two enzymes were able to divide the isolates

into 2 groups in the *in silico* experiments. The real digestions were successful; though, fragments from the *TspDT*I digestion are advised to separate on polyacrylamide gel or with capillary electrophoresis, because differences were not visible on agarose gel. Results of the BLAST search were ambiguous on varietas and species level, as well. Since the *rpb2* locus showed higher degree of polymorphism than the *tef1á*, it has the potential to be used in PCR-RFLP experiments for discrimination of strains or maybe even of varieties. Nonetheless, in the future it is necessary to involve more loci and molecular methods in phylogenetic experiments aiming the differentiation of *P. eryngii* strains on varietas level.

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# THE WHOLE GENOME SEQUENCE OF *VOLVARIELLA VOLVACEA* WILL FACILITATE RESOLUTION OF PROBLEMS LIMITING ITS COMMERCIAL EXPLOITATION

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## ABSTRACT

*Volvariella volvacea* is widely grown in southeastern Asia as a high quality human food source, and is one of most important cultivated mushrooms worldwide. However, developments in *V. volvacea* cultivation have been limited due to a low biological efficiency (i.e. conversion of growth substrate to mushroom fruit bodies), sensitivity to low temperatures, and an unclear sexuality pattern that has restricted the breeding of improved strains. The Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, has recently completed the sequencing of the whole genome of *V. volvacea* strain V23-1, a single-spore isolate derived from *V. volvacea* strain V23. The sequence, which has been deposited in the NCBI database, is assembled into 62 scaffolds with a total genome size of 35.7 megabases (Mb), and contains 11,084 predicted gene models. Comparative analyses based on the whole genome sequence have been made with other basidiomycete mushrooms with regard to the mating type system, polysaccharide degrading enzymes and lignin oxidizing enzymes. Transcriptional analysis of the loss of hyphal viability following exposure to low temperature (4 °C) has also been undertaken. The *V. volvacea* genome contains numerous genes encoding enzymes involved in the degradation of cellulose, hemicellulose and pectin. Furthermore, the molecular structure of the mating type system is similar to that of the bipolar system of basidiomycetes, and indicates that *V. volvacea* has a secondary homothallic life cycle. The absence of genes encoding enzymes involved in initiating the biosynthesis of unsaturated fatty acids, trehalose and glycogen may be related in part to the sensitivity to low temperature exposure. Elucidation of the *V. volvacea* genome sequence will promote a deeper understanding at the molecular biological level of the mechanisms involved in substrate degradation and help to resolve problems limiting the industrial exploitation of this mushroom.

**Keywords:** *Volvariella volvacea*, whole genome sequence, sensitivity to low temperature exposure, mating type, polysaccharide degrading enzymes

## INTRODUCTION

*Volvariella volvacea*, also known as the Chinese mushroom and straw mushroom, is an edible fungus grown in tropical and subtropical regions. In the 18th Century, Buddhist monks of Nanhua Temple located in the Chinese Province of Guangdong, developed a primitive method of cultivating the straw mushroom to enrich their diet. Subsequently, *V. volvacea* was presented as a tribute to China's royalty [1, 2].

Although *V. volvacea* has been cultivated for approximately 300 years, there are still many associated production problems that greatly restrict the commercial exploitation of this mushroom. Mushroom yields in proportion to the dry weight of compost at spawning (biological efficiency) are only ~15% for straw substrates and 40% for cotton-waste 'composts' [3], which are very low values compared with many of the other major cultivated edible mushroom species such as *Agaricus bisporus, Lentinula edodes* and *Pleurotus* spp [4]. *V. volvacea* is also very sensitive to low temperatures, and the fungal mycelium will lose viability when exposed to temperatures below 15 °C. Furthermore, mushroom fruit bodies suffer chilling damage and undergo autolysis when stored at low temperatures (4 °C) [5], thereby restricting the shelf-life.

*V. volvacea* is generally recognized as a primary homothallic basidiomycete [1, 6, 7] although some single spore isolates are self-fertile (heterokaryotic) while others are self-sterile (homokaryotic) [8]. Moreover, *V. volvacea* hyphae are

multinucleate [9] and do not form clamp connections which, in other fungi, serve as morphological markers to distinguish self-fertile from self-sterile mycelia. These traits are problematic for the development of a simple and efficient method for *V. volvacea* cross-breeding.

Here, we report the complete genome sequence of the monokaryotic *V. volvacea* strain, V23-1. These data will facilitate analysis of the cellulase system and elucidate the sexual pattern of the mushroom. Furthermore, in order to help resolve a major problem restricting the industrial exploitation of *V. volvacea*, we have compared transcript profiles of mycelium exposed to low temperature with those of non-exposed mycelium in order to better understand the mechanism(s) underlying the mushroom's sensitivity to cold.

## MATERIALS AND METHODS

### Strains and culture conditions

*V. volvacea*, dikaryotic strain V23, was obtained from the Key Laboratory of Agricultural Genetics and Breeding of the Shanghai Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences. Single spore isolates were obtained by separation of basidiospores of *V. volvacea* strains V23 and PY on potato dextrose agar (PDA) plates and identified as sterile homokaryons by the cultivation test.

### DNA extraction, genome sequencing, assembly and annotation

Genomic DNA of *V. volvacea* was extracted by an improved cetyl trimethyl ammonium bromide (CTAB) method and sequenced using the Roche 454 GSFLX (Roche, USA) and Illumina Solexa GAIIx (Illumina, USA) platforms. Following pre-processing, the Roche 454 reads were assembled into a primary assembly using Roche Newbler software and then scaffolded with Illumina paired-end and mate-pair reads using Velvet software. Gene model prediction was undertaken by combining four (GeneMark, Augustus, Fgenesh and Geneid) software types.

### RNA extraction

Fungal mycelium from cultures of *V. volvacea*, strain V23, was grown in 250 ml flasks containing 100 ml potato dextrose broth (PDB) at 32 $^{o}$C for 4 days, and the fungal mycelium exposed to 4 $^{o}$C at zero time and for 2 and 4 hours. After harvesting, mycelia were lyophilized and total RNA was extracted using the Trizol regent according to the manufacturer's instructions.

### Protein family classification

Carbohydrate-active enzymes (CAZy) were classified by local BLASTp searching against a library of catalytic and carbohydrate-binding module enzymes constructed from the CAZy database (http://www.cazy.org/). The distribution of sequences encoding putative lignin-modifying enzymes (LME) in the fungal genome was determined by comparison with LME-encoding sequences in the NCBI database. To build up the LME database, our custom program compiled by perl was used to choose the sequences based on the BLASTp result (BLASTp, cut-off e-value > $1e^{-50}$) from the obtained sequences. LME families were classified by BLASTp against the LME database.

## RESULTS AND DISCUSSION

### Genome sequencing and general features

The *V. volvacea* whole genome sequence was determined using Roche 454 GSFLX sequencing and Illumina Solexa sequencing, respectively. The combined sequencings generated 190×coverage of the genome, which was assembled into 62 scaffolds with an N50 of 388 kb and a total size of 35.7 Mb (Table 1). The size of the *V. volvacea* genome is similar to the genomes of several other species assigned to the Agaricaceae including *Schizophyllum commune* (38.5 M)[10], *Coprinopsis cinerea* (37 M) [11] and *P. ostreatus* (35 M) [12], but is bigger than that of another straw rotting fungus,

**Table 1.** Features of the *V. volvacea* genome

| General features | 0 |
|---|---|
| Size of assembled genome (Mb) | 36.45 |
| GC content (%) | 48.86% |
| Length of classified repeats (%) | 2.25 Mb (6.18%) |
| Number of predicted gene models | 11,084 |
| Average gene length (with intron) (bp) | 2,087 |
| Average transcript length (bp) | 1,572 |
| Number of single-exon genes | 1,066 |
| Average number of exons per multi-exon gene | 7 |
| Average exon size (bp) | 229 |
| Average intron size (bp) | 88 |

*Agaricus bisporus* (30.2 M) [13].

Annotation of the assembled genome sequence generated 11,084 gene models, 76.43% of which were supported by EST data. The average transcript length was 1,572 bp, and an average of six introns per multi-exon gene. The average exon and intron sizes were 252.3 bp and 83.2 bp, respectively (Table 1).

## Carbohydrate active enzymes

*V. volvacea* is a kind of straw rotting fungi and relies mainly on the degradation of cellulose to obtain the bio-energy. In the genome of *V. volvacea* a total of 357 CAZyme-coding gene homologs were identified. This value is higher than the average number of 305 of other basidiomycetes. The glycoside hydrolases (GH) superfamily in the genome of *V. volvacea* contained 224 homologs, belonging to 42 families. CE, GT, PL and CBM superfamilies had each 28, 66, 18 and 21 homologs, distributed in 8, 24, 3 and 6 families, respectively (Fig. 1). *V. volvacea* has the highest number of PL modules (18) compared to other basidiomycetes. We made a detailed comparative analysis of the number of CAZy families related to plant polysaccharide degradation in *V. volvacea* and other basidiomycete genomes (Fig. 2). This analysis shows *V. volvacea* has 47, 101 and 57 candidate CAZymes related to the degradation of cellulose, hemicellulose and pectin, respectively. These values are respectively higher than the average (36, 77 and 35) of other basidiomycetes. The *V. volvacea* genome also has a larger number (57) of enzymes for degradation of β-1,3-1,4-glucan, which widely distributed

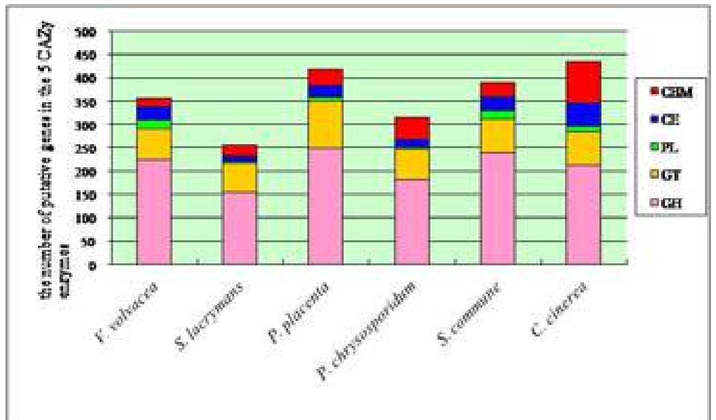


**Figure 1.** Comparison of the number of five CAZy gene groups in *V. volvacea* and other basidiomycetes fungi genomes. (From: Bao *et al*. [21])

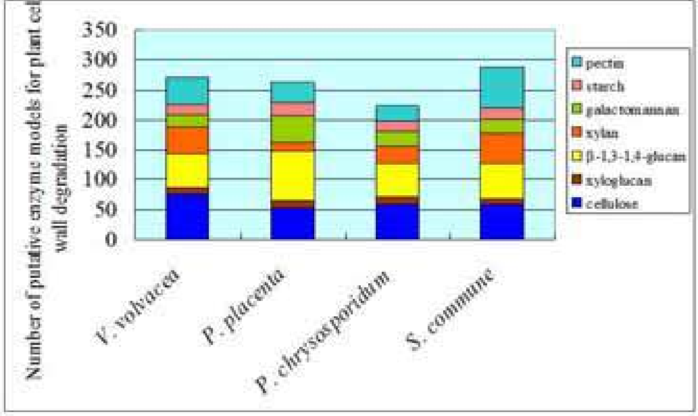


**Figure 2.** Number of putative enzyme models involved in plant cell wall degradation in *V. volvacea* and other basidiomycetes fungi genomes. (From: Bao *et al*. [21])

as non-cellulosic matrix phase polysaccharides in cell walls of grasses and cereal species. The set of CAZymes in the *V. volvacea* genome indicated the strong ability to digest the complex plant cell wall, especially for the pectin (57, average 37) and the xylan (56, average 31) (Fig. 2), suggesting *V. volvacea* mainly depend on pectin and xylan digestion to obtain the energy.

## Fungal oxidative lignin enzymes

*V. volvacea* is regarded as a straw-rotting fungus and appears to lack an effective ligninolytic enzyme system [14-16], which may account in part for its low biological conversion rate. Fungi degrade lignin by secreting multiple isoenzymes of three heme peroxidases (lignin peroxidases (LiPs), manganese peroxidases (MnPs), and hybrid enzymes known as versatile peroxidases (VPs)) and laccase. The *V. volvacea* genome contains two putative MnP and two putative VP gene encoding sequences, but lacks sequences encoding for LiP-encoding genes. Interestingly, the genome also contained 11 genes (*vv-lac1* to *vv-lac 11*) encoding laccase homologues, six of which were identical with the laccase genes obtained previously using PCR [17, 18]. Ten of the eleven were distributed within a 216 kb spanning region of scaffold 6 and arranged into a cluster supported by hypergeometric analysis. All the laccase genes except *vv-lac 11* were congregated in shorter chromosomal regions compared to *A. bisporous* and *C. cinerea* (Fig. 3), suggesting these genes may have been generated by duplication in recently evolutionary events. *VV-lac 11* is located at scaffold 8 and exhibits a high similarity with

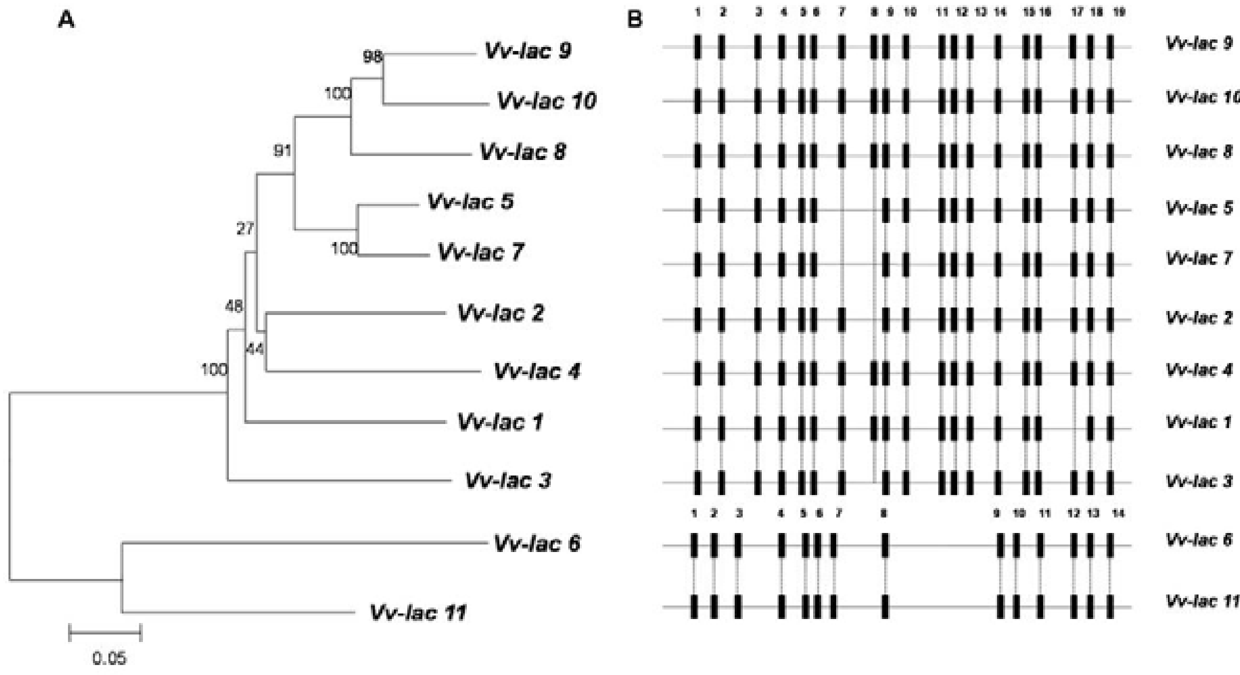


**Figure 3.** (A) Neighbor-joining tree of the deduced amino acid sequences of *V. volvacea*laccase genes. (B) Distribution of intron positions in *lac1-lac11*define two gene subfamilies. Black bars indicate intron positions. Dotted lines link the same introns. (From: Bao *et al*. [21])

*vv-lac6*, indicating their paralogous relationship (Fig. 3). Phylogenetic analysis revealed that nine *V. volvacea* laccase genes (*vv-lac1-5* and *vv-lac7-10*) are congregated within the same cluster (Fig. 3A), and share 17 (from a total of 19) conserved intron positions (Fig. 3B).

## Mating type loci and sexuality patterns

Scanning of the genome of the single spore-derived *V.volvacea*, strain V23-1, revealed two homeodomain (HD) genes, *VVO_04854* and *VVO_05004* (designated *vv-HD1*$^{-V23-1}$and *vv-HD2*$^{-V23-1}$) located 271 bp apart on scaffold 07. Both encoded HD proteins having a high similarity with homologues from the bipolar basidiomycetes, *A. bisporus* and *Pholiota nameko*, and the tetra polar basidiomycetes, *C. cinerea* and *S. commune*. We theorized that *V. volvacea* has one *A* mating type locus containing a pair of *HD* genes and accordingly, designed a pair of specific primers (VVmipF11:5'-

GTGACTGCTATGGAACACATTGGAC and VVmipR13: 5'- TCGGAGGAAGCGGGTCCACTACA) based on the DNA sequence surrounding the mating type *A* locus (designated *A*1) of strain V23-1 to amplify the *A* locus (designated *A*2) of another single spore-derived *V. volvacea* strain, V23-18. A pair of genes, *vv-HD1$^{-V23-18}$* and *vv-HD2$^{-V23-18}$* (Accession number: JX157875) was identified within the *A*2 locus, and the encoded HD1 and HD2 proteins from the spore monokaryons V23-1 and V23-18 showed 48% and 49% similarity, respectively.

In order to establish the efficacy of molecular marker-assisted cross-breeding techniques in generating improved *V. volvacea* cultivars, we designed four primer pairs based on the sequences of the *A* mating type genes (*HD1* and *HD2*) of the parental strains V23 (*A* mating type locus alleles *A1* + *A2*) and PY (*A* mating type locus alleles *A3* + *A4*), respectively. Of 124 single spore isolates obtained from strain V23, 101 were confirmed as homokaryons, 35 of which were mating type *A1* and 66 mating type *A2*. The remaining 23 were heterokaryons carrying both *A1* and *A2* mating type loci. Of 88 single spore isolates from strain PY, 72 were confirmed as homokaryons, of which 41 were mating type *A3* and 31 mating type *A4*. Sixteen were heterokaryons carrying both *A3* and *A4* mating type loci. Cross-breeding between 72 compatible pairs generated 58 hybrids, three of which were designated high quality hybrids based on agronomic traits determined by cultivation experiments.

## Transcriptional analyses of the 'cold shock response'

In order to identify the cellular responses occurring during low temperature (4 °C) exposure of *V. volvacea*, we carried out high-throughput sequencing of mRNA expression in fungal mycelium exposed to 4 °C at zero time, and after 2 and 4 hours. Differences in gene expression levels between 0 h and 2 h, and 0 h and 4 h were then compared using | log 2 (fold-change)|≥ 0.5 and FDR <0.001 as the threshold for significant changes.

Previously, yeast genes involved in carbohydrate energy reserves, especially trehalose and glycogen biosynthesis, were reported to be induced after exposure to 4 °C [19,20], suggesting that biosynthesis and accumulation of these reserve carbohydrates may be necessary for cold tolerance and energy preservation [19]. Analysis of gene expression associated with starch and sucrose metabolism (PATH: ko00500) revealed that 32 genes were expressed at 0, 2 and 4 h (Fig. 4). However, no significant differences in the expression levels of the majority of these genes were observed and, while several genes were significantly under-expressed, none were significantly over-expressed. Among the former, expression of *VVO_02898* and *VVO_08127*, which respectively encode homologues of TPS1 (trehalose phosphate synthase) and

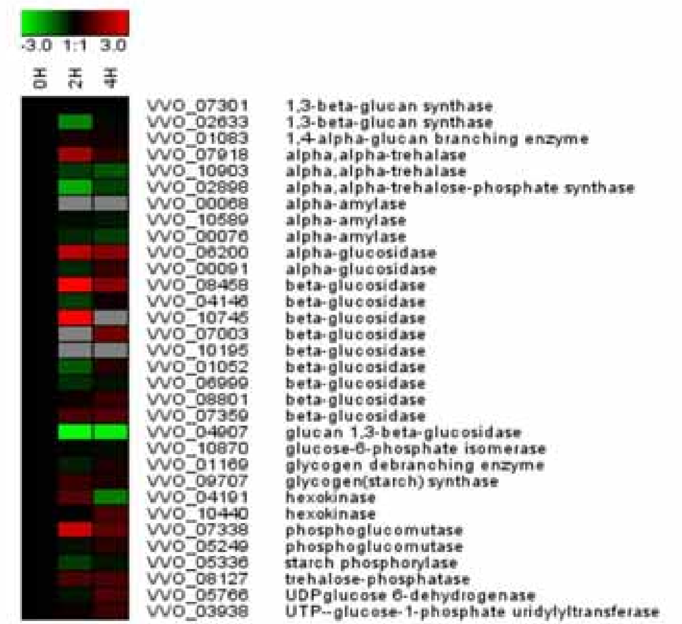


**Figure 4.** Heatmap showing expression levels of genes in starch and sucrose metabolism pathways after 0, 2 and 4 h exposure to 4 ºC. (From: Bao *et al*. [21])

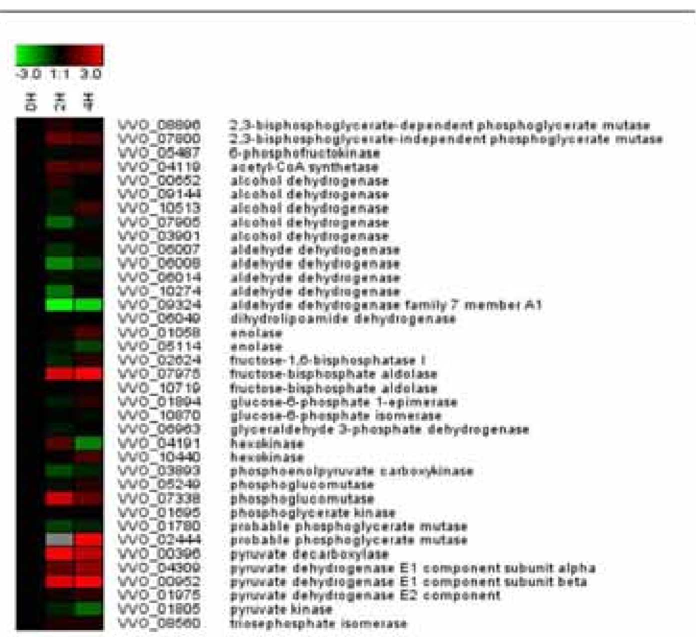


**Figure 5.** Heatmap showing expression levels of genes in glycolysis and gluconeogenesis pathways after 0, 2 and 4 h exposure to 4 ºC (From: Bao *et al*. [21])

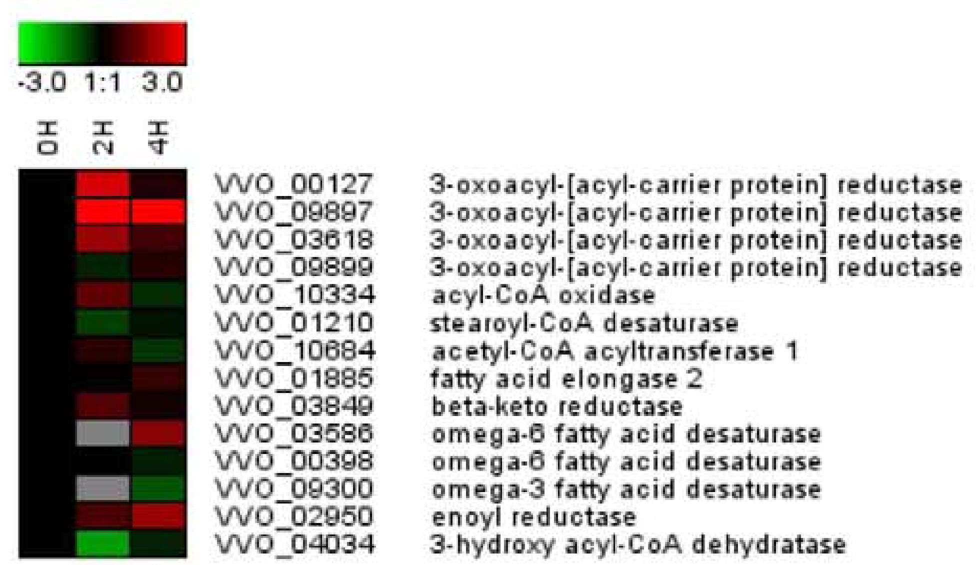


**Figure 6.** Heatmap showing expression levels of genes in unsaturated fatty acid biosynthesis after 0, 2 and 4 h exposure to 4 ºC. (From: Bao *et al*. [21])

TPS2 (trehalose-6-phosphate phosphatase) in yeast, was either repressed (-2.032, 9.01E$^{-8}$) or showed no significant change after 2 h exposure to 4 ºC. Expression of genes associated with glycolysis and gluconeogenesis metabolism (PATH: ko00010), including *VVO_06963* (encoding glyceraldehyde 3-phosphate dehydrogenase), *VVO_03893* (encoding phosphoenol pyruvate carboxyl kinase) and *VVO_09324* (encoding aldehyde dehydrogenase family 7 member A1), was again either repressed or showed no significant change after 2 h and 4 h exposure to 4 ºC (Fig. 5). Simiarly, three key genes involved in unsaturated fatty acid biosynthesis (PATH: ko01040): *VVO_01210* (encoding stearoyl-CoA desaturase), *VVO_00398* (encoding omega-6 fatty acid desaturase) and *VVO_10684* (encoding acetyl-CoA acyltransferase 1), exhibited no significant increase after exposure to 4ºC (Fig. 6). However, expression of *VVO_00127* (encoding 3-oxoacyl-[acyl-carrier protein] reductase, FabG) was induced at 2 h (log2 (fold change) = 2.48, FDR=7.22E$^{-14}$) but showed significant change at 4 h (0.40, 0.56).

In summary, increased expression of genes encoding enzymes involved in unsaturated fatty acid, trehalose and glycogen biosynthesis recorded elsewhere was not evident in *V. volvacea*. However, no up-regulation of these genes was observed in our genome-wide expression analysis following low temperature exposure of *V. volvacea*. This may, in part at least, explain why the mushroom hyphae becomes non-viable at low temperatures although the underlying mechanism remains unclear.

## ACKNOWLEDGMENTS

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# IDENTIFICATION OF THE WRKY TRANSCRIPTION FACTORS IN *AGARICUS BISPORUS* (WHITE BUTTON MUSHROOM)

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## ABSTRACT

With the changing environmental conditions, organisms can reprogram their transcriptome through transcription factors. WRKY transcription factors are a class of sequence-specific DNA binding transcription factors found almost exclusively in plants and are key regulators of gene expressions. So far, only two WRKY homologues have been identified from non-plant species, *Giardia lamblia* and *Dictyostelium discoideum*. Some WRKY proteins exist as chimeric proteins combining NBS-LRR (nucleotide binding site - leucine rich repeat) proteins and WRKY domains. During the study, WRKY domain was searched in silico in the genome sequence of *Agaricus bisporus* and also the priming sites were determined. Two WRKY and one Nucleotide Binding Site (NBS) primers amplified the WRKY domains in white button mushroom. The sequences of the amplicons were BLAST compared against *A. bisporus* genome. Surprisingly, *Agaricus* genome showed the presence of WRKY domain at multiple sites and also different type of WRKY domains could be identified in the genome. Also the NBS (associated with WRKY domain) primer for disease resistance gene amplified fragment in the *A. bisporus* showing the presence of disease resistance genes in the genome. This is a first report of presence of WRKY domain (specific to plants) in *A. bisporus* genome. Characterization of WRKY domain in *A. bisporus* is under progress.

## INTRODUCTION

To cope with variable environmental conditions, organisms have evolved a great capacity to extensively reprogram their transcriptome in a highly dynamic and temporal manner through an integrated network of transcription factors. The WRKY transcription factors are a group of regulatory proteins predominantly involved in stress responses [1]. Together with other transcriptional regulators, WRKY proteins enable plants to better adapt to the changing environment and respond properly to internal and external stimuli. WRKY factors are key regulators, both positive and negative, of both biotic and abiotic stresses, seed development, seed dormancy and germination, development and senescence. The WRKY protein family contains a highly conserved motif spanning about 60 amino acids in all the family members [2]. Within this domain, there is an almost invariable heptapeptide signature WRKYGQK at the N-terminus and a novel Zincfinger-like structure at the C-terminus. The WRKYGQK is the most dominant form of the signature followed by WRKYGKK and WRKYGEK [2, 3), however, there are at least 35 variants of this motif present in plant and non-plant species. The WRKY domain can be characterized as WRRY, WSKY, WKRY, WVKY, or WKKY. WRKY proteins preferably bind to the consensus sequence TTGACC/T, the so-called W-box, which is usually enriched in the promoter region of WRKY target genes such as stress responsive genes.

Members of the WRKY family can be classified into three groups according to the number of WRKY domains and the pattern of the Zinc finger motif in the WRKY protein [2]. Generally, group-I WRKY transcription factors contain two WRKY domains with distinct functions. Previous studies have demonstrated that the C-terminal WRKY domain mediates sequence specific binding to the target DNA [4, 5, 6]. It has been proposed that the N-terminal WRKY domain increases the affinity or specificity of these proteins to the target sites. Group II and III WRKY transcription factors contain one WRKY domain with a $C_2H_2$ zinc finger motif and $C_2H_XC$ zinc finger motif. Based on a phylogenetic analysis of the WRKY family, the members of group II can be divided into five subgroups: IIa, IIb, IIc, IId, and IIe [2].

Since the cloning of a WRKY gene cDNA from *Ipomoea batatas* [6], a large number of WRKY protein genes have been cloned from different plant species [7, 8, 9]. So far, only two WRKY homologues have been identified from non-plant species, *Giardia lamblia* [10] and *Dictyostelium discoideum* [11]. Some WRKY proteins exist as chimeric proteins combining NBS-LRR (nucleotide binding site - leucine rich repeat) proteins and WRKY domains [12, 13, 14].

The present work intended to study the presence of WRKY transcription factors in Macro-fungi and their role in stress response in the mushroom.

## MATERIAL AND METHODS

### Database search

WRKY gene sequences were obtained by multiple BLAST searches of NCBI database (http://www.ncbi.nlm.nih.gov) using the WRKY domain sequences. The NBS sites related to WRKY transcription factors were also identified and sequences were obtained from the database. The whole genome sequence of *Agaricus bisporus* was obtained from NCBI database and the whole genome was scanned for the presence of WRKY protein domain and their nucleotide binding sites. Primers were designed for the amplification of the WRKY factors and tested in silico for their success using Genious 6.0.

### DNA isolation

Genomic DNA was extracted from the fruit body of *A. bisporus* following standard cetyl trimethyl ammonium bromide (CTAB) isolation protocol. The LN2 frozen fruit body was ground with mortar and pestle to powder form. Lysis was done using preheated CTAB buffer (2% CTAB, 20 mM EDTA, 100 mM Tris-Cl at pH: 8.0, 1.4 M NaCl, and 0.2% marcaptoethanol) @ 5ml per gram tissue followed by chloroform/iso-amyl alcohol (24:1) extraction twice. The supernatant was taken after centrifugation and DNA was precipitated with 0.66 volume of cold isopropanol, collected by centrifugation or spooled out. DNA was washed and air dried briefly and 250-500 µl of TE was added and left overnight before adding 1 µl (10ng/ml) RNase to each 1 ml TE/DNA mixture and incubating for 45 minutes at 37ÚC. DNA was spooled out, air dried and re-suspended in 0.5 to 1 ml T.E. (8-24 hours; final concentration c. 0.1 to 1 µg/ul) and stored frozen at -20 ÚC.

### PCR amplification of WRKY transcription factors

Two WRKY primers pairs i.e. A7G1F + A7G1R and W3 F + W3R along with One NBS primer pair M-13R1 + M1495R were used to amplify WRKY domain in *A. bisporus.* Out of the three primer pair identified in silico to have binding efficiency with WRKY domains in *A. bisporus* genome, two primer pair successfully amplified the WRKY domains. The sequences of the primers are: WRKY Primer sequences

A7G1F – 5' CCTTCTCCTTCCCTTCGACT 3'; A7G1R – 5' AATGATCTCGGTGAGGTCAGA 3'

Leucine rich repeat nucleotide binding site primers

M13R1F- 5' CGGCCAAGTCGTGCAAYVAKRTCRTGCA 3'; M131495R – 5 ' YTTNARNGCNARNGGNARNCC 3'

The successful primer pairs were tested in 6 strains, 3 fertile and 3 non-fertile single spore isolates of *A. bisporus*. PCR reactions were carried out in 15 µl. The mixture contained 1 µl $MgCl_2$ (25mM); 1.5 µl of 10X PCR Buffer; 1.2 µl 10mM dNTP mix; 0.2 µl 5U Taq DNA polymerase; 1 µl of 10 pM each primer and 50ng template DNA. The PCR conditions were denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30s; annealing at 56°C for 30s; elongation at 72°C for 1 min. The final elongation at 72°C is done for 10 min.

### Elution, sequencing and analysis of amplified WRKY fragment

The amplified fragments were eluted from the gel using Qiagen gel extraction kit and were sequenced. The sequences obtained were subjected to BLAST against *A. bisporus* genome sequences in NCBI database. The matching sequences were downloaded and subjected to phylogenetic analysis using MEGA 6.0 software.

## RESULTS AND DISCUSSION

The whole genome sequence of *A. bisporus* was downloaded from NCBI database and scanned *in silico* for the presence of WRKY transcription factor and its variants. The results showed the presence of a number of WRKY domains distributed throughout the genome of *A. bisporus*. The priming sites were determined *in silico* using Genious 6.0 and tested various available WRKY primer pairs for the successful annealing. Out of ten primer pair tested three showed positive priming during the *in silico* analysis. These primers were tested under wet lab conditions against a number of *A. bisporus* strains and their fertile and non-fertile single spore isolates.

The WRKY primers pair A7G1F & A7G1R and NBS primer pair M13R1 & M1495R were tested in six strains and their 3 fertile and 3 non-fertile single spore isolates. Both the primer pairs successfully amplified WRKY transcription factor domains (Fig. 1 and Fig. 2). It was observed that the primers amplified the domain at different locations of the genome. Furthermore, the amplicon size also varied between 500 to 2000 bp. The results clearly demonstrated the presence of the factors at more than one location and also the structure and composition of the domain varied from one another.

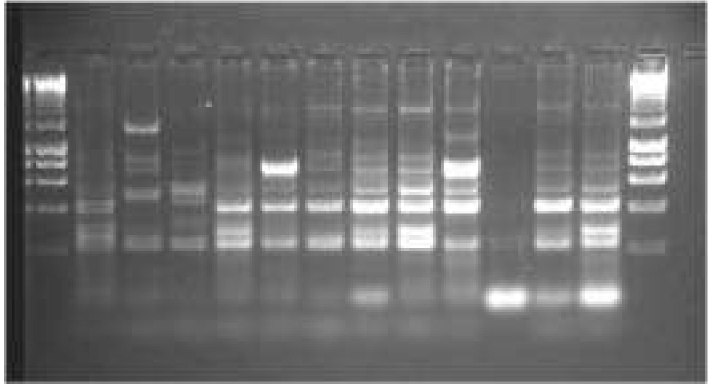

**Figure 1.** Amplification of WRKY transcription factor by primer pair A7G1F and A7G1R in different strains of *A. bisporus*

**Figure 2.** Amplification of WRKY transcription factor by primer pair M13R1 and M1495R in different strains of *A. bisporus*

The fragment were cut from the gel and eluted. The samples were sequenced using the WRKY primers and were subjected to BLAST against *A. bisporus* genome sequence from NCBI database (Fig 3). A total of 58 WRKY and related domains could be found distributed throughout the genome of *A. bisporus* (Fig 4).

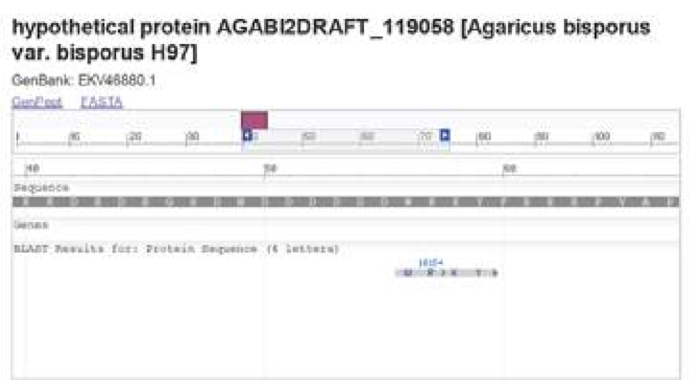


**Figure 3.** BLAST result of one of the WRKY transcription factor in *A. bisporus*

Earlier studies also showed the presence of different WRKY domains in plants and a few non-plant species. Different types of WRKY domains found in different plants and non-plant species are WRKYGQK, WRKYGEK, WRKYGKK, WKKYGQK, WQKYGQK, WSKYGQK, WSKYGQM, WTKYGQK, WNKYGQK, WKRKGQK, WVKYGQK, WRRYGLK, WRKYEDK, WRKYGKR, WRKYGSK, WEKFGEK, WRKYGQE, WKKYGHK, WRKCGLK, WRKYGQN, WKKYGYK, WKKYGED, WLKYGQK, WKKYEEK, WKKYGEK, WRKYGRK, WKKYGNK, WRKYGQT, WKKYGPK, WHKYGAK, WRKYGHK, WRKYGNK, WKNNGNT, WTKYDQR, WREYDQR, etc. (http://supfam.cs.bris.ac.uk/SUPERFAMILY/).

The presence of a number of WRKY like domains in the genome of *A. bisporus* indicates that different functions are to be performed by these genes. One of the Nucleotide binding site primers used in this study is of disease resistance gene. Presence of the domain also indicates the resistance to the biotic stress in mushrooms.

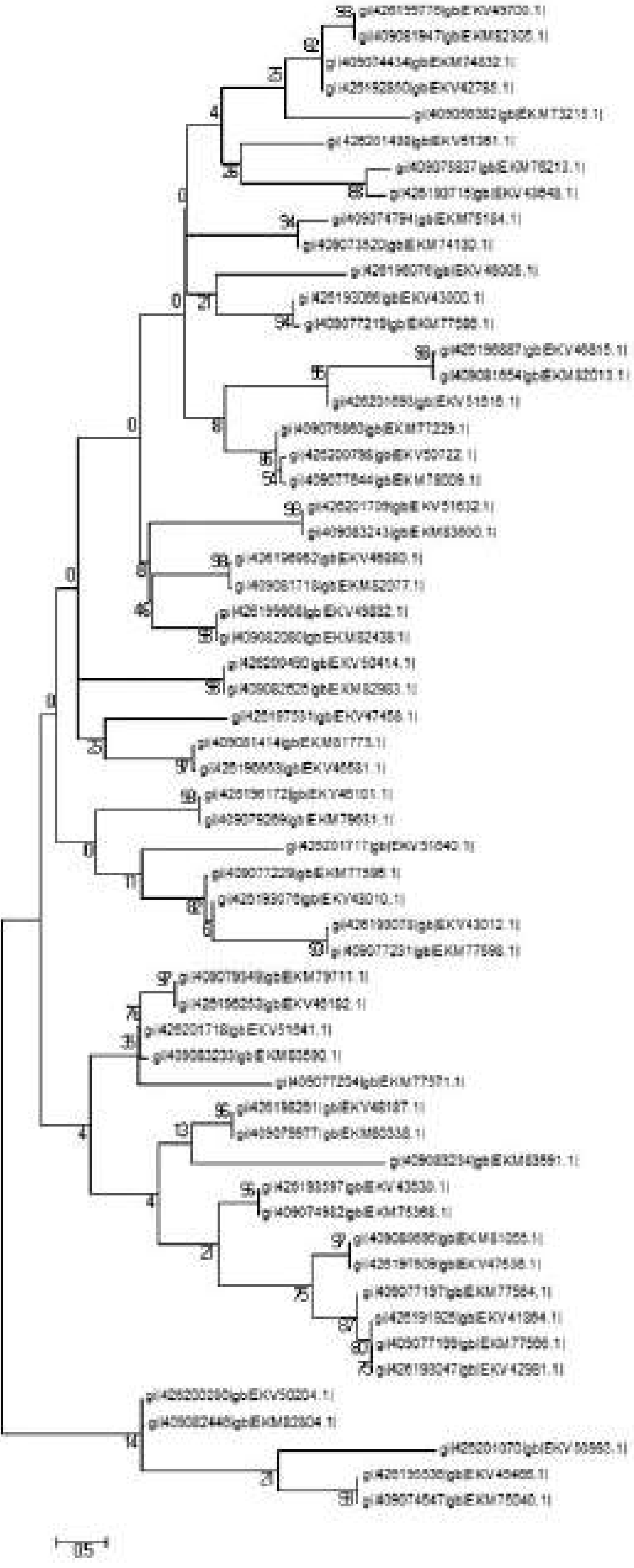

**Figure 4.** Phyologeny of WRKY Transcription factors distributed throughout the genome of *A. bisporus* using maximum likelyhood analysis

Transcriptional control is a major mechanism whereby a cell or organism regulates its gene expression. Sequence specific DNA-binding transcription regulators, one class of transcription factors, play an essential role in modulating the rate of transcription of specific target genes. In this way, they direct the temporal and spatial expressions necessary for normal development and proper response to physiological or environmental stimuli. Comparative genome analysis reveals that genes for transcription regulators are abundantly present in plant and animal genomes, and the evolution and diversity of eukaryotes seem to be related to the expansion of lineage-specific transcription regulator families. WRKY proteins are recently identified transcriptional regulators comprising a large gene family. The first cDNA encoding a WRKY protein, SPF1, was cloned from sweet potato (Ipomoea batatas). Numerous genes for WRKY proteins have since been experimentally identified from more than 10 other plant species, including *Arabidopsis thaliana* [5, 12], wild oats (*Avena fatua*) [15], orchardgrass (*Dactylis glomerata*) [16], barley (*Hordeum vulgare*) [17], tobacco (*Nicotiana tabacum*) [18-20], chamomile (*Matricaria chamomilla*) [21], rice (*Oryza sativa*) [17, 22], parsley (*Petroselinum crispum*) [7, 23], a desert legume (*Retama raetam*) [24], sugarcane (*Saccharum* hybrid cultivar) [25], bittersweet nightshade (*Solanum dulcamara*) [26], potato (*Solanum tuberosum*) [27, 28], and wheat (*Triticum aestivum*) [17]. In addition, over 70 WRKY genes were identified in the *Arabidopsis* genome by sequence similarity comparisons [29].

To date, WRKY genes have not been cloned from species other than plants. This is the first report on the presence of WRKY domains in higher basidiomycetes mushrooms. The characterization of the WRKY domains in mushroom is under progress.

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# FUNGAL UNSPECIFIC PEROXYGENASES: A NEW GENERATION OF OXYGEN-TRANSFERRING BIOCATALYSTS

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## ABSTRACT

The oxygenation of organic molecules is a challenging task in synthetic chemistry and therefore biocatalytic approaches using oxygen-transferring enzymes have come into the focus of chemists and biotechnologists. Fungal peroxygenases represent a unique enzyme type that selectively transfers oxygen from peroxides (R-OOH) to numerous substrates such as benzene derivatives, polycyclic aromatic hydrocarbons, *N*- and *S*-heterocycles, linear and cyclic alkanes, alkenes as well as to complex drug molecules and pesticides. Peroxygenases are heavily glycosylated heme-thiolate proteins that are actively secreted by fungi. Over 1,000 putative peroxygenase-like sequences, which form at least two distinct clusters, can be found in genetic data bases indicating the widespread occurrence of such enzymes in the whole fungal kingdom including true fungi and fungus-like heterokonts. Thus, peroxygenases represent, on the phylogenetic level, a fungi-specific superfamily of hemeproteins.Their catalytic cycle combines the pathways of heme peroxidases and cytochrome P450 monooxygenases. Due to their high stability and the use of cheap peroxides as co-substrate, peroxygenases could become a powerful biocatalytic tool for applications in organic synthesis and other fields.

**Keywords:** EC 1.11.2.1, heme-thiolate, oxyfunctionalization, *Agrocybe, Marasmius*

## INTRODUCTION

The term "peroxygenase" refers to enzymes that transfer a peroxide-borne oxygen atom to a substrate molecule. Biocatalysts preferably catalyzing such reactions are nowadays classified in a separate enzyme sub-subclass (EC 1.11.2) with five members that are all heme proteins (*www.chem.qmul.ac.uk/iubmb/enzyme/EC1/11/2/*). The name peroxygenase for an enzyme first appeared in the literature in 1977 in an article of Ishimarua and Yamazaki describing a hemeprotein that catalyzed the hydroperoxide-dependent hydroxylation of indole, phenol and aniline in microsomes of pea seeds [1]. Since the end of the 1980s, the term peroxygenase has been in use also for cytochrome P450 enzymes in the context of the so-called "peroxide shunt" pathway that is a side activity of some P450 monooxygenases [2]. The first fungal peroxygenase was discovered in the agaric mushroom *Agrocybe aegerita* by Upadhyay and co-workers in 1995 and described, for the time being, as an "alkaline lignin peroxidase" that oxidized veratryl alcohol to veratraldehyde [3].Ten years later, this enzyme was recognized as a heme-thiolate protein that acts on diverse aryl alcohols, halides [4] and simple aromatics such as toluene and naphthalene [5]. In 2011, after several renaming, this enzyme type was classified as unspecific peroxygenase (UPO) under EC 1.11.2.1 in the enzyme nomenclature system [6]. Nowadays, such enzymes of different fungal origin are usually abbreviated by using the first letter of the fungal genus, the first two letters of the specific epithet and the acronym UPO (e.g. *Aae*UPO = unspecific peroxygenase of *Agrocybe aegerita*).

## OCCURRENCE OF UNSPECIFIC PEROXYGENASES

The UPO-model fungus *A. aegerita* (syn. *Agrocybe cylindracea*) belongs to the family Strophariaceae (formerly Bolbitiaceae) and is commonly known as the Black poplar mushroom. The fungus grows preferably on wood of poplars (*Populus* spp.) and other broad-leaved trees, causes an unspecific white rot and tolerates high amounts of phenols [7]. It is found in Europe, North America and Asia and prefers warm and mild climates. *A. aegerita* is a popular edible mushroom in Mediterranean countries, especially in Italy (ital. Pioppino or Piopparello), where it is also commercially cultured [8].The second UPO was described for the Ink-cap *Coprinellus* (*Coprinus*) *radians*, a wood- and mulch-dwelling fungus that

belongs to the family Psathyrellaceae closely related to the Strophariaceae [9]. As *Aae*UPO, *Cra*UPO was found to oxidize aryl alcohols, toluene, naphthalene and bromide [10]. The third enzyme of this type, *Mro*UPO, is produced by the boreo-subtropical Pinwheel mushroom (*Marasmius rotula*) that preferably colonizes twigs and belongs to the family of Marasmiaceae [11]. *Mro*UPO hardly oxidizes halides, exhibits a less pronounced oxygenating activity for aromatic rings but instead can oxidize bulkier substrates such as steroids [12]. In addition to these three well-studied UPO producers, we have identified several other mushroom species secreting UPOs, for example, *A.parasitica, A. chaxingu, A. alnetorum, Agaricus bisporus, Coprinus* sp. DSM 14545, *Coprinopsis verticillata, Auricularia auricula-judae, Mycena galopus*. Eventually, recombinant UPOs from *A. aegerita* (r*Aae*UPO) and the genome-sequenced model fungus *Coprinopsis cinerea* (r*Cci*UPO) have been recently expressed at laboratory scale in *Saccharomyces cerevisiae* [13] and *Aspergillus oryzae* [14].

More information on the occurrence of UPOs was obtained from genetic databases after the first UPO genes had been sequenced [15]. BLAST searches in Gene Bank (http://www.ncbi.nlm.nih.gov/nucleotide) and other public sequence databases revealed more than one thousand homologous nucleotide sequences encoding putative UPO proteins. The majority of these sequences belongs to the Basidiomycota (~35%) and Ascomycota (~60%) and the remaining 5% to the Mucoromycotina ("Zygomycota"), Chytridiomycota and Glomeromycota as well as to Oomycota of the genus *Phytophthora* (fungus-like heterokonts). No indication was found for the presence of UPO genes in plants including green

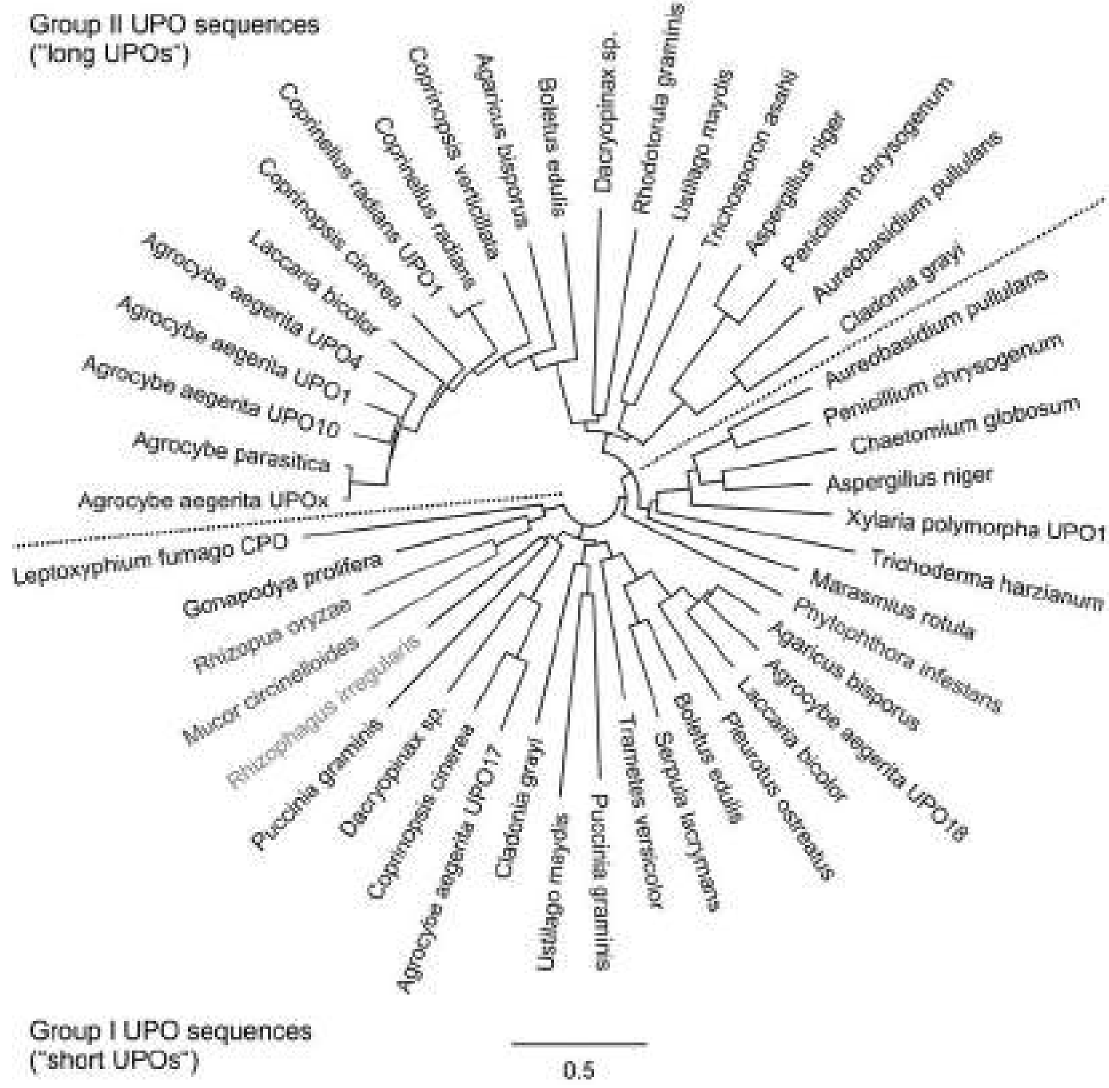


**Figure 1.** Neighbor-joining phylogentic tree based on 47 fungal UPO sequences from diverse fungi using Jukes-Cantor genetic distances. The dotted lines separate UPO sequences of groups I and II.

algae (Viridiplantae), animals (Metazoa), protists (Amoebae etc.) or prokaryotes (Eubacteria, Archaea). This indicates that UPOs are an evolutionary old, fungi-specific superfamily of proteins [15]. Furthermore, the presence of UPO genes in several *Phytophthora* species supports the hypothesis that an extensive horizontal gene transfer had taken place between phytopathogenic Ascomycota and Oomycota early in the evolution [16]. Fig. 1 illustrates the diversity of UPOs by a phylogenetic tree covering 47 sequences of 30 representative fungal species. The tree comprises examples of the above mentioned taxonomic entities as well as of different eco-physiological groups of fungi (litter decomposers, white-, brown- and soft-rot fungi, ectomycorrhizal fungi, phytopathogens, molds, yeast-like fungi and lichens). Last but not least, molecular screenings for UPO transcripts have indicated that these enzymes are ubiquitous in forest soils and leaf-litter [17].

## PRODUCTION AND CHARACTERIZATION OF UPO PROTEINS

UPOs can be produced using wild-type strains of the above mentioned fungi (e.g. *A. aegerita* or *M. rotula*) in liquid culture (agitated flasks or stirred-tank bioreactors). Growth media must be rich in carbon and nitrogen and are usually based on complex substrates such as soybean flour, peptone, yeast extract or grass pellets. The exact composition of the growth medium and the growth conditions (agitation, aeration, pH control, etc.) must be optimized for each particular species/strain. For example, our model organism *A. aegerita* needs slurries of soybean meal and bactopeptone [4],while *C. radians* prefers mixtures of glucose and soybean meal [10] and *M. rotula* soluble soybean peptone, yeast extract and glucose [11]. UPO production in liquid culture starts when the fungus switches from primary to secondary metabolism between days 5 and 10. Currently, the highest yields of UPO protein are gained with *M. rotula* that produces more than 400 mg $l^{-1}$[11]. Since UPOs are extracellular enzymes, crude preparations can be obtained by ultrafiltration of the culture liquids. Further purification is achieved by multistep fast protein liquid chromatography (FPLC) using anion, cation and mixed-ion exchangers as well as molecular sieves [18]. Typically, several UPO forms are obtained that slightly differ in their physico-chemical properties. They can represent both true isoenzymes and differently glycosylated forms of the same protein.

The four UPOs characterized so far, i.e. *Aae*UPO, *Cra*UPO, *Mro*UPO and r*Cci*UPO, contain protoporphyrine IX (heme) as prosthetic group, which is linked via the iron to an exposed cysteine (proximal hemeligand). This specific structural feature is responsible for the characteristic UV-Vis spectra of native UPOs and their reduced carbon monoxide complexes, whose maxima (Soret bands) range from 415-420 nm and from 445-450 nm, respectively, and thus strongly resemble P450 enzymes [19]. UPOs are highly glycosylated proteins (10-40% sugars) with up to seven *N*-glycosylation sites of the high mannose type [6,15]. Their molecular masses and isoelectric points vary from 32 to 46 kDa and 3.8 to 6.1, respectively [6].

## MOLECULAR PROPERTIES OF UNSPECIFIC PEROXYGENASES

The crystal structure of the major UPO form of *A. aegerita* was solved and reveals a compact globular shape with ten *alpha*-helices, five very short *beta*-sheets, a disulfide bridge in the C-terminal part as well as a magnesium in vicinity to a heme propionate residue [20] (Fig. 2, 3).

A more detailed analysis of all available UPO sequences, including that of chloroperoxidase (CPO, EC 1.11.1.10) from *Caldariomyces (Leptoxyphium) fumago* (a related heme-thiolate peroxidase with 27% sequence identity to *Aae*UPO), revealed that there are two large clusters of UPO-like proteins [15, 21]. They differ among others in size, which is why they have been designated as "short and long UPOs" (or group I and II UPOs) [15] (see also Fig. 1). The short UPOs of group I have a molecular mass around 30kDa and are found in all fungal phyla.The long UPOs of group II, with a mass around 45kDa,occur only in basidiomycetes and ascomycetes. *Mro*UPO[11] and CPO [22] belong to group I, and *Aae*UPO, *Cra*UPO and r*Cci*UPO to group II [6]. Both UPO groups bear a highly conserved cysteine exposed by two prolins (PCP-motif) as proximal heme ligand as well as a glutamate that acts as acid-base catalyst in peroxide activation/cleavage and two further acidic residues (ExD-motif) that are involved in the binding of stabilizing magnesium [15, 20]. Differences in the active sites of group I and II UPOs exist with respect to the alkaline amino acid that stabilizes the negative charge of the UPO intermediate "compounds zero"; it is histidine in the case of short UPOs and arginine in the long UPOs. Exemplarily, Fig. 2 shows the conserved amino acids in the active center of *Aae*UPO [6, 20].

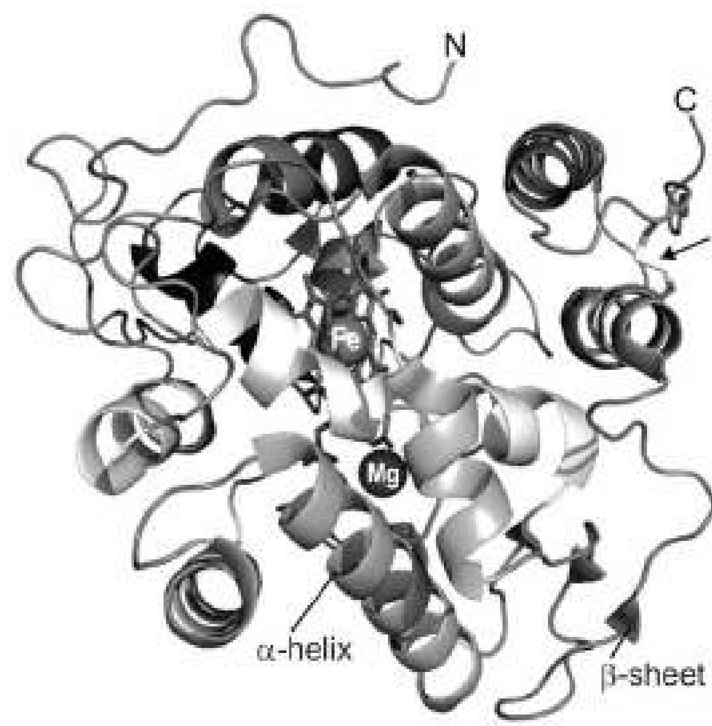


**Figure 2.** Ribbon model of *Aae*UPO. The black arrow indicates a disluphide bridge near the C-terminus (modelled by M. Pecyna based on the crystal structure data of Piontek *et al.* [20]).

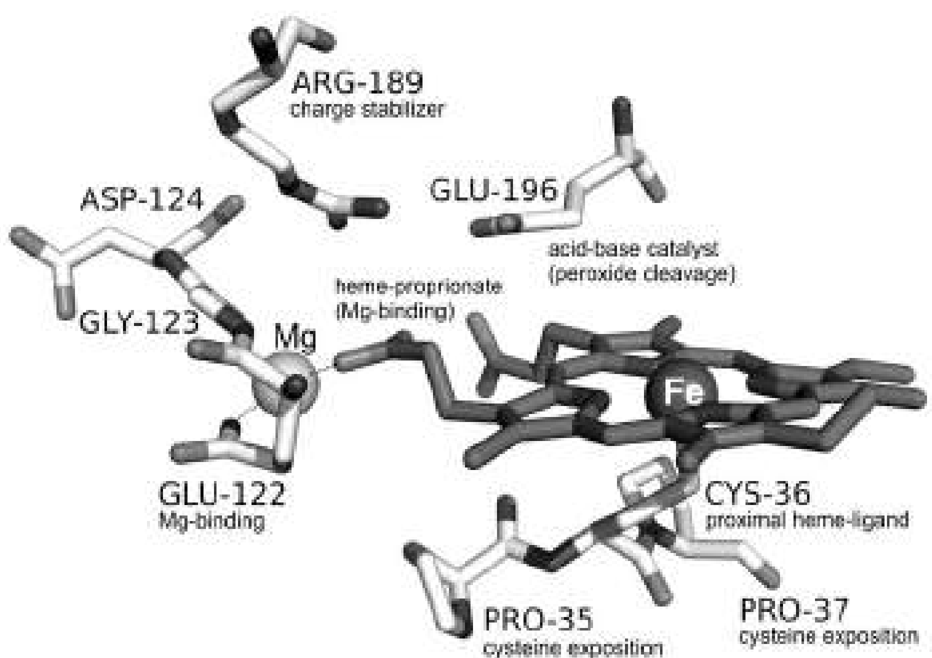


**Figure 3.** Arrangement of conserved amino acid residues and heme in the active site of *Aae* UPO (modified according to [6])

UPOs are organized in gene clusters (multigene families); ongoing own transcriptome studies on *A. aegerita* have indicated the presence of at least 16 UPO sequences, which probably include several gene variants, and in *M. rotula*, even over 50 UPO sequences have been found (MJ Pecyna and H Kellner 2014, unpublished results). In the genome of the common white button mushroom (*A. bisporus*), as much as 24 putative UPO sequences were identified and proposed to be related to the metabolism of humic substances [23].

## CATALYTIC PROPERTIES OF UPOs

An overview on UPO-catalyzed reactions is given in Fig. 4. The portfolio of reactions includes alkane and alkyl hydroxylation, epoxidation of alkenes and aromatics, heteroatom oxygenation, *O*- and *N*-dealkylation as well as radical formation. All in all, the number of UPO substrates have been estimated to exceed 300 and it is expected that even more substrates will be found along with the discovery of new UPOs [6].

The reaction cycle of UPOs combines elements of the catalytic cycles of P450s and heme peroxidases, in which compounds I and II are the key reactive intermediates that catalyze either two-electron oxidations resulting in oxygen atom incorporation, or one-electron oxidations resulting in the formation of free substrate radicals [6, 19, 24]. In other words, UPOs oxygenate

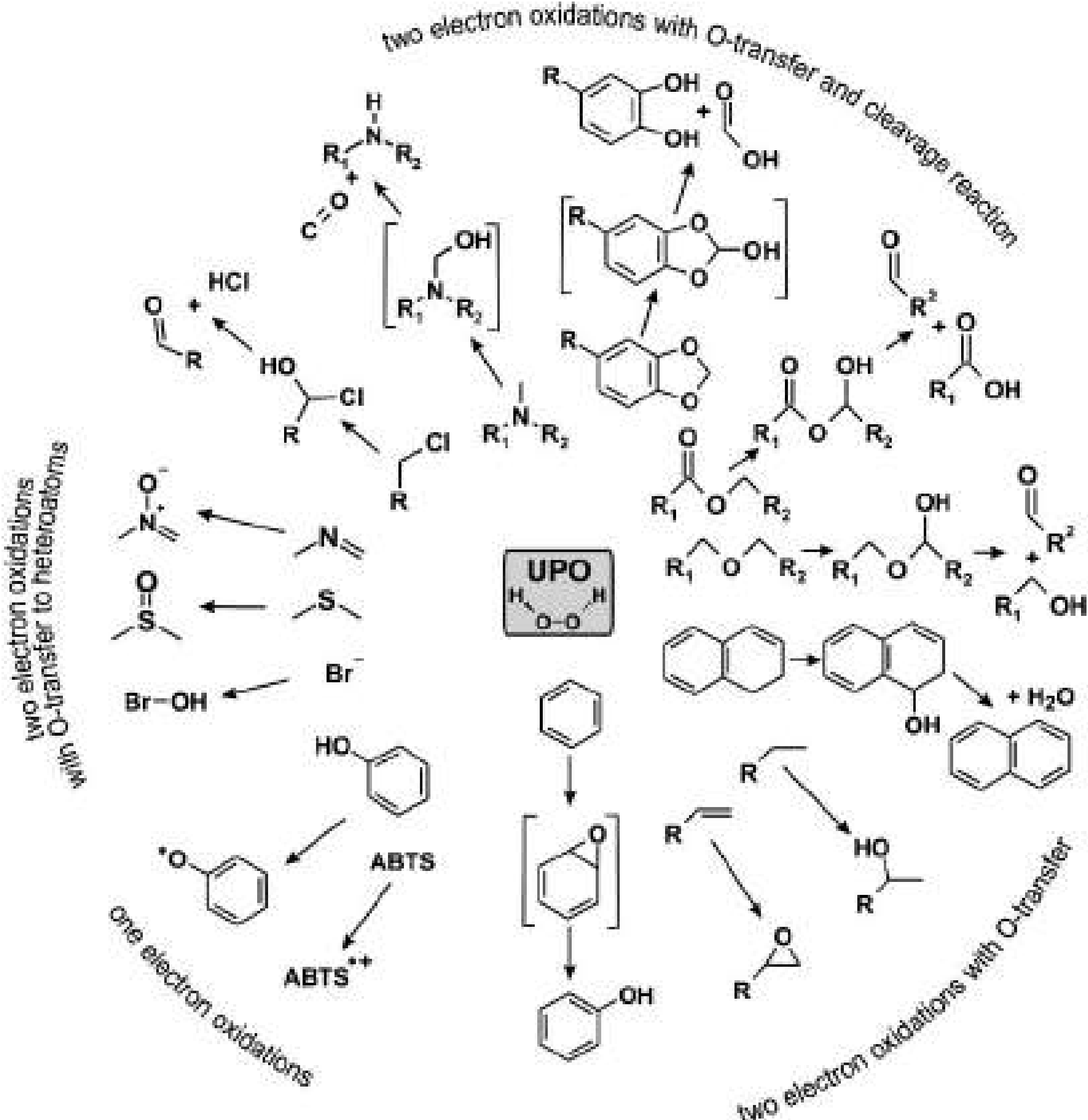


**Figure 4.** Summarizing overview on the reactions catalyzed by fungal UPOs

diverse substrates in a similar manner as P450s (mono-peroxygenase route) and oxidize phenolic compounds and ABTS (peroxidase route) like prototypical heme peroxidases [6, 25].

There are several spectrophotometric assays for the detection of the oxygenating activities of UPOs. They are based on the ability of UPOs to oxidize aryl alcohols to aldehydes, to *O*-dealkylate ethers or to oxygenate aromatic rings (Fig. 5). The oxidation of veratryl alcohol to veratraldehyde at neutral pH is used for routine measurements [3,4]. It starts with the

incipient hydroxylation of the benzylic carbon to give veratraldehyde hydrate (a *gem*-diol) that is in equilibrium with veratraldehyde absorbing at 310 nm. Veratraldehyde is also formed in the second assay that follows the cleavage of methyl veratryl ether (*O*-demethylation) leading to an unstable hemiacetal intermediate that spontaneously breaks down under release of methanol ($CH_3OH$) [26]. Demethylenation is a special case of *O*-dealkylation and performed with 5-nitro-1,3-benzodioxole, the oxidation of which results in the release of formic acid (HCOOH) and the formation of 4-nitrocatechol (followed at 425 nm) [27]. Aromatic ring oxygenation via initial epoxidation and subsequent spontaneous re-aromatization (phenol formation) can be monitored with naphthalene as a substrate at 303 nm [28]. One-electron oxidations catalyzed by UPOs are assayed with classical peroxidase substrates such as ABTS or 2,6-dimethoxyphenol [4]. It is also possible to determine UPO activities with chromatographic methods (HPLC, GC) as it was shown for the oxidation of pyridine, ethylbenzene, benzene, cyclohexane and methylbutene [29-33].

UPOs catalyze the hydroxylation of various linear, branched and cyclic alkanes as well as of alkyl groups (e.g. attached to aromatic rings). Due to the low solubility of alkane substrates, reactions are usually performed in the presence of co-

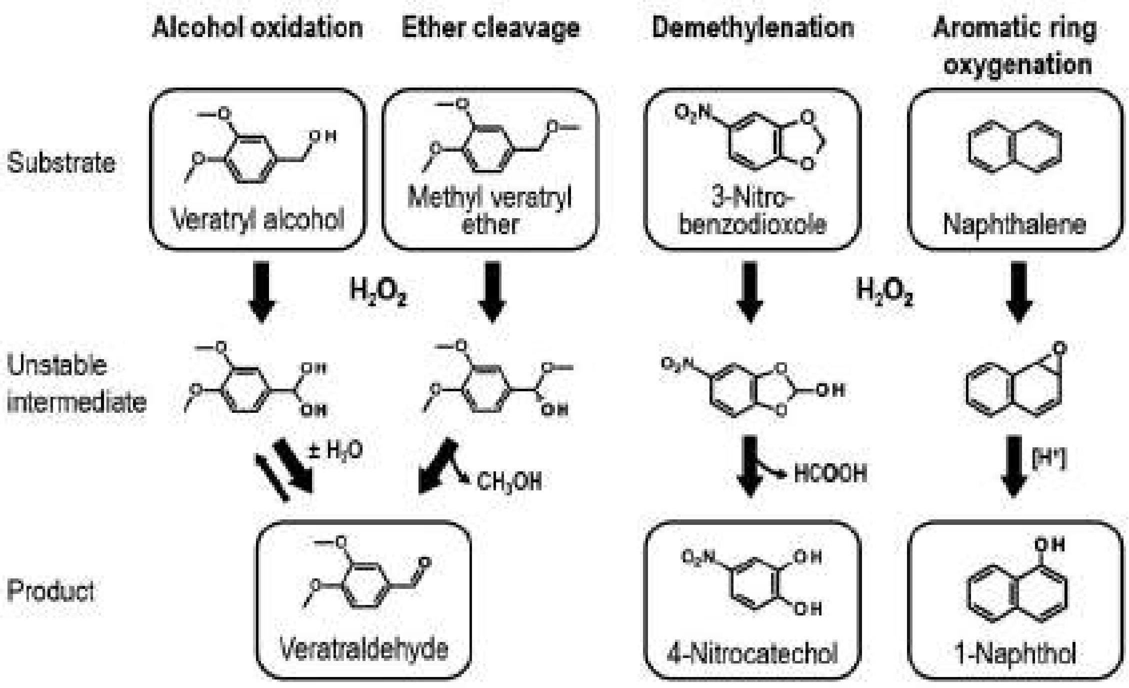


**Figure 5.** Reactions used to assay UPO activities [4, 26, 27, 28]

solvents (e.g. acetone 4% to 60% vol/vol). The size of linear alkane molecules that were found to be hydroxylated at *omega*-2 and -3 position ranges from gaseous propane to viscous *n*-hexadecane [32], and fatty acids were even oxidized up to a chain length of $C_{20}$ (arachidic acid) [14]. Branched alkanes are hydroxylated by UPOs as well and preferably at the tertiary carbons. Cyclic alkanes from cyclopentane to cyclooctane are oxidized to form the corresponding cycloalkanols [32]. In general, alcohols formed can be subject to over-oxidation to the corresponding carbonyls (ketones, aldehydes), and aldehydes, in turn, can be oxidized to carboxylic acids (Fig. 4) [34].

UPOs oxidize alkenes and alkenyls, in which both epoxidation and hydroxylation of the double bond's adjacent carbons (allylic hydroxylation) occurs (Fig. 4). In a study comprising 20 alkenes, among them propene and linear 1-alkenes up to $C_8$, branched alkenes such as 2,3-dimethyl-2-butene, cyclohexene, butadiene and limonene, were oxidized by *Aae*UPO in that manner [33]. A special case of allylic hydroxylation is the formation of naphthalene hydrates (i.e. 1- and 2-hydroxy-1,2-dihydronaphthalene) during the UPO-catalyzed oxidation of 1,2-dihydronaphthalene. The arene hydrates formed decay into naphthalene via spontaneous aromatization (Fig. 4). This reaction sequence represents a simple pathway for the selective synthesis of aromatic hydrocarbons via arene hydrates of conjugated cyclic dienes or cycloalkenyl benzenes [35].

Aromatic oxygenation has been studied in detail with naphthalene and benzene as substrates [28, 31, 36]. Naphthalene is regioselectively epoxidized by different UPOs to form naphthalene 1, 2-oxide that hydrolyzes in the presence of protons ($H^+$) to 1-naphthol as the major product (Fig. 5). Other polycyclic aromatic hydrocarbons such as fluorene, anthracene, phenanthrene, pyrene and dibenzofuran were also found to be subject to UPO-catalyzed oxidation leading to mixtures of mono- and polyhydroxylated products [37]. Benzene oxidation proceeds via initial epoxide formation and subsequent re-aromatization to form phenol; further oxygenation is typical and gives mixtures of hydroquinone, catechol and 1,2,4-trihydroxybenzene [31]. Phenolic products formed can be in turn substrates of the peroxidative activity of UPOs (one-electron oxidation), which leads to undesired phenoxyl radicals (Fig. 4). This can be prevented by adding radical scavengers such as ascorbic acid to the reaction mixture. Re-reduction of phenoxyl radicals is of particular relevance when polyphenolic substrates such as flavonoids are attempted to peroxygenation [38].

UPOs catalyze *O*- and *N*-dealkylations of diverse ethers and secondary/tertiary amines, respectively. The mechanism involves, in both cases, initial hydroxylation of one of the heteroatoms' adjacent carbons (e.g. methyl or methylene groups) giving rise to unstable intermediates (hemiacetals, hemiaminals), which spontaneously cleave under release of water. Thus, hemiacetals yield alcohols/phenols and aldehydes, and hemiaminals generate primary or secondary amines and aldehydes. In both cases, the aldehydes indicative for this mechanism can be detected by their corresponding 2,4-dinitrohydrazone adducts [12, 26]. Ether cleavage occurs between aromatic and aliphatic molecule parts, e.g. in alkyl aryl ethers (e.g. 1,4-dimethoxybenzene) or in alicyclic and aliphatic ethers (e.g. tertrahydrofuran, diisopropyl ether). Substantial *N*-dealkylation (~60%) was observed during *N*-methylaniline oxidation by *Aae*UPO [39].

UPOs are also capable of transferring oxygen to organic heteroatoms such as sulfur and nitrogen (Fig. 4). For example, the heterocycledibenzothiophene is oxidized at the sulfur atom to form the corresponding sulfoxide and sulfone [44]. In a similar reaction, *Aae*UPO was found to enantio selectively oxidize the side chain of thioanisole into the corresponding (*R*)-sulfoxide with high efficiency [40]. Pyridine and halo-, nitro- and cyanopyridines are oxidized by *Aae*UPO exclusively at the nitrogen atom to form the respective pyridine *N*-oxides [29].

*Aae*UPO shows strong bromide oxidation but, in contrast to CPO, only very low chloride oxidation, even though (according to studies of compound I) its redox potential is higher than that of CPO [41]. The oxidation of halides (X) is actually also an oxygen transfer reaction yielding reactive hypohalites (OX) that in turn can halogenate organic substrates such as phenols [5, 19]. In contrast to *Aae*UPO, *Mro*UPO has almost no bromide oxidizing activity, indicating that not all peroxygenases have specific halide binding sites [11].On the other hand, halogens bound to carbon atoms undergoing hydroxylation are released as the corresponding halides, because the geminal halohydrins initially formedare unstable. The oxidation of benzylchloride by *Aae*UPO that yields benzaldehyde and chloride is an example of such a reaction (Fig. 4) [39].

The promiscuity of UPOs in oxyfunctionalization reactions becomes evident when the oxidation of pharmaceuticals and drugs is examined. All reactions mentioned above have been observed in this context, and more than 60 different pharmaceuticals and a number of illicit drugs were shown to undergo oxidative modification by UPOs [12, 42]. Examples are the painkillers diclofenac (phenyl hydroxylation) and ibuprofen (isopropyl hydroxylation), the antitussive dextromethorphan (*O*-demethylation), the *beta*-blocker propranolol (naphthyl hydroxylation), the $K^+$-channel blocker tolbutamide (benzylic hydroxylation), the anti-inflammatory aminophenazone (*N,N*-desmethylation) and the antiviral drug osaltamivir (ester cleavage). Among the drugs (of abuse) that are oxidized by UPOs are MDMA ("Ecstasy", demethylenation), LSD (aromatic hydroxylation), THC (methylcyclohexenyl hydroxylation) as well as cocaine and codeine (*N*-desmethylation). Last but not least, UPOs have also successfully been used to prepare specifically labeled human drug metabolites and drug-drug interaction probes [43].

## CONCLUSION

Fungal unspecific peroxygenases can at least approach the catalytic versatility of cytochrome P450 enzymes and may suitably supplement existing oxyfunctionalization tools in biotechnological applications. Some examples that are currently

under development are biosensors for aromatic compounds [44] as well as new procedures for the synthesis of pesticide precursors [45], drug metabolites [46], chiral alcohols [47] and even bulk chemicals such as cyclohexanone [48].

Despite all the progress in understanding the catalytic mechanisms of UPOs and collecting their molecular data, the natural function of these enzymes in fungal organisms is not fully clear yet. Of course, the surpassing catalytic versatility may suggest their involvement in all kinds of detoxification reactions (e.g. of plant ingredients, phytoalexins, microbial toxins, xenobiotic compounds), but also other functions cannot be ruled out, e.g.,their involvement in lignin and humus modification or in biosynthetic pathways [49].

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# A STUDY ON BIOACTIVE FLUORESCENT COMPOUNDS FROM SELECTED MUSHROOMS AND ITS ANTIMICROBIAL ACTIVITY

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## ABSTRACT

Mushrooms have long been appreciated for their flavor, texture, medicinal and nutraceutical attributes. The present study deals with fluorescent bioactive compounds from *Armillaria mellea* (MTCC 409) and *Omphalotus olearius* (MTCC 2790). The cultures were obtained from MTCC, Chandigarh and pure mycelial cultures were developed. Time scale studies for the production, extraction, and estimation of fluorescent dyes were carried out and evaluated for their antibacterial activities. *A. mellea* and *O. olearius* grown in potato dextrose broth recorded the maximum mycelial dry weight of 5.90 ± 1.12g/50mL and 0.57 ± 0.025 g/50mL respectively on 28th day. *A. mellea* and *O. olearius* mycelium (1000 mg) recorded maximum crude fluorescent dyes in acetone (25.5%) and methanol (55.3%) respectively. Acetone extracts of *A. mellea* and *O. olearius* recorded highest zone of inhibition (0.4 cm and 1cm) against *E. coli* and *P. aeruginosa,* respectively.

**Keywords:** *Armillaria mellea, Omphalotus olearius,* fluorescent dyes, antimicrobial activity

## INTRODUCTION

Microorganisms are considered good source of valuable bioactive metabolites. The most important metabolites such as, penicillin, cyclosporine A, adriamycine antibacterial, antiviral, antitumor as well as anticoagulant properties have been reported in mushroom [1]. Fluorescent-labeled molecules are being used extensively for a wide range of applications in biological detection and diagnosis [2]. Bioluminescence has been widely exploited as marker system for detection and tracking of cells in the environment and as biosensors for the detection of pollutants [3]. Few of the fluorescent mushrooms in nature include *Armillaria fuscipes, A.gallica, A. mellea, A.tabescens, Dictyopanus foliicolus, D.pusillus, Filoboletu spallescens, F. yunnanensis, Mycena chlorophos, Neonothopanus gardneri, N. nimbi, Omphalotus illudens, O. japonicas, O. olearius, Panellus gloeocystidiatus,* and many have been evaluated for their medicinal, nutraceutical and fluorescent properties [4].

*A. mellea* contains the bioactive compounds such as polysaccharides and sesquiterpene aryl esters reported to have anti oxidation, immunopotentiation, anti-vertigo, anti-aging, anti-microbial and anti-bacterial activity [5, 6]. *O. illudens* contains the bioactive compounds as illudinic acid, illudin S and M, having antibacterial activity [7] and applications for treating cancer diseases [3]. *O. olearius* and *Lampteromyces japonicas* contain the bioactive compound which are like cytotoxic, tricyclic sesquiterpene and illudin S, those are reported to have anticancer activity [8]. Among the several fluorescent mushrooms, in this study *A. mellea* and *O. olearius* were utilized and fluorescent dyes were, extracted, estimated and tested for antimicrobial potential.

## MATERIALS AND METHODS

### Culture collection, maintenance and production of fluorescent dyes from *A. mellea* and *O. olearius*

Fluorescent mushrooms, *A. mellea* (MTCC 409) and *O. olearius* (MTCC 2790) were procured from microbial type culture collection (MTCC), Chandigarh, India. The pure culture mycelium was initiated and sub cultured at intervals of every 15 days. The cultures were maintained on the malt based media recommended by MTCC. Apart from the recommended media, the growth and metabolites production was also tested in potato dextrose broth.

**Extraction of intracellular fluorescent dyes**

Dried mycelium of *A. mellea* and *O. olearius* was taken and extracted by boiling for 4 hrs in 100 ml distilled water, acetone and methanol, respectively. The extracted dye were condensed and characterized.

**Anti-bacterial activity of bioactive compounds**

Loopful of bacterial culture was inoculated in the nutrient broth and incubated at 37 °C for 12-14 hrs to get the log phase. The fluorescent dyes extracted from selected mushrooms were tested for their efficacy towards selected bacteria by agar diffusion method [9]. Bacterial cultures, *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Proteus vulgaris,* and *Pseudomonas aeruginosa* were used for the anti bacterial assays. Nutrient agar plates were prepared, bacterial cultures grown on nutrient broth was aseptically transferred using a sterilized cotton swab and swabbed on the surface of the NA plates and allowed to dry for 1h. Wells of size 0.5 cm in diameter was cut in the agar plates using a sterile cork borer. The extracts from fluorescent mushrooms (*A. mellea* and *O. olearius*) were added at concentrations of 25µl, 50µl, 75 µl and 100 µl into the wells of the inoculated plates. After 24 - 48 hrs of incubation at 37 °C these were observed for the development of zone of inhibition. Sizes of the inhibited zone were measured and recorded. All the assays were carried out in triplicates. Petriplates containing respective solvents (100 µl) in the wells with bacteria were maintained as blank.

## RESULTS AND DISCUSSION

Among the two media, potato dextrose broth showed maximum mycelial dry weight of 5.90 ± 1.12g/50ml and 0.57 ± 0.025 g/50 ml for *A. mellea* and *O. olearius* respectively on 28th day.

**Extraction of intracellular fluorescent dyes from *A. mellea***

Mycelium of *A.mellea* and *O. olearius* yielded good amount of crude fluorescent dyes from distilled water and different solvents and the extracts were processed for the determination of antibacterial activity. *A. mellea* and *O. olearius* mycelium (1000 mg) recorded maximum crude fluorescent dyes in acetone (255 mg) and methanol (553mg) respectively.

**Antibacterial activity for *A. mellea* and *O. olearius***

*A. mellea* recorded zone of inhibition against both gram positive (*S.aureus*) and gram negative bacteria (*E. coli, P. aeruginosa* and *P. vulgaris*).

Acetone extract of *A. mellea* recorded the minimal zone of inhibition against *E. coli, S. aureus, P. vulgaris, P. aeruginosa* and *S. typhi.* Methanol extracts (Mycelia) of *A. mellea* recorded the zone of inhibition against *E. coli* (0.4 cm), *S. typhi* (0.3 cm), *S. aureus* (0.3 cm), *P. aeruginosa* (0.3 cm) and *P. vulgaris* (0.3 cm). Water extracts did not record any anti-bacterial activity.

Acetone extracts of *O. olearius* recorded the zone of inhibition against *E.coli* (0.4 cm), *S. typhi* (0.5 cm), *S. aureus* (0.6 cm), *P. aeruginosa* (1 cm) and *P. vulgaris* (0.6 cm), methanol extracts showed activity against *E. coli* (0.5 cm), *S. typhi* (0.3 cm) and *P. aeruginosa* (0.3 cm). Water extracts recorded the zone of inhibition against *S. typhi* (0.3 cm). Donnelly *et al.* [10,11] isolated two new sesquiterpene aryl esters, 4-o-methylmelleolide and judeol, both having strong antibacterial activity against gram positive bacteria. Armillaric acid also exhibited marked inhibitory activity against gram positive bacteria and yeast [10]. Momose *et al.* [13] isolated three compounds, melleolides K, L and M. Melleolides K had of antimicrobial activity against gram positive bacteria, yeast and fungi.

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# ANTI-FATIGUE EFFECTS OF *AGARICUS BISPORUS* EXTRACT IN RATS

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## ABSTRACT

Mushroom antioxidants help in scavenging the free radicals formed during heavy exercise, so the addition of mushroom extract to curd may provide combinatorial effect. The present study was undertaken to investigate the anti-fatigue properties of *Agaricus bisporus* extract (ABE). Antioxidant properties of the extract were screened and the results showed increased free radical scavenging, reducing power and metal chelating property with increasing concentration of ABE. Total phenolic content was 7.5821 mg tannic acid equivalent /g extract (or) 27.8 mg quercetin/g of extract, and total flavanoid content was 2.2385 mg quercetin/g of extract. HPLC profile of the polyphenols in the mushroom extracts revealed the presence of chlorogenic acid and p-coumaric acid. The anti-fatigue activity of ABE was measured using animal treadmill exercise. Endurance exercises reduced the levels of glycogen in control group. However, ABE supplementation enhanced liver and muscle glycogen levels ($p<0.05$). Lactic acid (LA) levels in muscle tissue were significantly higher in control exercise group when compared to sedentary group ($p<0.05$). The ABE supplemented group showed lowered LA levels in muscle tissue when compared to control rats ($p<0.01$) showing the efficient usage of glucose during exercise by the rats. In control exercised group of rats, the values of malondialdehyde (MDA) concentration were significantly higher in muscle and liver when compared with sedentary group. The ABE treatment decreased the MDA levels in muscle (37.7%) and liver (16.2%) to that of control group showing the *in-vivo* antioxidant property of the extract. In conclusion, the extract will have beneficial effect *w.r.t* its anti-fatigue property.

**Keywords:** *Agaricus bisporus* extract (ABE), anti-fatigue, malondialdehyde, exercise

## INTRODUCTION

Fatigue may be because of both physical and mental stress. Physical fatigue is the inability to continue to do work [1]. Severe and continuous exercise may elevate the formation of reactive oxygen species (ROS) elevating oxidative stress [2,3]. An elevated level of malondialdehyde (MDA), a lipid peroxidation product has been reported by many workers during the course of exercise [4]. The public have become much more health-conscious and an increased proportion of people now exercise routinely to prevent lifestyle-related diseases and to enhance their physical fitness. Moderate exercise is useful for preventing illness and mental stress, but over exercise itself can be a form of stress and cause fatigue or various types of damage to the organs. Fatigue is the symptom, which indicates that the health is either about or already subjected to harm [5]. Free radical formation and subsequent lipid peroxidation of bio-membranes might be noxious biochemical responses to either physical or emotional stress [6]. It has been demonstrated that exercise leads to increase in free radical formation thus causing oxidative damage to membranes (lipid peroxidation), thus, fatigue causes various disorders in relation to bio-regulatory, autonomic nervous, endocrine and immune system [7]. However, there is no detailed information available concerned with anti fatigue properties of mushrooms. The present study was undertaken to investigate the anti-fatigue properties of *Agaricus bisporus* extract by using animal treadmill and development of a fermented yogurt product using the same extract.

## MATERIALS AND METHODS

### Mushroom extract preparation

**Preparation of raw mushroom powder:** Mushrooms were purchased from the local market in Mysore, and were stored at 4 °C for 24 hours. The mushrooms were then cleaned, washed and cut into small pieces. The pieces were then freeze dried in alyophilizer until completely dry. The dried mushrooms were then powdered and sieved through a sieve (16 mesh screen). The mushroom powder was collected in sterile sample bags and stored in the dark at room temperature [8].

**Preparation of mushroom extract powder:** 5 g of the freeze-dried mushroom powder was added to 60 ml of 80% ethanol and heated to 60 °C using a water bath and left for extraction with shaking at regular intervals for 1 hour. Then the mixture was filtered through country filter paper and the residue was extracted with additional 60 ml of 80% Ethanol, as described above. The filtrate collected through each of the three extractions were pooled and well blended. The extract was then flash evaporated and freeze dried. The mushroom extract powder was collected in sterile sample bags and stored in dark at room temperature [8].

**HPLC analysis of mushroom phenolics in hydro-alcoholic extract**

Phenolic acids of hydro-alcoholic extract were analyzed according to the method of Wulf and Nagel on a reversed phase Shimpak C18 column (4.6 x 250 mm), using a diode array detector (operating at 650 nm). The Shimpak C18 HPLC column was obtained from Shimadzu Corp. A solvent system consisting of water/acetic acid/methanol (80:5:15) (v/v/v) was used as mobile phase at a flow rate of 1 ml/min. Phenolic acid standards such as gallic acid, *p*-coumaric acid, chlorogenic acid, sinapic acid, protocatechol acid, ellagic acid, and rutin were used for the identification of phenolic acids present in the hydro-alcoholic extract of mushroom. Quantification of phenolic acids was achieved by the absorbance recorded in the chromatograms related to external standards at 650 nm.

***In-vitro* antioxidant assays**

**1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity:** The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of DPPH. This spectrophotometer assay uses the stable radical DPPH as a reagent. The DPPH free radical scavenging activity of the extracts was measured according to the method of Brand-Williams *et al.* [9] . Stock solution of each mushroom extract (50 mg/mL) was diluted to a concentration in the range of 0.1 to 50 mg/ml. For the test, 3.9 ml of 0.06 mM DPPH radical (Sigma) was added to 0.1 ml of mushroom extract. Reaction mixture was vortexed and absorbance was measured at 515 nm using a spectrophotometer with methanol as the blank. The decrease in absorbance was monitored at 0 min, 1 min, 2 min, and every 15 min until the reaction has reached a plateau. The time taken to reach the steady state was determined by one-way analysis of variance (ANOVA). The DPPH free radical scavenging activity, expressed as percentage of radical scavenging activity, was calculated as follows:

Radical scavenging activity (SA) = $\{(A_0 - As)/A_0\} * 100$

Where, $A_0$ is the absorbance of 0.06 mM methanolic DPPH only whereas *As* is the absorbance of the reaction mixture.

**$Fe^{2+}$ Metal-chelating ability:** Metal- chelating ability of mushroom extract was assessed using the method of Decker and Welch [10]. Mushroom extract (1mg/ml) was mixed with 3.7 mL of distilled water. It was then reacted with a solution containing 0.1 ml 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. After 10 min, the absorbance of the reaction mixture was measured at 562 nm.

% Metal-chelating ability = [1-(O.D. of the sample/ O.D. of the control)] x100

**Reducing power ability:** Reducing power of the mushroom extracts was determined according to the method of Athukorala *et al*. [11]. The reducing power can be determined by the method of 1.0 ml extract was mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50 °C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance was measured at 700 nm. Ascorbic acid, butylated hydroxyanisole (BHA), á-tocopherol, trolox [12] or butylated hydroxytoluene (BHT) [13] can be used as positive control.

**Determination of total phenolic compounds:** Total phenolic compounds in the ethanol extracts were determined using Folin–Ciocalteu method [14]. One ml of the extract was added to 10.0 ml distilled water and 2.0 ml of Folin-Ciocalteu phenol reagent. The mixture was allowed to stand at room temperature for 5 min and then 2.0 ml of 20% sodium

carbonate was added to the mixture. The resulting blue complex was measured at 680 nm. Catechin was used as a standard for the calibration curve. The phenol compound was calibrated using the linear equation based on the calibration curve. The contents of the phenolic compound were expressed as mg catechin equivalent/g dry weight.

**Determination of total flavonoid concentration:** The $AlCl_3$ method [15] was used for determination of the total flavanoid content of the sample extracts. Aliquots of 1.5 ml of extracts were added to equal volumes of a solution of 2% $AlCl_3$ (2 g in 100 ml methanol). The mixture was vigorously shaken, and absorbance at 367 nm was read after 10 min of incubation. Quercetin was used as a standard for the calibration curve. The flavonoid compound was calibrated using the linear equation based on the calibration curve. The contents of the flavarnoid compound were expressed as mg quercetin equivalent/g dry weight.

**Animal studies**

**Experimental design:** Animal experiment was carried out as per guidelines of Institutional Animal Ethics Committee. Twenty five male mice of body weight 35-40 g were selected from the stock colony of this institution based on uniform food intake and body wt gain, placed individually in SS cages, exposed to 12 hr dark and light cycle maintained at 27± 2 °C with free access to drinking water and synthetic diet (15% protein, 10% fat from peanut oil with recommended levels of minerals and vitamins). These animals were divided into five groups consisting of five mice each, group I sedentary control; group II exposed to exercise but without feeding mushroom extract; group III sedentary controls, but forced fed with 100 mg equivalent of mushroom extract/mouse/day, daily in the morning at 12 Noon after fasting the mice for 3 hours (the diet cups were removed at 9 AM), group IV and group V were forced fed with 15 mg and 100 mg equivalent of mushroom extract, respectively for 21 days and subjected to treadmill exercise, for a fixed time.

**Tread mill experiment:** The mice were subjected to treadmill experiment for a period of 2-3 minutes daily. The speed was adjusted based on the ability of the mice to maintain its running pace constant.The speed and inclination was increased gradually to max.15 m/min and max. 5°, respectively, based on the performance of the mice.

**Determination of number of shocks:** After the mice have been trained to run on tread mill, they were evaluated for their performance under pre- determined set of conditions. The mice were made to run on the tread mill at a specific speed (10 min/min), inclination (5 V) and time duration (7 min). The mice which get tired and unable to run, stops running and sits on the track, which then hits the shock grid and receives a shock of 1 V, every time it comes into contact with the grid. The number of shocks received by each mouse for a particular period of time was recorded, which can be related to the fatigue experienced by mouse.

**Determination of biochemical variables:** On 28th day, a 7 min treadmill exercise was performed by group III and IV mice, 1 hr after the last treatment administration and were sacrificed immediately after exercise by cervical dislocation. Blood samples were collected from the retro orbital sinus. Blood glucose was determined immediately using a portable glucose analyser. Serum was separated after centrifuging the blood at 1000 rpm for 5 minutes at 4 °C. The effects of mushroom extract on liver and muscle tissues were analyzed for TBARS.

**Thiobarbituric acid reactive substances (TBARS):** The liver and muscle tissues were homogenized in ice-cold Tris–HCl buffer (pH 7.4, 20 mM) to produce a 1:2 (w/v) tissue homogenate, which was centrifuged at 3000*g* for 10 min. An aliquot (0.5 ml) of the supernatant was incubated with trichloroacetic acid (10 % w/v, 0.5 ml), followed by thiobarbituric acid (TBA, 2%, 2 ml), and the mixture was then heated at 80°C for 30 min. The reaction mixture was cooled and centrifuged at 8000 *g* for 20 min to remove the precipitated protein. The colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at *ë* = 532 nm. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = [(A\text{-}B)/A] \times 100$$

Where, *A* and *B* were the absorbance of the control and the compound solution, respectively.

## RESULTS AND DISCUSSION

### Antioxidant properties of *Agaricus bisporus* extract

**DPPH scavenging activity:** DPPH is nitrogen cantered free radical that shows strong absorbance at 517 nm. Deep violet coloured of DPPH solution changes to yellow in presence of DPPH radical scavengers. DPPH radical accepts an electron to become a stable diamagnetic molecule. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet color in the form of $EC_{50}$ values. Lower $EC_{50}$ value represents higher antioxidant activity. To evaluate the free radical scavenging activity, the mushroom extracts were subjected to scavenge DPPH by donating an electron. Results confess that scavenging activity increased with increasing concentration of *A. bisporus* extract. The results of DPPH radical scavenging activity revealed that % inhibition of all extracts increased with increasing concentration. The results are given in Table 1.

**Table 1.** DPPH scavenging activity

| Sample | $IC_{50}$ (mg) |
|---|---|
| BHA | 0.030 |
| BHT | 0.025 |
| Vitamin C | 0.045 |
| *Agaricus bisporus* extract | 0.968 |

**Table 2.** Metal chelating efficiency

| Sample | $IC_{50}$ (mg) |
|---|---|
| EDTA | 0.130 |
| *Agaricus bisporus* extract | 0.221 |

**Metal chelating efficiency:** Iron is known to generate free radicals through the Fenton and Haber–Weiss reaction. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalysing transition metal in lipid peroxidation. It is reported that chelating agents, which form *s*-bonds with a metal, are effective as secondary antioxidants since they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. Metal chelating efficiency of the extracts was estimated over a wide range of concentration (0-500 ppm) and also compared with EDTA, the standard chelating agent (Table 2.). EDTA showed 98% chelation at $\geq$ 400 ppm. The results of $Fe^{2+}$ chelating ability revealed that % inhibition increased with increasing concentration. The results are:

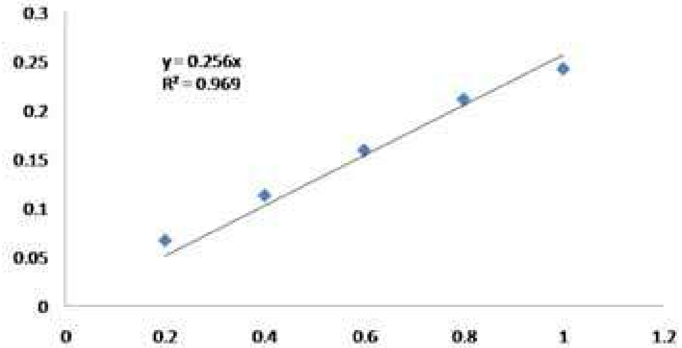


**Figure 1.** Reducing power ability of hydro-alcoholic extract of *A.bisporus*

**Figure 2.** Total phenolic content of hydro-alcoholic extract of *A.bisporus*

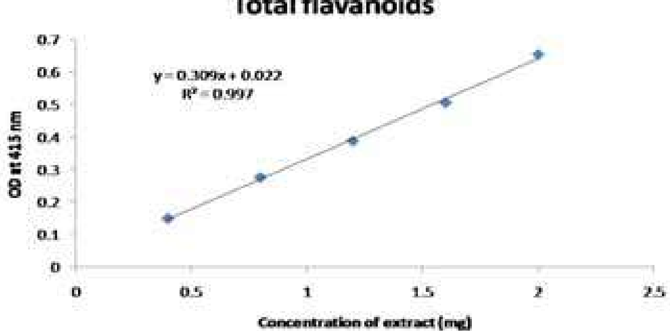


**Fig. 3.** Total flavanoid content of hydro-alcoholic extract of *A.bisporus*

**Reducing power ability:** Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide ($Fe^{3+}$) to form potassium ferrocyanide ($Fe^{2+}$), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. Reducing power was 46.84 mg Ascorbic acid equivalent / g of mushroom extract. The reducing power of the *A. bisporus* extract increases with the increase in concentration (Fig. 1). All the methods have proven the effectiveness of *A. bisporus* extract compared to the standard antioxidant, ascorbic acid.

**Phenolics and flavanoid contents in *A. bisporus* extract:** Total phenolic content was 7.5821 mg Tannic acid equivalent / g extract (or) 27.8 mg quercetin/ g of extract (Fig. 2) and total flavanoid content =2.2385 mg quercetin / g of extract (Fig. 3).

**Determination of polyphenol contents in *Agaricus bisporus* extract by HPLC:** HPLC profile of the polyphenols in the mushroom extracts revealed the presence of chlorogenic acid and p-coumaric acid (Figs. 4 and 5).

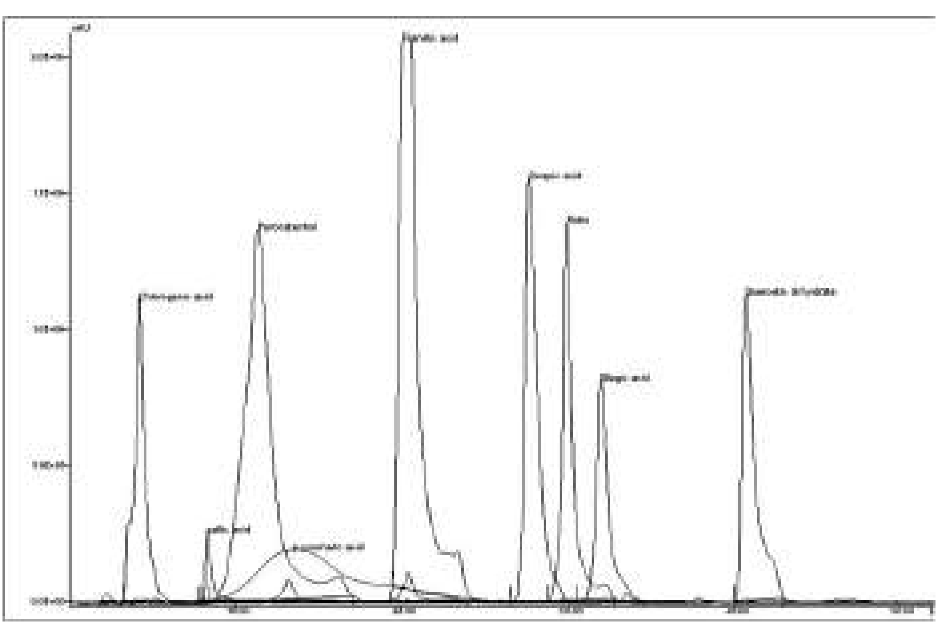

**Figure 4.** HPLC profile of standard polyphenols at 650 nm

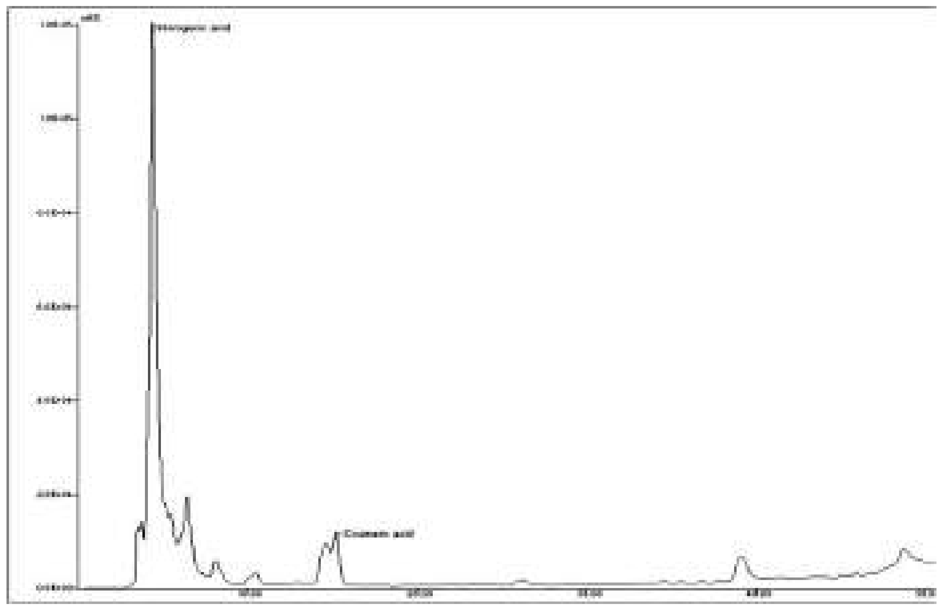

**Figure 5.** HPLC profile of *Agaricus bisporus* extract at 650 nm

**Anti-fatigue properties of *Agaricus bisporus* extract**

**Effect of *Agaricus bisporus* extract on physical endurance:** The anti-fatigue activity of *Agaricus bisporus* extract was measured by calculating the number of shocks received by the animal during its treadmill exercise. This is because only the animals with less endurance capacity will stop running and receives more number of shocks; i.e., animals receiving less number of shocks are having more endurance capacity. The numbers of shocks received by the animals gradually decreased from day 1 to day 6 with *A. bisporus* extract treatment when compared to control group ($p < 0.05$). Minimum number of shocks were recorded on day 6 of the *A. bisporus* extract treatment (2 numbers of total shocks) to that of control group (12 numbers of total shocks), (Table 3).

**Table 3.** Endurance capacity of *A. bisporus* extract assessed by number of shocks during the period of treadmill test

| | Total Number of shocks from all 6 mice | | | | | |
|---|---|---|---|---|---|---|
| | **1st day** | **2nd day** | **3rd day** | **4th day** | **5th day** | **6th day** |
| Group II (Exercise control) | 26 | 27 | 18 | 16 | 16 | 12 |
| Group IV (15mg extract + exercise) | 33 | 19 | 15 | 6 | 4 | 2 |
| Group V (100mg extract + exercise) | 16 | 7 | 5 | 3 | 2 | 2 |

**Effects of *Agaricus bisporus* extract on lipid peroxidation:** Thiobarbituric acid-reactive substances (TBARS)/ MDA, represents lipid peroxidation. In control exercise group the values significantly increased MDA concentration in muscle (43.7%) and liver (14.6%) when compared with sedentary group. The *A. bisporus* extract treatment decreased the MDA levels in muscle (37.7%) and liver (16.2%) to that of control group (Table 4).

**Table 4.** Determination of TBARS in Muscle and Liver

| **Group** | **Feeding and Exercise pattern** | **Muscle TBARS ( x $10^{-5}$ m MDA/g)** | **Liver TBARS ( x $10^{-5}$ m MDA/g)** |
|---|---|---|---|
| Group I | Control | 31.28 | 3.47 |
| Group II | Exercise | 52.16 | 5.36 |
| Group III | Extract 100 mg | 32.54 | 4.04 |
| Group IV | Test 15 mg + Exercise | 42.38 | 4.75 |
| Group V | Test 100 mg + Exercise | 23.38 | 3.3 |

## CONCLUSION

Anti-fatigue natural agents may be essential for persons involved in heavy exercise, such as athletes and other sports personnel, soldiers, industrial employees engaged in severe physical work prompting to utilization of available/stored energy. Continuous physical work may lead to anaerobic condition resulting in accumulation of blood lactic acid, increased toxic products of lipid peroxidation in blood and liver, which in turn may reduce overall physical performance. Dietary macronutrients especially the carbohydrates definitely provide the immediate energy required for carrying out the physical activity, however, it can lead to the accumulation of lactic acid [16, 17]. It is known that severe and prolonged physical activity increases oxidative stress causing elevated free radicals / reactive oxygen species (ROS) concentration that may decrease cellular function / integrity. Endogenous antioxidants namely vitamins A, E and C may neutralize these radicals under normal condition. However, supplementation of these vitamins is essential to neutralise excessive ROS. On the other hand polyphenol with many phenolic functional groups may possess better antioxidant activity compared to that of vitamin A, E and C. As such there is a paucity of information regarding the role of *Agaricus bisporus* in enhancing the physical endurance. In the present study we force fed *A. bisporus* extract to groups of animals and subjected them to exercise for evaluating the antioxidant potency. The results obtained demonstrated that the elevated level of free radicals as indicated by increased levels of lipid peroxidation [18-19] in liver of group III animals was reversed in group IV and V animals, which were force fed with *A. bisporus* extract suggesting an anti-oxidant role of the ingested polyphenols [20,21,22].

Accumulation of lactic acid in blood has been shown to decrease the physical efficacy [17]. In aerobic glycolysis NADH may be thought of a high energy compound whereas in anaerobic glycolysis its free energy oxidation is dissipated as heat [17]. Results clearly show significant decrease in number of total shocks from day one onwards in group IV and V to that of group III. As the exercise days continued, animals received less number of shocks in both the groups, which may be attributed to learning process of animals. This endurance might be attributed to *A. bisporus* extract supplementation. During the course of severe exercise, glycogenolysis may be predominant for adequate supply of energy [17]. From the present study the antifatigue property of *A. bisporus* extract was clearly established. This was achieved through decreased levels of lipid peroxidation in tissue which also support the antifatigue property of the *A. bisporus* extract.

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# COMPARATIVE STUDY ON THE PRODUCTION, PURIFICATION AND CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM OYSTER MUSHROOMS, *PLEUROTUS FLORIDA* AND *HYPSIZYGUS ULMARIUS* AND THEIR APPLICATIONS

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## ABSTRACT

Mushroom polysaccharides have attracted great deal of attention due to many health benefits such as immunomodulation, anticancer activity, prevention and treatment of cardiovascular diseases, antiviral and antimicrobial effects. The present work involved the production and purification of extracellular polysaccharides (EPS) from two oyster mushrooms, *Pleurotus florida (PF)* and *Hypsizygus ulmarius (HU)* and investigation of their effect in relation to antioxidant and anticancer activities. Several parameters such as pH, temperature and media for the growth of mycelial culture and for the production of EPS were optimized to be 4.5, 27 °C and GPKM media, respectively. Purification of exopolysaccharides was carried out by DEAE Sephacel (anion exchange chromatography) and high yield of exopolysaccharides were obtained as 0.726 mg/ml, and 0.665 mg/ml for PF-EPS and HU-EPS, respectively. The samples were further subjected to characterization, antioxidant and anticancer studies. Characterization by NMR showed relative peaks corresponding to polysaccharides. The results of antioxidant assay for the two polysaccharide samples (PF-EPS and HU-EPS) by phosphomolybdenum method was found to be 20.57 and 21.93 ìM AAE/g of tissue, respectively. Anticancer potential of purified polysaccharides were assessed by MTT assay on MCF breast cancer cell lines and two samples, PF-EPS and HU-EPS exhibited percentage of cell viability at 66.48% and 47.63%, respectively. On the whole comparatively, HU-EPS demonstrated encouraging results in terms of anticancer potential.

**Keywords:** *Pleurotus florida, Hypsizygus ulmarius,* exopolysaccharides, anticancer

## INTRODUCTION

Polysaccharides derived from mushrooms have emerged as an important class of bio-active substances. Polysaccharides isolated from macrofungi had already been considered to have antitumour activity. Schizophyllan, Lentinan and Krestin (mushroom polysaccharides), which were in Japan and China, are used for antitumour activity since it stimulates T and B lymphocytes, monocytes and macrophages leading to secretion of TNF-$\alpha$ (Tumour Necrosis Factor) or interleukins in both cell culture and humans.

Cultivated mushrooms have been limited in application since it consumes more time for cultivation and they have the ability to accumulate many toxic metals such as cadmium, lead, arsenic, copper, nickel, silver, chromium and mercury from contaminated soil. Thus, polysaccharides were produced from submerged culture under controlled environment and has been a best alternative [1]. Exopolysaccharides have been found in many food and pharmaceutical applications. A wide variety of applications includes thickening and stabilizing agents in chemical industry, immuno stimulating and antitumour agents for clinical use. It has also the ability to heal and protect skin against infection. It is also used for enhancing collagen biosysnthesis and increasing cell proliferation [2].

Polysaccharides from mushrooms are reported to have free radical scavenging activity, superoxide radical scavenging, reducing properties, lipid peroxidation inhibition, suppression of proliferation and oxidative stress etc. Polysaccharides isolated from different mushrooms genera are capable of providing antitumor activity eg., *Agaricus*, *Calocybe, Ganoderma, Grifola, Inonotus, Lentinus, Phellinus, Pholiota, Pleurotus,*etc., These include the activation of macrophages, T lymphocytes and natural killer cells, which are able to secrete inflammatory mediators of cytokines such as the tumor

necrosis factor, α-interferon, α interleukin. Polysaccharides can depress the E-selectin protein and gene expression, which inhibit tumor cell to cell adhesion. Other mechanism include antiproliferative effects, apoptosis induction and differentiation of the tumor cells [3].

*Pleurotus florida (PF)* and *Hypsizygus ulmarius (HU)* are the two edible oyster mushrooms used in the present study. Many bio active compounds have been reported from the fruiting body of *PF* and *HU.* The most important materials among these were the polysaccharide fractions obtained mainly from the fruiting body and also partly from the mycelium of PF and HF.

*Pleurotus* species are commonly called as Oyster mushrooms. It is a lignocellulolytic fungus that grows naturally in the temperate and tropical forests on dead and decaying matter and it is in second grade among the important cultivated mushrooms in the world.There are about 40 species coming under *Pleurotus* mushroom, among the 25 species are commercially cultivated [4].

*Hypsizygus ulmarius* (elm oyster mushroom) is a high yielding mushroom for which commercial cultivation technology has been released and is gaining popularity. Previous reports suggests that this mushroom is rich in antioxidants and proved for its anti-diabetic activity [5].

The present study aims to compare the production, purification and characterization of exopolysaccharides of two edible oyster mushrooms, *Pleurotus florida (PF)* and *Hypsizygus ulmarius (HU)* and their application in relation to antioxidant and anticancer activities.

## MATERIALS AND METHODS

### Sample Collection and cultivation

Bed spawn of both PF and HU were collected from TNAU (Tamil Nadu Agricultural University, Coimbatore) and used for exopolysaccharide production. The collected spawn was grown over paddy straw at 25 °C for 25 days. Grown mushrooms were harvested after 25 days.

### Isolation of exopolysaccharide from PF and HU mycelial broth

The exopolysaccharides present in the culture broth of PF and HU was extracted following the methodology of Xu *et al.* [6]. For the extracellular product isolation, the mycelial cells were removed from culture broth using a filter paper. The filtrate was concentrated to one-fifth of its original volume with a rotary evaporator under reduced pressure and mixed with 4 volumes of chilled 95% (v/v) ethanol, then stirred vigorously and left at 4 °C overnight. The precipitate was collected by centrifugation at 5000 rpm for 20 min and lyophilized (exoploysaccharide, EPS - I). The EPS-I was further treated with the Sevag reagent to remove the protein, yielding the EPS-II fraction.

**Screening of media:** Spawn was cultured in PDA plate and small piece was cut using knife and inoculated in respective broth.The mycelia of PF were grown in YMG liquid media prepared by dissolving 1 g of yeast extract, 3 g of malt extract, and 2 g of dextrose in 100 ml distilled water. Mycelial plugs of PF and HU were grown in YMG media for 20 days at 25 ºC and were seeded to liquid media under aseptic conditions. The flasks were placed on a rotary shaker at 120 rpm, 25 ºC. (Modified Yeon-Ran Kim, 2003) [7]. The seed medium was also used as the basal medium consisting of 30 g/l glucose, 5 g/l peptone, 5 g/l $KH_2PO_4$, 1 g/l $MgSO_4.7H_2O$, (modified Baojing Yuan *et al.* [8] for screening of media.

**Optimization of pH and temperature:** Screened media was taken for optimal pH and temperature. pH optimization was carried out at 3.5, 4, 4.5, 5, 5.5, respectively [9]. Temperature optimization was carried out at 25, 27, 30, 32 °C [10].

**Mass production of exopolysaccharides:** With the screened media and optimized pH and temperature, mycelial culture of both PF and HU was cultivated in large volume for extraction of exopolysaccharides [6] and assayed using phenol sulphuric acid method for total carbohydrates.

### Purification of exopolysaccharides

Purification of exopolysaccharides was carried out using DEAE Sephacel anion exchange chromatography [11]. Fractions are gradient eluted with 0.5 M NaCl at the rate of 5ml/min. Collected fractions were assayed for total carbohydrates by phenol sulphuric acid method. Eluates showing highest concentration of exopolysaccharides were pooled and lyophilized.

### Determination of nuclear magnetic resonance of exopolysaccharides

The $^1$H nuclear magnetic resonance (NMR) spectra of exopolysaccharides in $D_2O$ were obtained with 300MHz Bruker NMR Spectrometer [12].

### Evaluation of total antioxidant capacity (TAC) by Phosphomolybdenum method

The total antioxidant capacity of the exopolysaccharides was evaluated by the phosphomolybdenum method according to the procedure described by Prieto *et al.* [13]. The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acidic pH. A 0.2 ml extract was combined with 2 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. The antioxidant activity was expressed as the number of gram equivalent of ascorbic acid. The calibration curve was drawn using ascorbic acid.

### Evaluation of anticancer activity by MTT assay

A colorimetric assay based on the MTT method by Sara silva *et al.* [14] was used to measure the growth inhibition of MCF breast cancer cell lines in microtitre plates of 96 wells containing DMEM medium. Appropriate concentrations of exopolysaccharides (1mg/ml) were added to each well ($2 \times 10^5$ cells per well) and the cultures were incubated with $CO_2$ incubator with 5% $CO_2$ at 37 °C for 48h. Cell viability was determined by addition of 20µl of 3 mg/ml MTT to each microtitre well and after 4h of incubation, the supernatant was removed and 50 µl of dimethyl sulfoxide was added to each well to solubilize the precipitate. The survival rate of MCF breast cancer cell lines was assayed by measuring the optical density in a microtitre plate reader at 650 nm. Results were expressed as % of cell viability.

$$cell\ viability\ \% = \frac{test}{sample} * 100$$

All assays were carried out in triplicates and the results are expressed as mean ± standard of three replicates.

## RESULTS AND DISCUSSION

### Media screening

In order to produce exopolysaccharides (EPS) from PF and HU, culture media has to be optimized with certain parameters. Screening of media was carried out for culturing the mycelia in broth. From the Fig. 1, it was shown that YGM media produces polysaccharide (0.068 mg/ml) in mycelial powder, but at long incubation period (20 days). On the other hand, GPKM media produces the polysaccharide in mycelial broth (0.089 mg/ml) within 7 days of incubation. In order to carry out further study, GPKM was prefered, which gives high yielding exopolysaccharides. However, work on *Grifola frondosa* in YMG media displayed polysaccharide extraction at 7.73 mg/l [7]. Similar studies by Duan Yan-Quing *et al*. [15] in GPKM media on *A.aegirita* Mo-Aa exhibited the yield at 3.72g/l.

## Optimization of pH and temperature

While working with mushroom mycelia, it became an important criteria to optimize the pH and temperature, since mushrooms belong to the family of fungi. Generally fungi prefer acidic pH and low temperature. Here, the study was carried out to find the pH and temperature for better yield of mycelial growth and exopolysaccharides production. From Fig. 2, it was inferred that with slight change in pH, growth of the mycelia was affected. At the pH 3, 5 and 4, the mycelial growth was

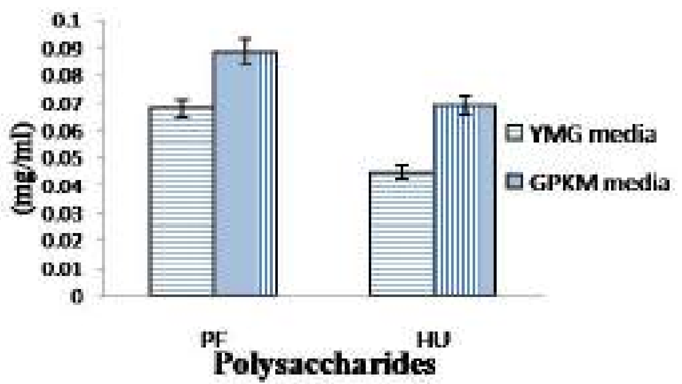


Figure 1. Screening of media for mass production of *Pleurotus florida* and *Hypsizygus ulmarius*

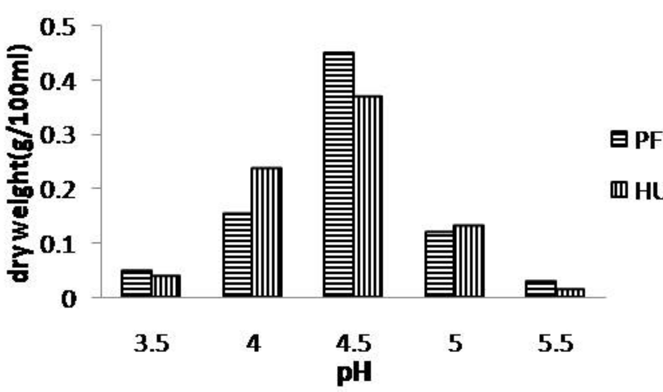


Figure 2. Optimization of pH for mycelia growth

limited, and in the pH 5 and 5.5 was tend to be negligible. However, at pH 4.5, there was reasonable quantity of mycelial growth. Since mycelial growth was directly proportional to the EPS, pH 4.5 is optimized for further studies. Other researchers reported optimum pH for the growth of *Alternania alternata* to be 3.0 [10] and study by Zhicai Zhang *et al*. [9] showed that there is no significant difference from pH 5 to 7.5 for the growth of mycelia *Tremella aurantialba.*

Temperature was also an important factor to be considered, while working on mushrooms, since it prefers comparatively low temperature than bacteria. From figure 3, it was clearly noticed that at temperature 25 °C, there was limited growth and at 32 °C, lot of bacterial growth was noted during the growth of mycelia. Thus optimum temperature for the growth of mycelial culture was found to be 27 °C. Our results are in concurrence with studies by Shamy and Nehad [10] on *Alternaria alternata* who showed that optimal temperature to be 30 °C.

## Mass production of exopolysaccharides

In order to get bulk product, mass production step was necessarily carried out with the screened media, optimized pH and temperature. Production of exopolysaccharide (EPS) was carried out by method of Xu *et al.* [6] for both PF and HU.

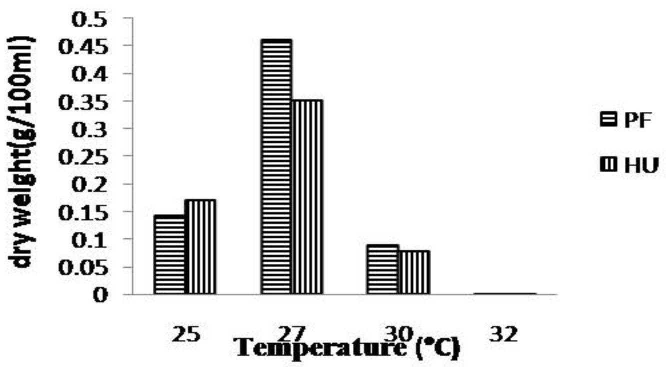

Figure 3. Optimization of temperature for mycelial growth

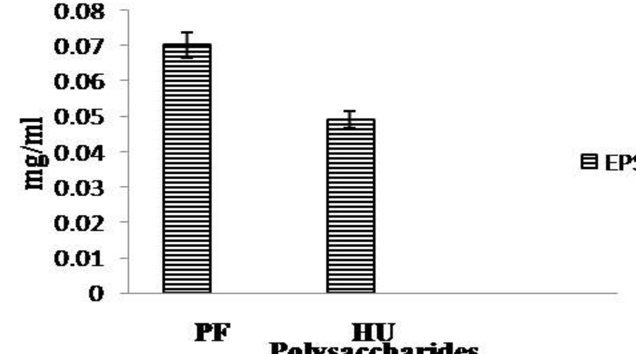


Figure 4. Mass production of exopolysaccharides

Figure 4. depicts the mass production of exopolysacharides. The mass production was carried out for samples namely *P. florida* (PF) and *H. ulmarius* (HU), exopolysaccharides (EPS), respectively. These obtained exopolysaccharides were used for further purification.

## Purification of exopolysaccharides

Purification by DEAE Sephacel anion exchange chromatography was carried out to remove the residual proteins. The unbound fractions containing exopolysaccharides was collected by eluting the column with sodium phosphate buffer of pH 8. Collected fractions were checked for total carbohydrates and high polysaccharide fractions were pooled, dialyzed and lyophilized [11].

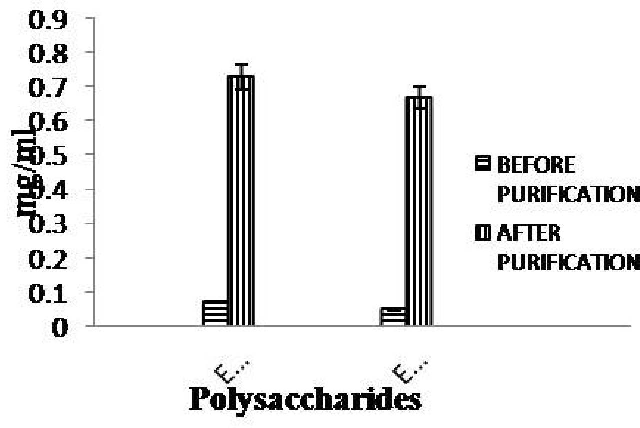


**Figure 5.** Comparison of Exopolysaccharides yield

After purification, exopolysaccharide production of both PF and HU was increased by 10 and 13 fold, respectively compared to the crude exopolysaccharides (Fig.5). Thus, these polysaccharides namely PF-EPS and HU-EPS were used for further studies such as characterization and application studies based on the yield. Similar studies by Sara Silva *et al.* [14] from *Letinula edodes* showed 0.037 g/l of exopolysaccharide after purification.

## Characterization of exopolysaccharides by NMR

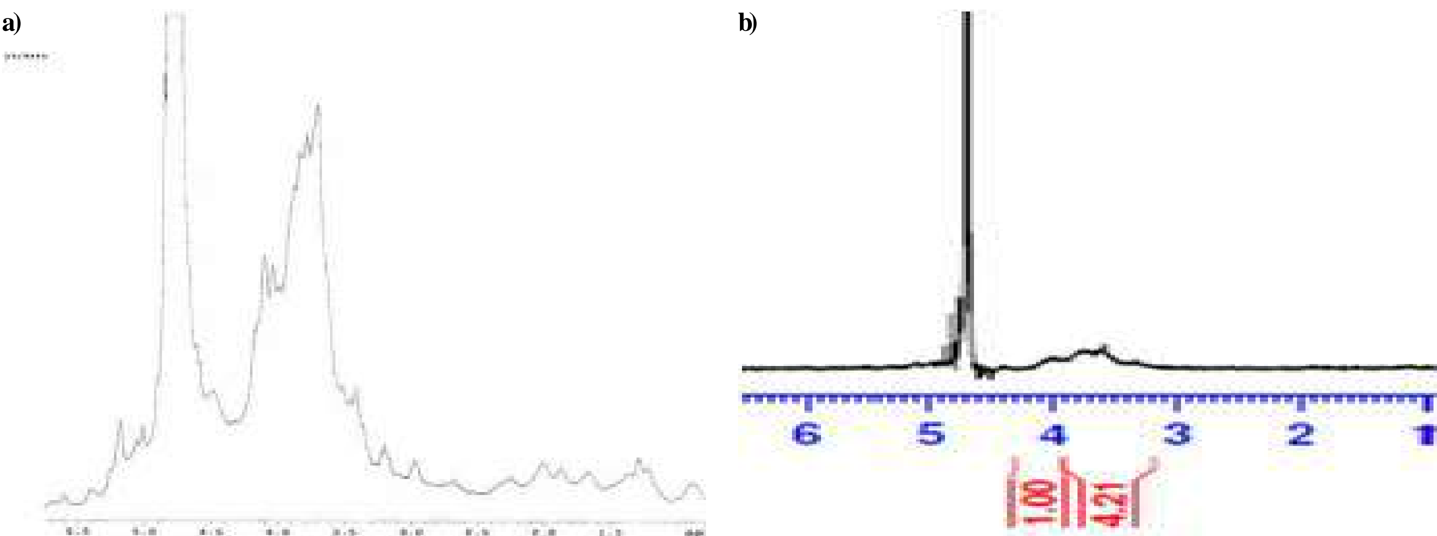


**Figure 6.** NMR spectrum of a) PF exopolysaccharides b) HU exopolysaccharides

$H^1$ NMR spectra for the two polysaccharide samples were analyzed by comparing with previous similar works. As per spectral Fig. 6 and 7, the peaks at 5.10 and 4.51 ppm, is characteristic of the $\alpha$ and $\beta$ linkages respectively [12]. The anomeric region 4.3- 4.9 ppm were found to be characteristic of $\beta$ anomers.

The ring proton regions 3.0-4.2 ppm showed overlapping peaks and were assigned to protons of carbons C2 to C5 (or C6) of the glycosidic ring [16], such that axial, equatorial protons and $CH_2OH$ protons are appearing as multiplet.

## Evaluation of total antioxidant capacity (TAC) by phosphomolybdnum method

Antioxidant compounds are present in many of fruits and vegetables naturally. Nowadays in the emerging field, antioxidant compound is necessarily produced in excess separately in order to act against free radicals, since free radicals destroy the

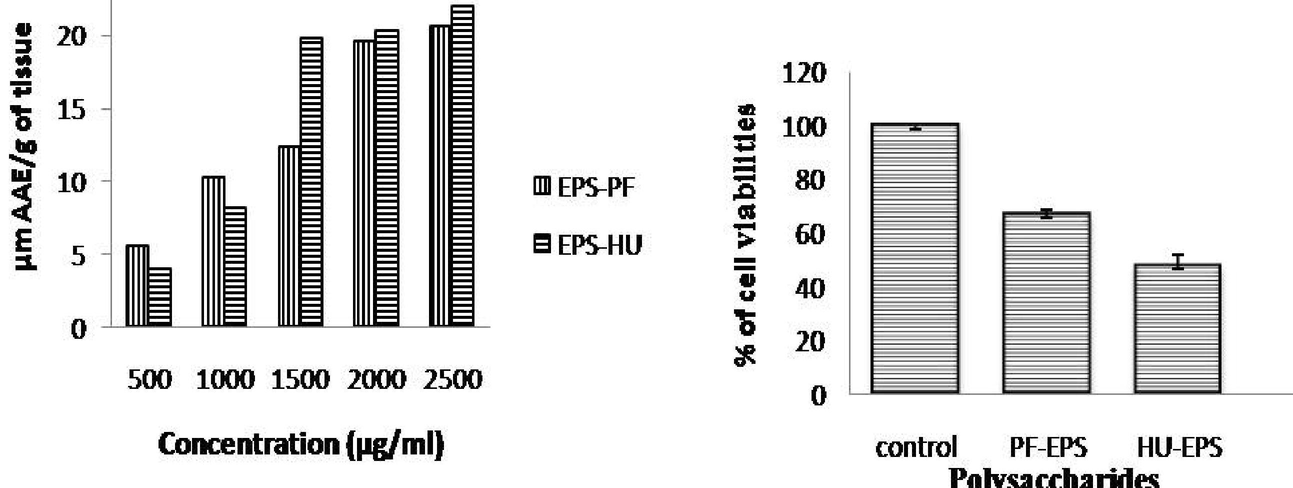


**Figure 7.** Total Antioxidant Capacity (TAC) of mushroom exopolysaccharides

**Figure 8.** Cytotoxic effect of the polysaccharide samples on MCF-7 cancer cells

body's macromolecules such as protein, DNA, RNA, etc. In the present study, antioxidant assay was carried out in order to evaluate the antioxidant capacity of these two purified polysaccharide samples. The results of the assay were expressed as micromolar of ascorbic acid equivalents, which was then tabulated (Fig. 8).

At 2500 µg/mL, PF fruiting body polysaccharide showed higher ascorbic acid equivalents. Out of these two exopolysaccharides, the antioxidant activity was more or less similar as 20.57, 21.93 µM AAE/g of tissue for PF-EPS and HU-EPS respectively. Previous work by Adebayo *et al*., (2012) on *Pleurotus pulmonarius* showed 25.6 µM AAE/ g for 1mg/ml of fruiting body polysaccharides.

**Evaluation of Anticancer activity by MTT assay**

In recent years, occurrence of breast cancer is gradually increasing. It is a type of cancer originating from breast tissue,most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Emerging trend was to find the alternative medicine to cure these types of cancer in early stages, since breast cancer had caused 4,58,503 deaths worldwide in 2008. Thus, the present work has been extended to MCF breast cancer cell lines by observing cell viability on treatment with purified exopolysaccharides. Anticancer activity was done by MTT assay for purified polysaccharide samples at concentration of about 1mg/ml.

Results showed that HU-EPS has low cell viability towards the MCF breast cancer cell lines at about 47% compared to other polysaccharides. Studies by Thetsrimuang *et al.* [17] on MCF breast cancer cell lines by crude polysaccharides from *Lentinus polychorus* at 1mg/ml concentration have shown cell viability of 55%. Similar work by Larissa *et al.* [18] in *Hypsizygus marmoreus* showed inhibition of tumor growth upto 59%.

## CONCLUSION

The present study aimed at the exopolysaccharides(EPS) production from PF and HU with screened media (GPKM), optimized pH (4.5) and temperature (27 °C) respectively. Purification of exopolysaccharides was carried out by DEAE Sephacyl anion exchange chromatography and has a yield upto 0.726 mg/ml, and 0.665 mg/ml, respectively. These exopolysaccharide samples were subjected to further analysis such as characterization by NMR, antioxidant and anticancer activity. Characterization by NMR showed relative peaks corresponding to polysaccharides such as OH and CH stretch, glycosidic linkages etc., respectively. Antioxidant assay was carried out for exopolysaccharide samples (PF-EPS and HU-EPS) and results obtained as 20.57, 21.93 µM AAE/g of tissue, respectively and the dosage for anticancer studies were fixed at 1mg/ml. MTT assay was carried out on MCF breast cancer cell lines to assess the anticancer activity of the purified exopolysaccharides. The anticancer activity of two samples PF-EPS and HU-EPS was denoted as percent viability of

66.48% and 47.63%, respectively. Hence, the present study showed that HU-EPS displayed high anticancer effect comparatively. Mushroom polysaccharides are yet to be explored for a lot of various pharmaceuticals for applications in near future.

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# PURIFICATION AND CHARACTERIZATION OF AN *N*-ACETYL-D-GLUCOSAMINE SPECIFIC LECTIN FROM THE AUSTRALIAN MUSHROOM *PSATHYRELLA ASPEROSPORA*

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## ABSTRACT

Australia has a spectacular biodiversity including animals, plants and fungi. Indigenous Australians have been using higher fungi traditionally as medicines and in religious practice for thousands of years. It has been estimated that there is a large number of different mushroom species present in Australia that are poorly explored and catalogued. Importantly, very little is known about the extent and diversity of lectins from Australian mushroom species [1]. *Psathyrella asperospora* (Family: Psathyrellaceae) (synonym *Lacrymaria asperospora*) is an Australian indigenous mushroom from which we have isolated an *N*-acetyl-D-glucosamine (GlcNAc) specific lectin [1]. *De novo* sequencing of *Psathyrella asperospora* lectin (PAL) using LC-MS/MS, identified 10 tryptic peptides that revealed substantial sequence similarity to the GlcNAc specific lectin from *Psathyrella velutina* (PVL) in both carbohydrate binding and calcium binding sites. Significantly, we also found that PAL has anti-proliferative effect on human colon cancer HT29 cells with an $IC_{50}$ of 0.48 μM that represents one of the most potent mushroom lectin yet reported [2]. Further characterization of PAL's anti-proliferative activity using propidium iodine staining revealed that it induced cell cycle arrest at $G_2$/M phase in a manner dependent on its ability to bind GlcNAc on the cell surface. Large scale purification of PAL has now been performed in order to fully characterise the carbohydrate binding specificity including its thermodynamic properties and structural determination using glycan arrays, isothermal calorimetry (ITC) and X-ray crystallography.

**Keywords:** *Psathyrella asperospora*; *N*-Acetyl-D-glucosamine (GlcNAc) specific lectin; mushroom lectin; anti-proliferation; $G_2$/M cell cycle arrest

## INTRODUCTION

Lectins are proteins, non-immunoglobulin in nature, capable of specific recognition and reversible binding to the carbohydrate moiety of glycoconjugates on the cell surface, resulting in cell agglutination and subsequent precipitation in a solution [3]. The specificity of lectins makes them an important tool in glycoproteins purification, identification and glycan analysis [4]. Lectins are ubiquitous in nature, occurring in plants, humans, animals, fungi, bacteria, viruses, and also in all foods, with their abundance being wider in mushrooms compared to plants [5]. Over the past few decades, a number of lectins have been isolated from mushrooms, and they have attracted considerable interest due to their various bioactive properties, including anti-proliferative [6-8], anti-tumour [9-11], mitogenic [6, 10, 12], immunomodulatory [9, 13, 14], hypotensive and vasorelaxing [15], and antiviral [8, 10, 16] activities.

Australia has a spectacular biodiversity including animals, plants and fungi. Indigenous Australians have been using fungi traditionally as medicines and in religious practice for thousands of years [17]. A large number of different mushrooms species are present in Australia that are poorly explored and catalogued. Importantly, very little is known about the extent and diversity of lectins from Australian mushroom species [1]. *Psathyrella asperospora* (Family: Psathyrellaceae) (Syn.: *Lacrymaria asperospora*) is an Australian indigenous mushroom, not used for food, that we recently reported to express an *N*-acetyl-D-glucosamine (GlcNAc) specific lectin [1]. The specie (*P. velutina*) of the same genus has been reported to express a GlcNAc/*N*-acetyl-neuraminic acid (Neu5Ac) specific lectin, referred to as PVL [18, 19]. PVL has now been

well characterized with respect to specificity and interaction kinetics [19, 20], and a 1.5 Å crystal structure of PVL complexed with GlcNAc is also available [21]. However, until now there has been no report of its pharmacological activity including cytotoxic or anti-proliferative activity.

Lectins with high affinity towards GlcNAc have been isolated and characterized from both vertebrates and invertebrates [3]. They have been found to be potent and selective inhibitors of human immunodeficiency virus and cytomegalo virus replication *in vitro* [22] as well as other human pathogens [23]. GlcNAc specific lectins are also known to be cytotoxic towards human hepatocellular carcinoma, human placenta choriocarcinoma and rat osteosarcoma cells [24].

A previous report showed that the GlcNAc specific lectin from the fruiting body of *P. asperospora* named PAL exhibited potent anti-proliferative activity against colon adenocarcinoma (HT29) cells. Further characterization of PAL's anti-proliferative activity showed that HT29 cells are arrested at $G_2$/M phase of the cell cycle, and that this effect can be halted through the addition of free GlcNAc. Here we report on the large-scale purification of PAL and the preliminary characterisation of the carbohydrate binding specificity using glycan arrays and structure determination by X-ray crystallography.

## MATERIALS AND METHODS

### Mushroom collection and reagents

The fruiting bodies of *P. asperospora* (Accession no. MEL 2061945) were collected in Melbourne, Australia, identified at the Royal Botanic Gardens, Melbourne and immediately frozen at -20 °C. Unless otherwise stated all the reagents were purchased from Sigma. Fructose was obtained from Ajax chemicals, lactose from OXOID Ltd, Neu5Ac from Jülich Chiral Solutions GmbH, chitin affinity sepharose from New England BioLabs, and rabbit erythrocytes from IMVS Veterinary Services Division.

### Large-scale PAL purification, molecular mass determination and protein estimation

*P. asperospora* lectin was purified as described by Rouf *et al*. [2]. The extraction, isolation and purification steps were carried out at 4 ºC except the final size exclusion chromatography step. Thawed fruiting bodies (200 g) was suspended in 550 ml PBS (pH 7.4) homogenized using a Waring blender and left overnight at 4 ºC with gentle shaking. The resulting homogenate was filtered through cotton gauze and centrifuged twice, first for 20 min at 1,500 x g, followed by another 20 min at 10,000 x g. Solid $(NH_4)_2SO_4$ was added to the resulting supernatant (crude homogenate) to a concentration of 40% and allowed to fully dissolve for 45 min. Following centrifugation at 12,000 x g for 25 min, $(NH_4)_2SO_4$ was added to the resulting supernatant to a final concentration of 80%, allowed to dissolve and centrifuged once again to obtain the 80% pellet. This pellet was resuspended in a minimal volume of 20 mM Tris buffered saline (TBS) (pH 8.5) and extensively dialyzed against the same buffer.

Subsequently, the dialyzed 80% $(NH_4)_2SO_4$ precipitate was briefly centrifuged to remove unsuspended/sedimented particles and then it was loaded onto a 10 ml chitin sepharose column equilibrated with TBS (pH 8.4) and the affinity adsorbed PAL eluted with TBS (pH 8.5) containing 50 mM GlcNAc and 10% (v/v) glycerol in 2 cycles. The GlcNAc eluted fraction was extensively dialyzed against TBS (pH 8.5) and applied to a Hiprep Sephacryl S-100 HR column (GE healthcare) (16 × 600 mm; bed volume 120 ml) equilibrated with TBS (pH 8.5). The purification of PAL was monitored at each step using a hemagglutination assay with rabbit erythrocytes. PAL purified in this manner was stored at -20 ºC in TBS (pH 8.5) containing 10% (v/v) glycerol. Protein estimation was performed using the BCA (Bicinchoninic acid) Protein Quantitation Kit as described by the manufacturer (Thermo scientific). Standard curves were prepared using bovine serum albumin (BSA) concentrations between 0 and 2 mg/ml. Samples and standards were read on a Viktor3 1420 Multilabel counter (PerkinElmer) at 595 nm. The molecular mass of purified PAL was determined using SDS-PAGE and size exclusion chromatography (SEC). SDS-PAGE was performed on a 10% (w/v) acrylamide gel as described by Laemmli, 1970 [25], and gels stained with Coomassie brilliant blue R-250. SEC was performed on a Hiprep Sephacryl S-100 HR column (GE healthcare) (16 × 600 mm; bed volume 120 mL) calibrated with Conalbumin (75 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Ribonuclease-A (13.7 kDa) and Aprotinin (6.5 kDa).

### Hemagglutination and Hemagglutination inhibition (HA & HA-I) assays

The hemagglutination assay was modified from that of previously described by Han *et al.* [26]. A serial two fold dilution of the samples was prepared. Each dilution was mixed with an equal volume (25 µl) of PBS and added to 50 µl of neuraminidase treated or normal 2% erythrocytes suspension (rabbit or human) at room temperature in a microtiter U-plate. PBS (50 µl) added to 50 µl of 2% erythrocytes was used as a blank and the results were observed after 1 hr when the blank was fully sedimented (appeared as a dot at the bottom of the well). The hemagglutination titer was defined as the reciprocal of the highest dilution of the lectin solution showing hemagglutination activity (HA-A) and was considered as one hemagglutination unit (HA-U protein). The specific HA activity was defined as the number of HA units per mg of protein (HA-U/mg).

Hemagglutination inhibition was performed as previously described by Liu *et al.* [27],with slight modification, with the inclusion of additional sugars including Lac, Gal (Fluka biochemica), Man, Ribose (Rib), Xyl (Aldrich), GalNAc and GlcNAc. A serial two fold dilution of the different sugars to give final concentrations ranging from 50 to 0.20 mM in PBS were pre-incubated with equal volume (25 µl) of sample (diluted to the previously determined hemagglutination titer) in U-plate for 30 min. Neuraminidase treated or normal 2% rabbit and human A, B and O erythrocytes solution (50 µl) was then added, and incubated at room temperature for a further 1 hr. The minimum inhibitory concentration (MIC) was then measured by determination of the minimum sugar concentration that was able to completely inhibit the HA-A, visualised as a clear sharp dot at the bottom of the well.

### Preparation of glycan arrays

Glycans sourced from Dextra Laboratories (Reading, UK) and Glycoseparations (Moscow, Russia) were functionalised and printed on activated SuperEpoxy 2 glass slides (Array It Microarray Technologies) as previously described by Day *et al*. [28].

### Fluorescence labeling of PAL

Purified PAL (200 ìg) was buffer exchanged against PBS, pH 7.4 using centrifugal filter device (<10 kDa; Amicon® Ultra Centrifugal Filters) and labeled with 10 ìl of Alexa Fluor® 647 succinimidyl ester (Life Technologies) (10 ìl dye is sufficient for about 1 mg protein). The reaction mixture was wrapped in aluminum foil and incubated for 1 hr at room temperature. Subsequently a minimum volume of 10 x TBS, pH 8.5 buffer was added to reconstitute the solution back into 20 mM TBS buffer and deactivate any remaining free dye. PAL activity was examined by HA and HA-I assay.

### Application to glycan arrays

Prior to use, all slides were blocked with 20 mM TBS containing 0.1% BSA and dried by centrifugation at 900 rpm for 5 min in an empty 50 ml tube. Labeled PAL (65 µl) was applied to the array contained by a Gene Frame, and the solution was evenly distributed with the aid of a Gene Frame Cover slip. After incubation for 15 min in dark at room temperature, the Gene Frame and coverslip was removed carefully in a bath of buffer solution (20 mM TBS buffer). Slides were then washed twice in a 50 ml tube containing 50 ml of fresh 20 mM TBS buffer and dried in an empty 50 ml tube by centrifugation at 900 rpm for 5 min. The slides were scanned using the Pro Scan Array Microarray 4-laser scanner. Fluorescence intensities of the array spots were measured using the Blue Argon 647 excitation laser set to the FITC setting (647 nm excitation and 517 nm emission). The slides were scanned prior to (prescan) and following each experiment. The data was analyzed using "Scan Array Express" (PerkinElmer) imaging software. The relative binding of each glycan was expressed as mean RFU (relative fluorescence units) of four replicates.

### Crystallization of PAL

PAL was co-crystallized in 2.4 M malonate pH 5 with GlcNAc [PAL: 9 mg/ml, GlcNAc: 2.15 mM (10x excess)] by vapour diffusion in 1 µl volume, using a sitting drop format. This condition, as well as others, were prepared and monitored by the EMBL crystallization robot, HTX laboratory, Grenoble. Crystals were observed after 2 weeks.

## RESULTS AND DISCUSSION

### Large-scale purification of PAL and sequence comparison with PVL

We previously showed that the crude homogenate from *P. asperospora* was able to hemagglutinate both rabbit and human blood types A, B and O erythrocytes. The hemagglutination activity was enhanced following neuraminidase treatment of human blood types A, B and O erythrocytes [1]. Neuraminidase treatment of rabbit blood had no effect on activity. Importantly, regardless of the blood types used and treatment prior to activity assays the only saccharide able to inhibit hemagglutination was GlcNAc, with minimum inhibitory concentrations (MICs) in the low mM range [1]. Therefore, in this study due to its high hemagglutination titer, untreated rabbit blood was used to monitor PAL activity during purification.

PAL was purified to homogeneity in three steps; 80% ammonium sulfate $(NH_4)_2SO_4$ precipitation, chitin affinity chromatography and Hiprep Sephacryl S100 size exclusion chromatography (SEC). From 200 g of frozen *P. asperospora* fruiting bodies 28.4 mg of PAL was purified at a purification fold of 9, and a recovery of 13.6 % (Table 1). After SDS-PAGE analysis (Figure not shown), it was found that the initial rise in UV absorbance (shorter peak) represented the presence of contaminating proteins and the second, shaper peak represented the purified PAL that was collected and pooled. The molecular mass of PAL as determined by SEC (Fig. 1B) was approximately 36.0 kDa, which correlated well with that determined by SDS-PAGE under reducing conditions (Fig. 1A, lane 6) of approximately 40 kDa. Accurate determination of molecular mass was afforded by DLS (d*ynamic light scattering*) analysis, with PAL determined to have a molecular mass of 41.8 kDa [2].

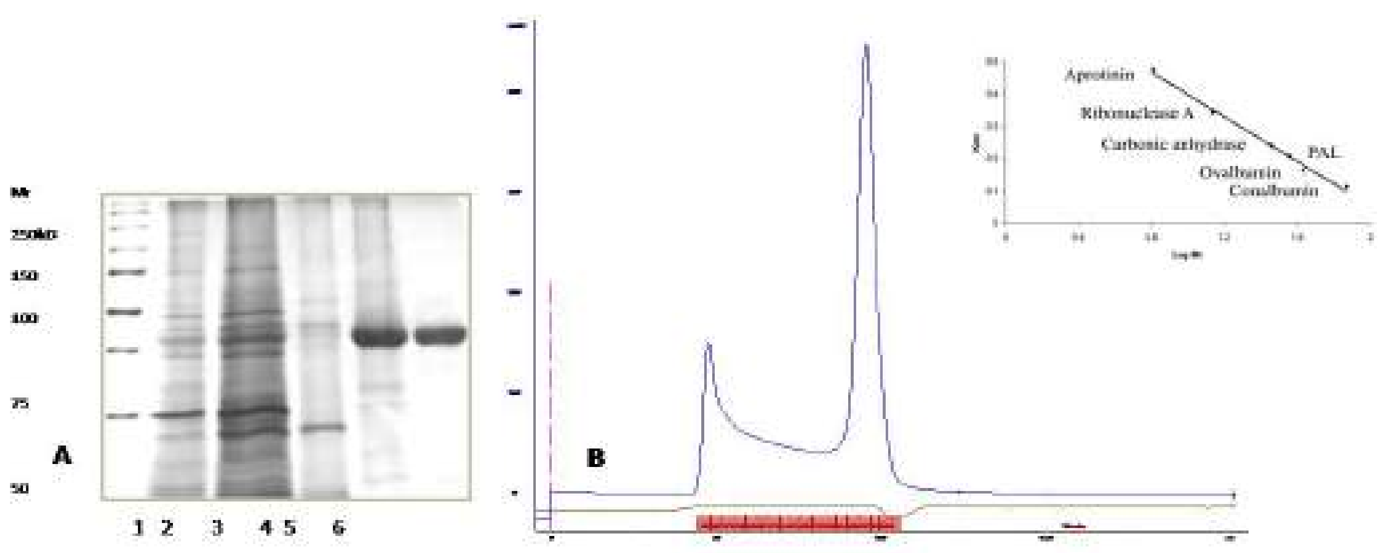


**Figure 1.** (A) SDS-PAGE of purified PAL: lane1 - Molecular weight markers; lane 2 - crude homogenate; lane 3 - 80 % $(NH4)_2SO_4$ precipitation; lane 4 – flow though of chitin affinity chromatography, lane 5 - Eluate of chitin affinity chromatography and lane 6 - pooled SEC fraction, (B) The chromatogram profile of size exclusion chromatography (SEC) on Hiprep Sephacryl S100 column. The calculated molecular weight by SEC of purified PAL was 35.96 KDa (insert).

**Table 1.** Purification of *P. asperospora* lectin (PAL)

| Fraction | Total protein (mg) | Total HA-A (HA-U) | Specific activity (HA-U/mg) | Purification fold | % Recovery |
|---|---|---|---|---|---|
| Crude homogenate | 1831.5 | 2475000 | 1351.4 | 1 | 100 |
| 80% $(NH_4)_2SO_4$ precipitate | 367.5 | 960000 | 2612.2 | 2 | 38.8 |
| Chitin affinity chromatography | 119.9 | 1080000 | 9011.3 | 7 | 43.6 |
| Size exclusion chromatography | 28.4 | 336000 | 11851.9 | 9 | 13.6 |

PAL was previously characterized by *de novo* sequencing using LC-MS/MS, with 10 tryptic peptides identified [2]. Mascot analysis revealed the 10 peptides to share sequence similarity to the *P. velutina* lectin (PVL, gi:78057570) which is GlcNAc specific [18, 29]. An NCBI-BLASTP search and ClustalW2 analysis of a 43 amino acid peptide sequence constructed from peptides 4, 6 and 7, TVALADLVGEGTGGVYLLRGGSLLLQVVKVLDNFGYNA-GGSVR, exhibited high identity to PVL (70% identity) (Fig. 2). Similarly, PAL peptides 1, 2, 3, 5, 8, 9 and 10 also showed high identity to PVL. Significantly, a number of these peptides overlapped with known carbohydrate (peptides 2, 3, 5, 7, 8, 9 and 10) and calcium (peptides 3 and 5) binding domains. Fig. 2 shows the multiple sequence alignment of PVL and the PAL tryptic peptides, with the carbohydrate and calcium binding domains as determined through the X-ray crystal structure of PVL being highlighted. Although highly conserved, some differences between PAL and PVL are evident. In particular, a number of PVL residues known to be involved in carbohydrate recognition and binding, Trp54, Glu191, Asn214, Asp270 and Try306 are, based on *de novo* sequencing of tryptic peptides, substituted in PAL with Ser, Val, Pro, Phe and Leu respectively. However, given the high affinity and strict specificity for GlcNAc that PAL exhibits, it would appear that these substitutions have no dramatic effect on the structure of the carbohydrate-binding sites. In addition, Leu183 in the PVL $Ca^{2+}$ binding domain is conservatively substituted with Ala in PAL (Fig 2), and a two-residue insertion (an Arg and Leu) was found in PAL between PVL residues Leu183 and Leu184. However all other residues in the PVL $Ca^{2+}$ binding consensus sequence, Asp-h-Thr-Gly-Asp-Gly-h-h-Asp, are conserved.

PAL and PVL [18] both bind GlcNAc in a divalent cation independent manner, however the crystal structure of PVL revealed two $Ca^{2+}$ binding sites consisting of nine residue loops with the consensus sequence Asp-h-Thr-Gly-Asp-Gly-h-h-Asp (where h is an hydrophobic residue) [21]. It is still unclear as to the exact function of $Ca^{2+}$ in PVL for mushroom metabolism, although it has been postulated that $Ca^{2+}$ binding may play a role in lectin stabilization in a similar way to that seen in integrins [21]. A sequence alignment of PVL and tryptic peptides of PAL revealed a high conservation of residues in $Ca^{2+}$ binding site 1, with only Leu183 substituted with an Ala in PAL, and a His in AAL-II. PAL sequence information was not obtained for $Ca^{2+}$ binding site 2. Therefore it would seem highly probable that the ability to bind $Ca^{2+}$ is a conserved feature of this family of GlcNAc specific lectins.

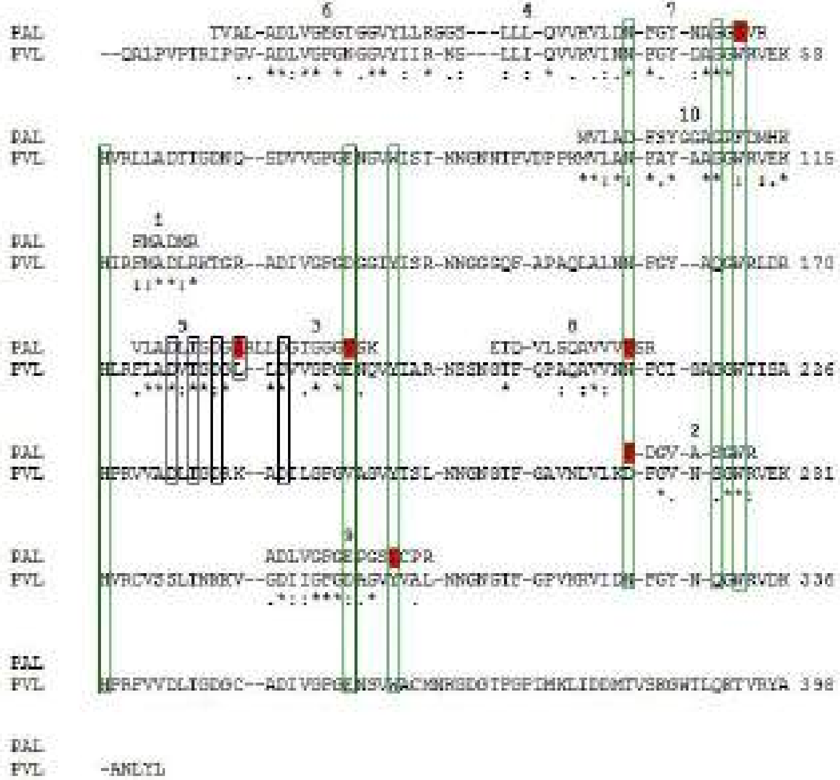

**Figure 2.** ClusterW2 sequence alignment of 10 PAL tryptic peptide sequences (numbered) and PVL (gi:78057570). Residues involved in carbohydrate and $Ca^{2+}$ binding in PVL are boxed in green and black respectively. Amino acids highlighted in red represent PAL residues not conserved in PVL.

## PAL carbohydrate specificity and preliminary X-ray crystallography

Mushroom lectins exhibit a broad specificity varying from simple sugars to complex saccharides and glycoproteins [6]. PAL showed strict specificity for GlcNAc among the saccharides tested, but also exhibited high binding affinity towards fetuin and mucin, which possess terminal Neu5Ac (Table 2). Similarly, the closely related PVL preferential binds free and oligosaccharides bearing non-reducing terminal GlcNAc structure [18] as well as terminal Neu5Ac residues on glycoproteins and oligosaccharides [19, 30].

As previously observed for the crude homogenate from *P. asperospora* [1], among the saccharides tested GlcNAc was the only capable of inhibiting the hemagglutination activity of purified PAL, with a MIC of 0.78 mM. Of particular interest was the lack of PAL hemagglutination inhibition exhibited by free Neu5Ac. The closely related PVL is known to bind free Neu5Ac in addition to GlcNAc, but only very weakly ($K_d < 10^{-3}$ M) [21]. However, PAL did show high binding affinity for mucin (MIC 0.002 mg/ml) and fetuin (MIC 0.0078 mg/ml), but not for asialofetuin even at 1 mg/ml concentration (Table 2), suggesting that sialoglycoconjugates may be a ligand for PAL.

**Table 2.** Inhibition of hemagglutination activity associated with purified PAL

| | Minimum inhibitory concentration (MIC) |
|---|---|
| **Saccharides*** | |
| Galactose | - |
| D-Glucose | - |
| GlcNAc | 0.78 mM |
| GalNAc | - |
| Lactose | - |
| D-Mannose | - |
| L-Arabinose | - |
| D-Ribose | - |
| D-Xylose | - |
| L-Fucose | - |
| D-Maltose | - |
| Neu5Ac# | - |
| Sucrose | - |
| **Glycoproteins**** | |
| á-acid glycoprotein | 0.125 mg/ml |
| Fetuin | 0.0078 mg/ ml |
| Asialofetuin | - |
| Mucin | 0.002 mg/ml |
| BSA | 0.5 mg/ml |
| Conalbumin | 1.0 mg/ml |
| Fibrinogen | 1.0 mg/ml |

* Saccharide concentration range from 0.2 to 50mM, # Maximum final concentration was 25mM, ** Glycoprotein concentration range from0.0005 to 1.0 mg/ml

To explore PAL's carbohydrate specificity in greater detail glycan array experiments were performed. PAL was labeled with Alexa Fluor® 647 succinimidyl ester and two different concentrations of PAL (50 and 100 ug/ml) directly applied to a glycan array slide that were printed as previously described [28]. Table 3 shows PAL binding to glycan structure present on the array, with strong binding (RFU of greater the 2500 coloured dark red) through to no binding (RFU less than 500 coloured white) indicated. Glycans shown in Table 3 are grouped as the function of their terminal residue, Gal, GlcNAc, Mannose, Fucose, Neu5Ac, and also include glycosaminoglycans (GAGs) and related structure. As expected, high binding of PAL to the glycans containing terminal non-reducing GlcNAc was observed. We showed previously that free Neu5Ac does not inhibit PAL hemagglutination activity but Neu5Ac-containing glycoconjugates (fetuin and mucin, see Table 2) do. The ability of PAL to bind glycosidically linked Neu5Ac was confirmed by glycan array analysis with strong binding observed to oligosaccharides containing Neu5Ac (Table 3). PAL binding was also found on GlcNAc containing terminal galactose structures. Among the 120 glycans, the GlcNAc and Neu5Ac containing sialylated oligosaccharide, Neu5Acá2-3Galâ1-3GlcNAcâ1-3Galâ1-4Glc, exhibited the highest binding with about 5000 RFUs. In addition, weak binding interactions of PAL can be observed in a number of GlcNAc containing mannosylated, fucosylated glycans, GAGs and other related structures. Similarly, another GlcNAc specific lectin, *Agrocybe aegerita* lectin-2 (AAL-2) showed high binding selectivity towards almost 30 glycans that possessed terminal non-reducing GlcNAc [29]. AAL-2 was not reported to bind to any sialic acid containing glycans, however, with no test performed on glycoproteins.

**Table 3.** PAL binding to glycan structures present on array

| Class | Glycan | ID |
|---|---|---|
| Terminal Galactose | Galβ1-3GlcNAc | 1A |
| | Galβ1-4GlcNAc | 1B |
| | Galβ1-4Gal | 1C |
| | Galβ1-6GlcNAc | 1D |
| | Galβ1-3GalNAc | 1E |
| | Galβ1-3GalNAcβ1-4Galβ1-4Glc | 1F |
| | Galβ1-3GlcNAcβ1-3Galβ1-4Glc | 1G |
| | Galβ1-4GlcNAcβ1-3Galβ1-4Glc | 1H |
| | Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc | 1I |
| | Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc | 1J |
| | Galα1-4Galβ1-4Glc | 1K |
| | GalNAcα1-O-Ser | 1L |
| | Galβ1-3GalNAcα1-O-Ser | 1M |
| | Galα1-3Gal | 1N |
| | Galα1-3Galβ1-4GlcNAc | 1O |
| | Galα1-3Galβ1-4Glc | 1P |
| | Galα1-3Galβ1-4Galα1-3Gal | 2A |
| | Galβ1-6Gal | 2B |
| | GalNAcβ1-3Gal | 2C |
| | GalNAcβ1-4Gal | 2D |
| | Galα1-4Galβ1-4GlcNAc | 2E |
| | GalNAcα1-3Galβ1-4Glc | 2F |
| | Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc | 2G |
| | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc | 2H |
| GlcNAc | GlcNAcβ1-4GlcNAc | 4A |
| | GlcNAcβ1-4GlcNAcβ1-4GlcNAc | 4B |
| | GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc | 4C |
| | GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc | 4D |
| | GlcNAcβ1-4MurNAc | 4E |
| Mannosylated | GlcNAcβ1-2Man | 5A |
| | GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Man | 5B |
| | Manα1-2Man | 5C |
| | Manα1-3Man | 5D |
| | Manα1-4Man | 5E |
| | Manα1-6Man | 5F |
| | Manα1-6(Manα1-3)Man | 5G |
| | Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man | 5H |
| Fucosylated | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc | 7A |
| | Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc | 7B |
| | Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc | 7C |
| | Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc | 7D |
| | Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc | 7E |
| | Fucα1-2Gal | 7F |
| | Fucα1-2Galβ1-4Glc | 7G |

| Category | Glycan | ID |
|---|---|---|
| Fucosylated | Galβ1-4(Fucα1-3)Glc | 7H |
| | Galβ1-4(Fucα1-3)GlcNAc | 7I |
| | Galβ1-3(Fucα1-4)GlcNAc | 7J |
| | GalNAcα1-3(Fucα1-2)Gal | 7K |
| | Fucα1-2Galβ1-4(Fucα1-3)Glc | 7L |
| | Galβ1-3(Fucα1-2)Gal | 7M |
| | Fucα1-2Galβ1-4(Fucα1-3)GlcNAc | 7N |
| | Fucα1-2Galβ1-3GlcNAc | 7O |
| | Fucα1-2Galβ1-3(Fucα1-4)GlcNAc | 7P |
| | SO3-3Galβ1-3(Fucα1-4)GlcNAc | 8A |
| | SO3-3Galβ1-4(Fucα1-3)GlcNAc | 8B |
| | Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc | 8C |
| | Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc | 8D |
| | Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-3GlcNAcβ1-3)Galβ1-4Glc | 8E |
| | Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3)Galβ1-4Glc | 8F |
| | Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc | 8G |
| | Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc | 8H |
| | Fucα1-3Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc | 8I |
| | Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3(Fucα1-2)Galβ1-4Glc | 8J |
| | Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc | 8K |
| | Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-4(Fucα1-3)GlcNAcβ1-3)Galβ1-4Glc | 8L |
| | Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc | 8M |
| | Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc | 8N |
| | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc | 8O |
| | GalNAcβ1-3(Fucα1-2)Galβ1-4Glc | 8P |
| | Galβ1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc | 9A |
| Sialylated | Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc | 10A |
| | Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc | 10B |
| | Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc | 10C |
| | Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc | 10D |
| | Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc | 10E |
| | Fucα1-2Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc | 10F |
| | Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc | 10G |
| | Neu5Acα2-6Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc | 10H |
| | Neu5Acα2-6Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc | 10I |
| | Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc | 10J |
| | Neu5Acα2-3Galβ1-4GlcNAc | 10K |
| | Neu5Acα2-6Galβ1-4GlcNAc | 10L |
| | Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc | 10M |
| | Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc | 10N |
| | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc | 10O |

| Group | Structure | Position |
|---|---|---|
| **Sialylated** | Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc | 10P |
| | Neu5Acα2-3Galβ1-4Glc | 11A |
| | Neu5Acα2-6Galβ1-4Glc | 11B |
| | (Neu5Acα2-8Neu5Ac)n | 11C |
| | (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)2Manβ1-4GlcNAcβ1-4GlcNAc-Asn | 11D |
| **GAGs and Related Structure** | Neocarratetraose-41, 3-di-*O*-sulphate (Na$^+$) | 12A |
| | Neocarratetraose-41-*O*-sulphate (Na$^+$) | 12B |
| | Neocarrahexaose-24,41, 3, 5-tetra-*O*-sulphate (Na$^+$) | 12C |
| | Neocarrahexaose-41, 3, 5-tri-*O*-sulphate (Na$^+$) | 12D |
| | Neocarraoctaose-41, 3, 5, 7-tetra-*O*-sulphate (Na$^+$) | 12E |
| | Neocarradecaose-41, 3, 5, 7, 9-penta-*O*-sulphate (Na$^+$) | 12F |
| | ΔUA-2S→GlcNS-6S Na$_4$ (I-S) | 12G |
| | ΔUA→GlucNS-6S Na3 (II-S) | 12H |
| | ΔUA→2S-GlcNS Na$_3$ (III-S) | 12I |
| | ΔUA→2S-GlcNAc-6S Na$_3$ (I-A) | 12J |
| | ΔUA→GlcNAc-6S Na$_2$ (II-A) | 12K |
| | ΔUA→2S-GlcNAc Na$_2$ (III-A) | 12L |
| | ΔUA→GlcNAc Na (IV-A) | 12M |
| | ΔUA→GalNAc-4S Na$_2$ (Δ Di-4S) | 12N |
| | ΔUA→GalNAc-6S Na$_2$ (Δ Di-6S) | 12O |
| | ΔUA→GalNAc-4S,6S Na$_3$ (Δ Di-disE) | 12P |
| | ΔUA→2S-GalNAc-4S Na$_2$ (Δ Di-disB | 13A |
| | ΔUA→2S-GalNAc-6S Na$_3$ (Δ Di-disD) | 13B |
| | ΔUA→2S-GalNAc-4S-6S Na$_4$ (Δ Di-tisS) | 13C |
| | ΔUA→2S-GalNAc-6S Na$_2$ (Δ Di-YA2S) | 13D |
| | ΔUA→GlcNAc Na (Δ Di-HA) | 13E |
| | (GlcAβ1-3GlcNAcb1-4)$_n$ (n=4) | 13F |
| | (GlcAβ1-3GlcNAcβ1-4)$_n$ (n=8) | 13G |
| | (GlcAβ1-3GlcNAcβ1-4)$_n$ (n=10) | 13H |
| | (GlcAβ1-3GlcNAcβ1-4)$_n$ (n=12) | 13I |
| | (GlcA/IdoAa/β1-4GlcNAcα1-4)$_n$ (n=200) | 13J |
| | (GlcA/IdoAa/β1-3(±4/6S)GalNAcβ1-4)$_n$ (n<250) | 13K |
| | ((±2S)GlcA/IdoAa/β1-3(±4S)GalNAcβ1-4)$_n$ (n<250) | 13L |
| | (GlcA/IdoAβ1-3(±6S)GalNAcβ1-4)$_n$ (n<250) | 13M |
| | HA-4 10 mM | 13N |
| | HA-6 10 mM | 13O |
| | HA-8 9.7 mM | 13P |
| | HA-10 7.83 mM | 14A |
| | HA-12 6.5 mM | 14B |
| | HA-14 5.6 mM | 14C |
| | HA-16 4.9 mM | 14D |
| | HA 30000 Da 2.5 mg/ml | 14E |
| | HA 107000 Da 2.5 mg/ml | 14F |
| | HA 190000 Da 2.5 mg/ml | 14G |
| | HA 222000 Da 2.5 mg/ml | 14H |
| | HA 1600000 Da 2.5 mg/ml | 14I |

>2500 RFU
1500-2500 RFU
500-1500 RFU
<500 RFU

Interestingly, PAL did not bind a number of GlcNAc-containing glycans, for instance the terminal galactose containing glycan, Galâ1-4GlcNAcâ1-6(Galâ1-4GlcNAcâ1-3)Galâ1-4Glc (Table 3). On the other hand, most mannosylated glycans on the array were bound by PAL (although at relatively low levels), this was not observed in our previous studies using free mannose in hemagglutination inhibition assay [1].

In preliminary crystallography experiments, PAL was co-crystallized in 2.4 M malonate, pH 5 with GlcNAc, with crystals being observed after 2 weeks. Fig 3 shows the PAL crystal that was used to obtain preliminary X-ray diffraction data to a resolution of 2.1 Å. Structure determination of PAL by molecular replacement using PVL as the search probe is currently underway.

**Figure 3.** Microscopic image of droplets of PAL crystal. Crystallization condition: 9 mg/ml PAL in 2.4 M malonate, pH 5 with 2.15mM GlcNAc

## CONCLUSION

Lectins are well known to possess cytotoxicity and/or anti-proliferative activity against cultured cells [6-8]. Our previous report described PAL as the first GlcNAc-specific mushroom lectin with potent ($IC_{50}$ 0.43 μM) anti-proliferative activity [2]. Given GlcNAc is known to be aberrantly expressed on the surface of cancer cells, our cytostatic highly selective GlcNAc specific PAL might have a potent application in cancer diagnosis or therapy. We have now successfully purified PAL on a large scale and have obtained more extensive carbohydrate specificity data using glycan array as well as generating PAL crystals that have diffracted to 2.1 Å.

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# ANTIOXIDANT AND FUNCTIONAL PROPERTIES OF β–GLUCAN EXTRACTED FROM EDIBLE MUSHROOMS *AGARICUS BISPORUS, PLEUROTUS OSTREATUS* AND *COPRINUS ATRAMENTARIUS*

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## ABSTRACT

β-glucan was extracted from three edible varieties of mushroom namely *Agaricus bisporus, Pleurotus ostreatus* and *Coprinus atramentarius,* using hot water extraction method and studies were carried out to investigate their structural, antioxidant and functional properties. The antioxidant activities were determined using different assays viz., DPPH (2,2-diphenyl-1-picryl-hydrazyl), reducing power, metal chelating ability and ABTS (2,2-Azino-bis,3- ethylbenzothiazoline-6-sulfonic acid). The FTIR was used to elicit the structural conformations of various β-glucans. The antioxidant activities varied significantly among all the sources of beta glucan, however, the beta glucan from *Coprinus atramentarius* showed highest values for DPPH (EC50=5.12±0.205), reducing power (3.75±0.195), chelating ability (2.89±0.256) and ABTS (3.5±0.503), where as β-glucan from *Pleurotus* showed the strongest lipid peroxidation inhibition (EC50 4.15±0.503) as compared to others. As far as the functional properties are concerned, *Coprinus* β-glucan also showed the highest swelling power, fat binding, foaming and emulsifying properties, however, all the functional properties of beta glucan varied significantly among all its sources. It was concluded that the β-glucan from *C. atramentarius* showed better antioxidant and functional properties as compared to β-glucan from *A. bisporus* and *P. ostreatus.*

**Keywords:** mushrooms, β-glucan, antioxidant, structure, functional properties

## INTRODUCTION

Human kind has been valued fungal sources, particularly mushrooms as an important edible and medical resource since times immemorial, and they are used as an attractive source for the development of drugs and nutraceuticals [1]. Mushroom belongs to a special group of macroscopic fungi. The antioxidant activity of mushroom extracts was found to be due to their polysaccharide content as well as to their total phenolic contents [2]. The free radical species produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes are causing a wide variety of pathological effects, such as DNA damage, carcinogenesis and cellular degeneration related to aging [3] and they also contribute to progressive decline of the immune system [4]. β-glucans obtained from mushrooms are recognized as biological response modifier (BRM) which are used for the treatment of cancer and various infectious diseases both in modern medicine and traditional chemotherapeutic drug [5]. Polysaccharides are potentially useful biologically active ingredients for pharmaceutical use, such as for immune regulation, for anti-radiation, anti-blood coagulation, anti-cancer, anti-HIV and hypoglycemic activities [6-7]. The mushroom derived polysaccharides lentinan, schizophyllan, and krestin have been accepted as immunoceuticals in Japan, Korea and China [8].

## MATERIALS AND METHODS

Wild and cultivated varieties of mushroom were procured from the Kashmir Valley. The cultivated varieties, namely *A. bisporus* and *P. ostreatus* were collected from Mushroom Research and Training Centre, Division of Plant Pathology, SKUAST-K, and the wild variety, namely *C. atramentarius* sample was randomly collected from the outskirts of Srinagar.

**Extraction of β-glucan from various mushrooms**

β-glucan was extracted from the various mushrooms following the method of Smiderle *et al*. [9]. Fresh fruiting bodies of *A. bisporus, P. ostreatus* and *C. atramentarius* were collected washed with distilled water, sliced and then dried in an oven at 30 ºC for 24 hours. The dried mushrooms were milled and submitted to successive cold and hot aqueous extraction, successively for 6h (×3:1,000 ml each).The cold extraction was performed aiming to separate other compounds, such as phenols, heteropolysaccharides, and glycogen. The hot aqueous extracts from each mushroom were evaporated to a small volume and the polysaccharides were precipitated by addition to excess ethanol (3:1;v/v) and centrifuged at 10,000 rpm, at 10 ºC, for 20 min. The sediment was dialyzed against distilled water for 24 (12-14 kDa cut off), concentrated under reduced pressure and freeze dried. The purification was performed by freeze thawing process [10]. The recovered fraction were dissolved in water and the solutions were submitted to freeze and thaw slowly untill complete separation of soluble and insoluble polysaccharides. The precipitates, obtained after centrifugation (10,000 rpm at 4 ºC, for 20 min), were treated with dimethyl sulfoxide (50 ml), for 2h, at 60 ºC, dialyzed against tap water for 24 and then resubmitted to the freeze-thawing process, giving rise to soluble fractions of β-D-glucans.

**Antioxidant activity assays**

**Assay for DPPH radical scavenging activity:** The radical scavenging activity of β-glucan was conducted by using the method previously carried out by Fu *et al*. [11]. The 0.1 mM solution of DPPH radical in ethanol was prepared and 2 ml of this solution was added to 2 ml of water solution containing different content of IOPS (1, 2, 4, 8, 10 mg). Briefly, the absorbance of solutions at 517 nm was measured using UV–vis spectrophotometer (UV-2450, Shmadzu, Japan). Vitamin C was used as the positive control. The DPPH radicals scavenging rate of sample was calculated as the following equation.

$$\text{Inhibition } \% = (A_{blank} - A_{sample})/A_{blank} \times 100\%$$

where $A_{sample}$ was the absorbance with sample and $A_{blank}$ was the absorbance without sample. Ascorbic acid was used as positive control.

**Ferric-reducing antioxidant power assay:** Reducing power was determined according to Oyaizu [12]. Each polysaccharide powder (0.1 to 20 mg/ml, 2.5 ml) in Milli-Q water was mixed with 2.5 ml, 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was vortexed and incubated at 50 ºC for 20 min. Then 2.5 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 2000g for 10 min. The upper layer (5 ml) was mixed with 5 ml of Milli-Q water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank using a UV/Vis spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. A higher absorbance indicates a higher reducing power. Ascorbic acid was used as the positive control.

**Chelating ability on ferrous ions:** Chelating ability was determined according to the method of Dinis, Madeira, and Almeida [13]. Each polysaccharide powder (0.1 to 20 mg/ml, 1 ml) in Milli-Q water was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against the blank. Blank was the solution with all reagents but without extract. A lower absorbance indicates a higher chelating ability. The EC50 value (mg extract/ ml) is the effective concentration at which ferrous ions were chelated by 50%. Citric acid and EDTA were used for comparison.

**ABTS assay :** For ABTS assay the procedure followed the method of Arnao *et al.* [14] with some modifications. The stock solutions included 7.4mM ABTSd+ solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS*+ solution with 60 ml methanol to obtain an absorbance of 1.170.02 units at 734 nm using the spectrophotometer. Fresh ABTSd+ solution was prepared for each assay. Fruit extracts (150 ml) were allowed to react with 2850 ml of the ABTS*+ solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer over the linear range of the standard curve.

**Functional properties of various fungal β-glucan**

Various functional properties of β-glucan are studied such as swelling power, fat binding capacity, emulsifying capacity and stability, foaming capacity and stability, bile acid binding capacity were studied.

**Swelling power:** The swelling power was determined according to the method described by Bae *et al.,* [15]. A mixture of 0.3 g of sample and 10 ml of distilled water was placed in a shaking water bath at 70 °C for 10 min, then transferred to a boiling water bath. After boiling for 10 min, the tubes were cooled with tap water for 5 min and centrifuged at 1700g for 4 min. Swelling power was expressed as the ratio of wet sediment weight to dry sample weight.

**Fat Binding Capacity:** *In-vitro* fat-binding capacity was determined according to the method reported by Lin and Humbert [16]. β-Glucan samples (0.2 g) were dispersed in soy oil (10 ml), and the mixtures were placed at room temperature ambient conditions for 1 h and agitated on a vortex mixer every 15 min. After centrifugation at 1600g for 20 min, the supernatant was decanted and the residue was weighed. The fat absorption was obtained from the amount of soy oil bound to 1 g of dry sample.

**Emulsifying properties:** 1% sample was homogenized with 5 ml of refined oil. The emulsions were then centrifuged at 1100 g for 5 min (5810, Eppendorf, Hamburg, Germany). Subsequently the height of the emulsified layer and the total contents in the tube were determined. The emulsion capacity was obtained through the following calculation.

Emulsion capacity (%) = (Height of the emulsified layer/ Total height of tube contents )× 100

Emulsion stability was evaluated by heating the emulsion for 30 min at 80 °C and centrifuging for 5 min at 1100 g.

Emulsion Stability (%) = (height of the emulsified layer after heating/ height of emulsified layer before heating) ×100

**Foaming capacity and Stability:** Aqueous dispersions (2% w/v db) of the sample were homogenized in a high speed homogenizer (Remi Instruments Division, Vasai, India) at 10,000 rpm for 1 min.

Foaming capacity was calculated as the per cent increase in volume of the sample dispersion. The foam stability was determined by measuring the foam volume with time and computing half-life.

Foaming capacity (%) = (volume after whipping – volume before whipping)/ ×100
(Volume before whipping)

Foam stability (%) = foam stability after standing time (60 min)/ ×100
Initial foam volume

## RESULTS

### Antioxidant assays

It was concluded from Fig. 1 and Table 1 that *Coprinus* β-glucan has the highest DPPH scavenging activity and reducing power as compared to *Agaricus* β-glucan and pleurotus β-glucan. The chelating ability and ABTS of *Pleurotus* β-glucan was more as compared to *Agaricus* β-glucan and *Coprinus* β-glucan. So far as the functional properties are concerned, *Coprinus* β-glucan has the highest swelling power, highest fat binding capacity, emulsifying properties and foaming properties.

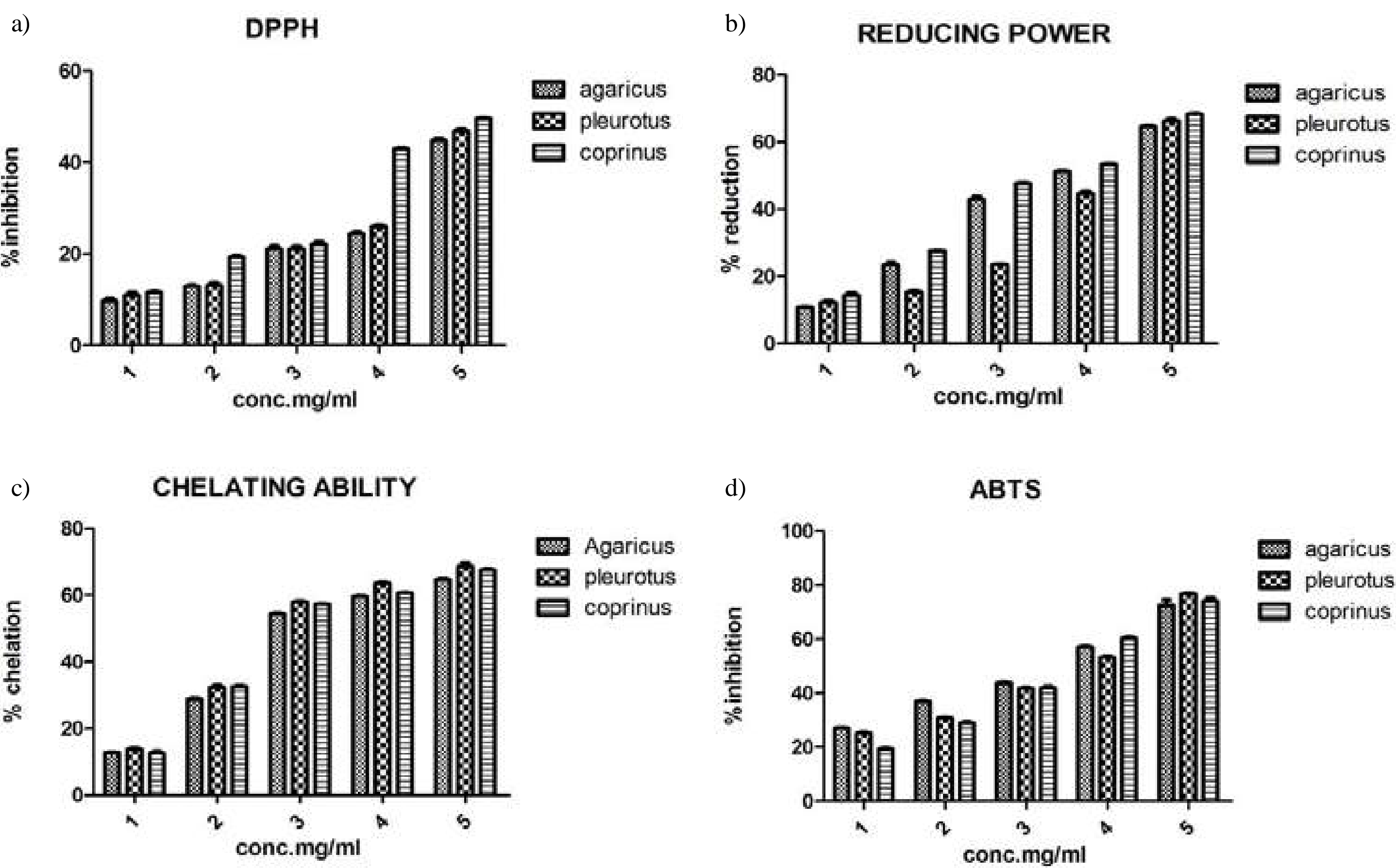


**Figure 1.** a) DPPH inhibition, b) reducing power, c) chelating ability and d) ABTS inhibition of *Agaricus, Pleurotus* and *Coprinus* sp.

**Table 1.** Functional properties of mushroom beta glucans

| Source | Swelling power. (g/g sample) | Fat binding capacity. (g oil/g sample) | Emulsion capacity (%) | Emulsion stability. (%) | Foaming capacity. (%) | Foaming stability. (%) |
|---|---|---|---|---|---|---|
| *Agaricus* | 3.45 ±0.19 | 5.38±0.27 | 64.26±0.01 | 94.64±0.96 | 9.80±0.04 | 6.06±1.27 |
| *Pleurotus* | 3.74±0.21 | 5.53±0.32 | 65.35±0.03 | 96.73±0.02 | 10.20±0.36 | 9.16±1.44 |
| *Coprinus* | 4.59±0.10 | 6.65±0.35 | 65.47±0.08 | 97.68±0.20 | 9.93±1.46 | 8.33±1.44 |

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# QUALITATIVE PHYTOCHEMICAL SCREENING, TOTAL PHENOLIC CONTENT AND *IN-VITRO* ANTIOXIDANT ACTIVITY IN METHANOLIC EXTRACTS OF *MORCHELLA ESCULENTA Fr.*

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## ABSTRACT

Among wild edible species of mushrooms, morels rank first in choice and delicacy and have been one of the highly prized wild edible mushrooms in the world. The bioactive components present in *Morchella esculenta* are responsible for the nutraceutical potential of the mushroom. The present study was carried out to assess the phytochemical screening, total phenolics and antioxidant activities of methanolic extracts of *M. esculenta*. Qualitative phytochemical analysis showed the presence of alkaloids, anthraquinone, anthocyanins, tannins, saponins, glycosides, flavonoids, terpenoids, phenols, carbohydrates as well as proteins and amino acids, only steroids are absent. The total phenolic content of methanolic extracts of morel was 238.52±0.021 (mg gallic acid equivalents per gram weight). The *in-vitro* antioxidant potential was analyzed by DPPH and hydrogen peroxide method. The DPPH scavenging activity was 85.2±0.371 % and peroxide was 84.1±0.281 % at 500 μg/ml concentration, comparable to that of ascorbic acid.

**Keywords:** morels, phytochemical screening, DPPH, hydrogen peroxide, antioxidant potential

## INTRODUCTION

Human beings have been constantly searching for food sources that can improve biological functions and make people healthier, fitter, and to live longer. Mushrooms are the fungi that have been used as food since times immemorial [1].Wild edible mushrooms fall under the category of non- timber forest products (NTFP) which have been untapped resources because a wide variety of wild mushrooms are still unexplored [2].Wild edible mushrooms have long history of medicinal usages in addition to their nutritional value and have a valuable source of biologically active compounds [3, 4]. From India, many species of edible fungi have been reported to be traditionally and regularly consumed by the local inhabitants without causality and fatality. Wild edible mushrooms having very good commercial value are species of *Morchella, Helvella, Hericium, Sparassis, Hydnum, Trapeinda, Clavaria, Ramaria, Boletus, Albatrellus, Cordyceps, Lactarius* and *Rusulla* etc. [5]. All these wild edible mushrooms are consumed as well as sold fresh, collected and dried for sale [6,7].

In the North-West Himalayan region of India many species of mushrooms are collected and consumed. Among the wild edible varieties of mushrooms, the morels (*Morchella* spp.) have been consumed the most because of the indigenous nutritional and health benefits. Morels are among the most highly prized of all the wild harvested mushrooms, and commonly called as 'Guchhi' in the Indian market. This mushroom is collected from the wild and is exported to many countries for its excellent culinary properties [9-12]. In North West Himalayas, seven different species of *Morchella* have been analyzed for their nutritional and nutraceutical potential [7].

In the recent years, much attention has been paid to the investigation of nutraceuticals from various edible mushrooms [13]. Although the nutritional facts, culinary and indigenous medicinal uses of morels are well accepted all over the world, but the medicinal qualities have yet to make into the mainstream. *M. esculenta* is one of the dominantly found morel species in North –West Himalayas, and the ethnobotanical data gathered also reveals that this mushroom species has great nutritional and indigenous health benefits. The present study evaluates the quantitative phytochemicals, total phenolic content, *in-vitro* antioxidant activity in methanolic extracts of *M. esculenta;* so that it's unexplored nutraceutical potential can further be exploited.

## MATERIAL AND METHODS

### Sample collection

The fresh mushroom species were collected from the Northwest Himalayan region of Shimla, India (31º 6' 12" N; 77º 10' 20" E). The fruiting bodies were thoroughly cleaned of extraneous matter and dried completely and coarsely grounded.

### Preparation of extracts

Grounded mushroom was extracted with solvent - methanol at room temperature prior to removal of solvent. 10 grams of the ground sample was mixed with six times of 99.6% methanol and kept for 24 hours. This process was repeated thrice and filtrates were collected. The filtrates obtained were concentrated under vacuum on a rotary evaporator (Buchi Rotary Evaporator, Model R-124) and stored at 4 °C for further use [14].

### Quantitative Phytochemical screening

Methanolic extracts of *M. esculenta were* used for qualitative screening of phytochemicals as per standard biochemical procedures. The preliminary tests for methanol extracts were performed to confirm the presence of alkaloids, anthraquinones, anthocyanins, carbohydrates, flavonoids, glycosides, phenols, proteins and amino acids, saponins, steroids, tannins and terpenoids [15].

### Estimation of Total Phenolic content

The total phenolic content in methanolic extracts of grounded sample was estimated by Folin-Ciocalteu reagent, as described by Singleton and Rossi [16]. 100 mg of gallic acid was dissolved in 100 ml ethanol to prepare Gallic acid stock solution (1000 µg/ml). Various dilutions of standard gallic acid were prepared from this stock solution. 1 ml aliquots of 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/ml of gallic acid solution were mixed with 5.0 ml of Folin-Ciocalteu reagent (diluted ten-fold) and 4.0 ml of sodium carbonate solution (75 g/l) and calibration curve was plotted. The absorbance was measured after 30 min at 20 °C at 765 nm. 1 ml extract was mixed separately with the same reagents and absorbance was measured at 765 nm after 1 hour. The total phenolic compound in the extract was determined using the formula:

$$C = C_1 \times V/m$$

C= Total content of phenolic compounds in mg/g in GAE (Gallic acid equivalent); $C_1$= The concentration of gallic acid established from the standard curve in mg/ml; V=The volume of extract in ml, m =Weight of extract in grams.

### *In-vitro* evaluation of antioxidant activity

*In-vitro* antioxidant activity of the extract of *M. esculenta* was determined by using two different methods: DPPH and hydrogen peroxide radical smethods.

**Free radical scavenging activity using DPPH method:** The free radical scavenging activity of extracts were measured by 1, 1-dipheny l-2-picrylhydrazyl (DPPH) [17]. 0.1 mM solution of DPPH in ethanol was prepared and 1.5 ml of this solution was added to 0.5 ml of extract solution in ethanol at different concentrations (50-300 µl/ml). The mixture was shaken and allowed to stand at room temperature for 30 minutes. The absorbance was then measured at 517 nm using a spectrophotometer (UV- VIS, Systronics). A blank without DPPH was used to remove the influence of the color of the extracts and an ethanolic solution of DPPH was used as a negative control. Ascorbic acid was used as a reference. All of the measures were carried out in triplicates. The DPPH radical scavenging activity was calculated using the following equation:

$$DPPH\ scavenging\ effect\ (\%) = \frac{Ao - As}{Ao} \times 100$$

where, $A_o$ is the absorbance of negative control, $A_s$ is the absorbance of sample respectively.

**Free radical scavenging activity using hydrogen peroxide:** The free radical scavenging activity of different extracts was determined by using hydrogen peroxide radical [18]. An aliquot of 0.6 ml of hydrogen peroxide (43Mm) and 1.0 ml of various concentrations of extracts prepared using phosphate buffer (200-400 μg/ml) were mixed followed by 2.4 ml of 0.1 M phosphate buffer (pH 7.4). The resulting solution was kept for 10 minutes and the absorbance was recorded at 230 nm. All measures were taken in triplicates. For each concentration, mixture without sample was taken as a control and a mixture without hydrogen peroxide was taken as a blank. Ascorbic acid was used as a standard compound. The percentage scavenging activity of hydrogen peroxide was calculated as:

$$Scavenging\ activity\ (\%) = \frac{Ao - As}{As} \times 100$$

where, $A_o$ is the absorbance of negative control, $A_s$ is the absorbance of sample respectively.

## Statistical Analysis

All of the measurements were performed in triplicates. The data is given either as ±SD or mean ± S.E.M.

## RESULTS AND DISCUSSION

Qualitative phytochemical analysis of *M. esculenta* extracts showed the presence of alkaloids, anthraquinone, glycosides, tannins, saponins, flavonoids, terpenoids, phenolics, carbohydrates, anthocyanins as well as proteins and amino acids and steroids were found (Table 1). The results revealed that *M. esculenta* may be used as potential sources of phytochemicals and thus can be used for designing drugs that can prove to be of keen interest in the treatment and prevention of diseases like cancer, tumor, heart diseases, etc.Very less work has been done on the phytochemical screening of morels. Duyilemi and Lawal [19] investigated the antibacterial activity and phytochemical screening of *Chrysophyllum albidum* leaves. Egwim *et al.* [20] studied the proximate composition, phytochemical screening and antioxidant activity of ten selected wild edible Nigerian mushrooms. Johnsy and Kaviyarasan [21] studied the preliminary phytochemical screening, antimicrobial and antioxidant activity of methanolic and aqueous extracts of *Lentinus sajor- caju*.

**Table 1:** Qualitative phytochemical screening of *M. esculenta* methanolic extracts

| S. No. | Phytochemical | *M. esculenta* extracts |
|---|---|---|
| 1 | Alkaloids | + |
| 2 | Anthraquinone | + |
| 3 | Anthocyanins | + |
| 4 | Proteins and amino acids | + |
| 5 | Carbohydrates | + |
| 6 | Phenols | + |
| 7 | Terpenoids | + |
| 8 | Sterols | - |
| 9 | Saponins | + |
| 10 | Glycosides | + |
| 11 | Flavonoids | + |
| 12 | Tannins | + |

**NOTE:** **(+)**: Shows the presence of phytochemicals; **(-)**: Shows the absence of phytochemical

The amount of total phenols was determined with Folin- Ciocalteu reagent. Gallic acid was used as standard compound. The standard curve of gallic acid concentrations and absorbance is shown in Fig. 1. The absorbance for various dilutions of gallic acid with Folin- Ciocalteu reagent and sodium carbonate were found.The total phenolic content of methanol extracts of *M. esculenta* was 238.52±0.0012 with absorbance at 765 nm was 2.59±0.0012 (mg gallic acid equivalents per gram weight). Data expressed as mean ± standard error of three samples analyzed separately.

**Figure 1.** Calibration curve of gallic acid

Antioxidant activity of sample extract at different concentrations was determined using two different techniques viz. DPPH method and $H_2O_2$ method. The DPPH is a stable organic free radical with an absorption maximum band around 515-528 nm [22] and is widely used for evaluation of antioxidant potential of compounds. Results are presented in Table 2 and Table 3. The results obtained from both the methods revealed that *M. esculenta* exhibits high antioxidant activity. At the concentration of 500µg/ml, the extracts showed 85.20% and 84.12% scavenging activity by DPPH and hydrogen peroxide method respectively. The methanolic extracts of *M. esculenta* showed good antioxidant activity as evaluated by both the methods.

**Table 2.** Percentage scavenging activity of the *M. esculenta* methanolic extracts using 1,1-diphenyl-2-picrylhydrazyl radical

| Concentration (µg/ml) | Percent scavenging activityof DPPH radical | |
|---|---|---|
| | Ascorbic acid | *Morchella* extract |
| 100 | 48.08 ± 0.136 | 32.33 ± 0.3142 |
| 200 | 58.59 ± 0.2423 | 32.95 ± 0.4307 |
| 300 | 75.45 ± 0.3153 | 56.88 ± 0.3142 |
| 400 | 76.89 ± 0.3579 | 76.89 ± 0.4307 |
| 500 | 87.89 ± 0.3724 | 85.20 ±0.3710 |

Values are the average of triplicate experiments and represented as mean ±S.E.M

**Table 4.** Percent scavenging activity of the *M. esculenta* methanolic extracts using hydrogen peroxide radical

| Concentration of extract (μg/ml) | Percent scavenging activity of $H_2O_2$ | |
|---|---|---|
| | **Ascorbic acid** | **Morchella extract** |
| 100 | 51.56 ± 0.1453 | 55.95 ± 0.0233 |
| 200 | 61.19 ± 0.5008 | 60.23 ± 0.0726 |
| 300 | 77.15 ± 0.0145 | 74.42 ± 0.0176 |
| 400 | 78.80 ± 0.1152 | 77.72 ± 0.0240 |
| 500 | 87.46± 0.1152 | 84.12 ±0.2810 |

Values are the average of triplicate experiments and represented as mean ±S.E.M

Various other scientists have also worked on the antioxidant activity of morels. Mau *et al*. [23] and Ferreira *et al*. [24] investigated the antioxidant properties of several wild and medicinal mushrooms. Mau *et al*. [25] investigated the antioxidant properties of ethanolic extracts from *Grifola frondosa, M. esculenta* and *T. albuminosus* mycelia. Gursoy *et al*. [26] studied the Antioxidant activities, metal contents, total phenolics and flavonoids of seven *Morchella* species. Wagay [27] studied the phenolic quantification and anti-oxidant activity of *M. esculenta*. Hamzah *et al.* [28] studied the phytochemical screening and antioxidant activity of methanolic extract of selected wild edible Nigerian mushrooms.

## CONCLUSION

The present finding highlights that consumption of diets rich in wild edible mushrooms is associated with various health benefits. The mushroom species due to presence of phenolic compounds have antioxidant activities and delay the oxidative damage in the human body. Arising from the awareness of the relationship between diet and disease has evolved the concept of nutraceuticals. In essence, mushrooms nutraceuticals are foods that are eaten not only to satisfy functional dietary needs but also elicit additional health needs.

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# SEM STUDY OF EXOSPORIAL ORNAMENTATION OF BASIDIOSPORES INGENUS *RUSSULA*, *LACTARIUS* AND *LACTIFLUUS* FROM NORTH-WEST HIMALAYA

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## ABSTRACT

Morphology of basidiospore surface in Basidiomycetes is used as an important taxonomic character. Different types of ornamentations exist in basidiospores of Russulaceous mushrooms. Every species has its unique ornamentation and is used as key character to separate some closely aligned taxa in genus *Lactarius*, *Lactifluus* and *Russula*. To study the spore surface diversity Scanning Electron Microscopy (SEM) studies were conducted where basidiospores were mounted on a double sided adhesive tape pasted on a metallic specimen-stub and after gold plating, the material was scanned at different magnification in high vacuum mode to observe pattern of spore ornamentation. SEM studies were carried out with JSM6610LV GEOL scanning electron microscope in 51 taxa of russulaceous mushrooms collected from North-West Himalaya spread over three investigated genera (*Lactarius, Lactifluus* and *Russula*). On the basis of SEM studies spores with eight varied ornamentation type on their surface were documented. These are: tuberculate type (warts completely isolated with no inter- connections); catenulate type (isolated warts with inter connections forming chain like rows or catenulations); winged type A (broad wings around the surface with isolated warts not forming reticulum (scattered or loose arrangement of wings); winged type B (winged all around the surface forming incomplete reticulum (compact arrangement of wings); incomplete reticulate type (wart to wart connections forming reticulum but isolated warts also exist in between); complete reticulate type (no isolated warts present), ridged type (when only 2-4 warts connected forming small to large ridges on the surface, no complete wing like structures formed. Isolated warts and catenulations) and rugulose type (very low warted spores).

**Keywords:** russulaceous, ornamentation, basidiospores

## INTRODUCTION

In 1924, Melzer for the first time discussed the importance of spore ornamentation [1].The study of structures of basidiospores is a primary requirement for the identification and classification of fungal species [2]. Scanning electron microscopy is a valuable technique for studying spore surface. Spore ornamentation is one of the important taxonomic characters for distinguishing species within *Russula, Lactifluus* and *Lactarius*. Josserand in 1940 gave a view that pattern is a molded structure inherent in spore itself [3]. Burge studied the spores in four *Russula s*pecies using SEM [4]. Hesler and Smith studied the spore ornamentation in 59 taxa of North American *Lactarius* and described 5 basic types of ornamentation on the basis of SEM [5]. Singer recognized 12 types of exosporial ornamentations in order Agaricales [6].

## MATERIALS AND METHODS

Fungal forays were undertaken to various localities of North West Himalayas between 2008- 2013 for the collection of russulaceous mushrooms. Spore prints were obtained from the fresh specimens. For scanning electron microscopic examination, the basidiospores were obtained from dry spores from the spore print that were directly mounted on a double sided adhesive tape pasted on a metallic specimen-stub and then spattered with gold coating. These gold plated spores were observed at different magnifications in high vacuum mode to observe pattern of spore ornamentation. SEM studies were carried out with JSM6610LV GEOL scanning electron microscope.

## RESULTS AND DISCUSSION

In the present work SEM studies of 51 taxa of Russulaceous mushrooms spread over three genera (Table 1, 2, 3) were undertaken. On the basis of SEM studies undertaken, following eight ornamentation types have been found to occur in Russulaceous mushrooms. (Figure 1)

1. **Tuberculate type:** Warts completely isolated with no inter connections

2. **Catenulate type:** Isolated warts with inter connections forming chain like rows or catenulations

3. **Winged type A:** Broad wings around the surface with isolated warts not forming reticulum (scattered or loose arrangement of wings)

4. **Winged type B**: Winged all around the surface forming incomplete reticulum (Compact arrangement of wings)

**Table 1.** Basidiospore Ornamentation Types in Genus *Lactifluus*

| | *Lactifluus* | Subgenus | Ornamentation type |
|---|---|---|---|
| *1.* | *Lf. volemus* var. *volemus* | *Lactifluus* | Complete Reticulum |
| *2.* | *Lf. sainii*sp. nov. | “ | Rugulose |
| *3.* | *Lf. luteolus* var. *luteolus* | “ | Catenulate |
| *4.* | *Lf. echinatus* | “ | Winged type A |
| *5.* | *Lf. echinatus* var. *versicuticularis* var. nov | “ | Winged type A |
| *6.* | *Lf. angustifolius* | “ | Rugulose |
| *7.* | *Lf. piperatus* | “ | Rugulose |

**Table 2.** Basidiospore Ornamentation Types in Genus *Lactarius*

| | *Lactarius* | Subgenus | Ornamentation type |
|---|---|---|---|
| *1.* | *L. subindigo* | *Piperites* | Incomplete reticulate |
| *2.* | *L. salmoneus*var.*curtisii* | “ | Incomplete reticulate |
| *3.* | *L. rubrilacteus* | “ | Winged type B |
| *4.* | *L. rubrifluus* | “ | Incomplete reticulate |
| *5.* | *L. deliciosus*var. *areolatus* | “ | Incomplete reticulate |
| *6.* | *L. deterrimus* | “ | Incomplete reticulate |
| *7.* | *L. sanguifluus* | “ | Incomplete reticulate |
| *8.* | *L. sanguifluus var. excentristipus* | “ | Incomplete reticulate |
| *9.* | *L. pubescens* | “ | Winged type B |
| *10.* | *L. yazooensis* | “ | Incomplete reticulate |
| *11.* | *L. marginozonatus*sp.nov. | “ | Incomplete reticulum |
| *12.* | *L. eburneus* var. *eburneus* | *Plinthogalus* | Incomplete reticulate |
| *13.* | *L. westii*var.*microsporus*var. nov. | “ | Winged type B |
| *14.* | *L. subisabellinus*var.*subisabelllinus* | “ | Incomplete reticulate |
| *15.* | *L. subdulcis*var. *hesleri*ivar. nov. | *Russularia* | Winged type B |
| *16.* | *L. aqueous* sp. nov. | “ | Winged type B |
| *17.* | *L. badiopallenscens* | “ | Incomplete reticulate |
| *18.* | *L. mukteshwaricus* | “ | Winged type A |

**Table 3.** Basidiospore Ornamentation Types in Genus *Russula*

| | *Russula* | Subgenus | Ornamentation type |
|---|---|---|---|
| 1. | *R. brevipes*var. *acrior* | *Compactae* | Incomplete reticulate |
| 2. | *R. romagnesiana*var.*acrida*var. nov. | " | Catenulate |
| 3. | *R. illota* | *Ingratula* | Catenulate |
| 4. | *R. pectinatoides* | " | Catenulate |
| 5. | *R. fertiilandica* var. nov. | " | Incomplete reticulate |
| 6. | *R. hetero-cutica*sp. nov. | *Heterophyllidia* | Ridged |
| 7. | *R. virescens* | " | Rugulose |
| 8. | *R. grisea* | " | Incomplete reticulum |
| 9. | *R. heterophylla* | " | Catenulate |
| 10. | *R. cyanoxantha*var.*variata* | " | Rugulose |
| 11. | *R. crustosa* | " | Incomplete reticulate |
| 12. | *R. mustelina* | " | Incomplete to complete reticulate |
| 13. | *R. pleurocystidia-innumerosa*sp. nov. | " | Catenulate |
| 14. | *R. cyanoxantha*var.*cynaxantha* | " | Catenulate |
| 15. | *R. vesca* | " | Catenulate |
| 16. | *R.cremeoavellanea* | *Incrustatula* | Catenulate |
| 17. | *R. olivacea* | " | Ridged |
| 18. | *R. rosea* | " | Catenulate |
| 19. | *R. lilacea* | " | Catenulate |
| 20. | *R. smaragdina* | *Russula* | Catenulate |
| 21. | *R. aquosa* | " | Ridged |
| 22. | *R. persicina* | " | Catenulate |
| 23. | *R. paludosa* | " | Catenulate |
| 24. | *R. peckii* | " | Tuberculate |
| 25. | *R. brunneoviolacea* | " | Winged type A |
| 26. | *R. curtipes* | " | Catenulate |

5. **Incomplete reticulate type**: Wart to wart connections forming reticulum but isolated warts also exist in between

6. **Complete reticulate type:** No isolated warts present

7. **Ridged type:** When only 2-4 warts connected, forming small to large ridges on the surface, no complete wing like structures formed. Isolated warts and catenulations prominent

8. **Rugulose type**: Very low warted spores

## CONCLUSION

Surface details of spores is a very important and useful tool in separating some closely aligned taxa. Amongst the various ornamentation type investigated Incomplete reticulate and Catenulate types are more common whereas Tuberculate type are least represented.

## ACKNOWLEDGEMENTS

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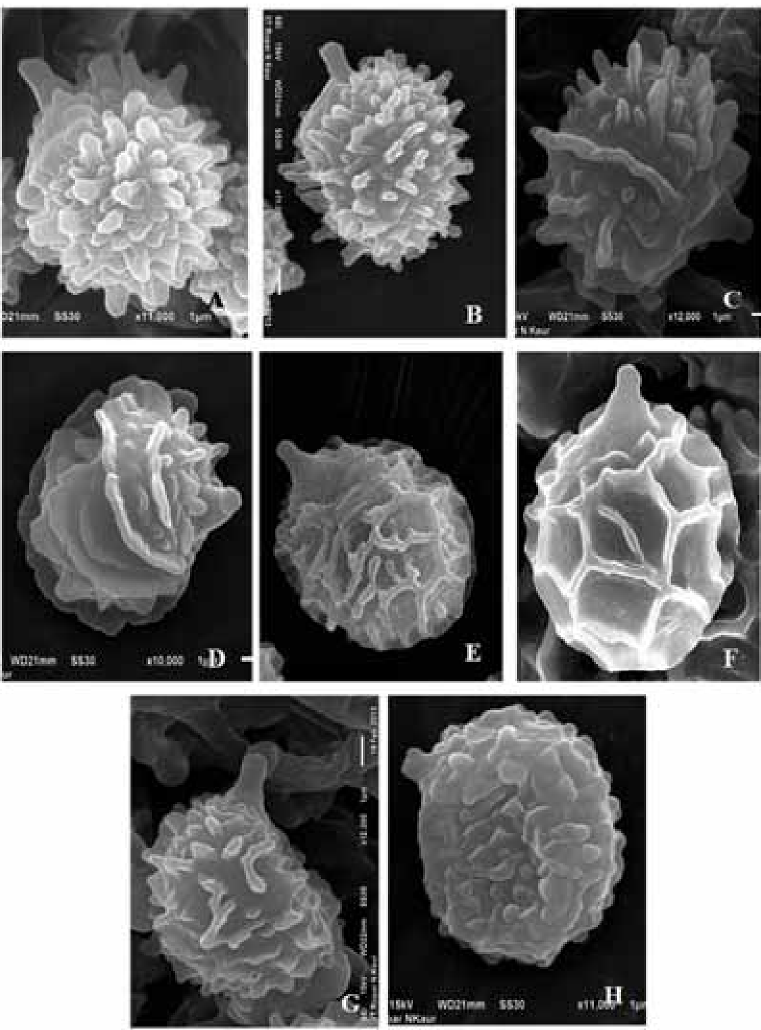

**Figure 1.** SEM Types: **A**. Tuberculate Type (*Russula peckii*), **B**. Catenulate Type (*R. cremeoavellanea*), **C.** Winged Type A (*Lactarius echinatus*), **D.** Winged Type B (*L. westii*), **E.** Incomplete Reticulate Type (*L. sanguifluus*), **F.** Complete Reticulate Type (*L. volemus*), G. Ridged Type (*R. aquosa*), **H.** Rugulose Type (*Lf. piperatus*)

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# LIGNOLYTIC ENZYMES OF *CALOCYBE INDICA*

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## ABSTRACT

*Calocybe indica* which is gaining popularity in the tropical parts of India and cultivated round the year while in subtropical parts it is preferred during summer seasons. The fruiting temperature requirement is from 28 $^0$C to 35 $^0$C. It is cultivated on wheat or paddy straw after chemical sterilization, hot water treatment or on pasteurized straw. The mycelial growth and fruiting takes place at higher temperature than the other cultivated mushroom like white button mushroom, shiitake mushroom or oyster mushroom. The lignocellulolytic enzymes responsible for growth on substrate and secretion of enzymes at higher temperatures of *C. indica* have not been thoroughly studied. So studies were undertaken to estimate influence of temperature on biomass production, changes in pH, total protein production, laccase activity, tyrosinase activity, aryl alcohol oxidase (AAO), manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) on wheat straw medium, saborauds medium and modified Czapek dox medium (10g/l glucose) after 6, 12, 18, 24 days. It was interesting to record that *C. indica* produced Versatile peroxidase on all three culture medium and maximum quantity was recorded on wheat straw media after 12 days at 35 $^0$C. Maximum protein was recorded on straw medium and least in Czapek dox medium. Wheat straw medium produced maximum laccase after 24 days at 30 $^0$C (187.38 u/ml) followed by 35 $^0$C and 25 $^0$C. Maximum biomass was recorded on saborauds medium at 300 $^0$C and 350 $^0$C after 24 days followed by wheat straw and Czapek dox medium. This is the first report of versatile peroxidase in *C. indica* in addition to *Pleurotus eryngii, P. pulmonarius, P .ostreatus*, *Bjerkandera* adusta and *Bjerkandera* sp. In all *Pleurotus* spp and Bjerkandera the production of VP is stimulated under peptone containing media. In the present case the wheat straw produced maximum VP followed by Sabourauds medium (peptone containing medium) and least in Czapeck dox medium.

**Keywords**: *Calocybe indica*, ligninolytic enzymes, oxidases, peroxidases, laccase, manganese peroxidase, lignin peroxidase, versatile peroxidase

## INTRODUCTION

*Calocybe indica* (milky mushroom) is an indigenous edible mushroom belonging to Lyophyllae of the family Trichlomataceae. This mushroom is identified on the bases of cyanophilic basidiospores and siderophyllous granules in basidia. It is found during rainy season in the indogangetic plains of the West Bengal (India) and in Bangladesh [1]. This mushroom becoming popular in tropical and subtropical parts of India due to its robust basidiomes, fibrous texture, attractive white colour, suitable for cultivation during warmer seasons at higher temperature, consistent yield and better keeping quality are the major reason for its popularity. Nutritionally also it has higher amounts of $B_{2,}$ niacin and rich in minerals like Na, K, P [2]. The fruit bodies contains eighteen fatty acids including essential fatty acids like eicosapentaenoic and docasohexaenoic acids [3]. The substrate requirement for *Calocybe indica* is also like *Pleurotus* spp. and various kinds of agricultural wastes including wheat straw, paddy straw, ragi maize, bajra, cotton stalks and leaves. Sugar cane bagasse, cotton and jute wastes, tea and coffee waste, dehulled maize cobs and coconut coir substrate can be utilized for cultivation of *Calocybe indica* [4, 5]. There is no need to compost the substrate for cultivation of *Calocybe indica* as the mycelium can degrade cellulose, hemicellulose and lignin by secretion of various extracellular enzymes. Doshi *et al*. [6] have reported pectinolytic and cellulolytic enzyme production from the fruit bodies of the *Calocybe indica*. However, no information is available on the ligninolytic oxidases and peroxidases enzyme, change in protein, biomass and role of temperature during incubation for enzyme secretion. Therefore studies were undertaken to estimate various ligninolytic enzymes of *Calocybe indica* in liquid culture medium inoculated at three different temperature conditions.

## MATERIALS AND METHODS

**Culture**: Culture of *C. indica* (DMRO- 38) was obtained from the "Gene Bank" of ICAR - Directorate of Mushroom Research, Solan.

**Liquid broth medium for growth and enzyme studies**:- Three culture media namely, wheat straw extract (50 g/l), Saborauds medium and Czapek dox medium (modified- 10g/l glucose instead of 30 g/l sucrose.) were prepared and 25 ml medium was transferred in 150 ml conical flasks. Each medium has 48 flasks and 16 flasks were incubated at 25, 30, 35 $^0$C in BOD under dark. Four samples were taken after 6, 12, 18 and 24 days for enzyme analysis.

**Biomass, pH and protein estimation**: Mycelial biomass was quantified on pre weighed Whatmann filter paper (No.1) by taking out culture filtrate from mycelium and washing twice with sterilized distilled water. The filter papers were oven dried (50 $^0$C) to a constant weight. The culture filtrate was used to estimate protein and change in pH and enzyme activity from each medium under different incubation temperature. Protein was estimated by following the method of Bradford [7] using bovine serum albumin (BSA) as protein standard. The enzyme samples were drawn using sterile pipette tips in 2ml sterilized micro centrifuge tubes. The enzymes samples were stored at ±4 $^0$C for enzyme estimation.

**Enzyme estimation**:- All the enzymes and protein samples were estimated using Perkin Elmer (Lambda-12) UV-VIS. Double beam spectrophotometer. One International Unit (U) of enzyme activity was defined as the activity that would change 1uMol of substrate or synthesize 1µM of product per minute. Enzyme activity divided by per mg protein gave enzyme activity per mg of protein. Laccase(p-diphenol oxidase, EC1.10.3.2): Laccase was estimated by following the method of Prilliger modified by Haars *et al*. [8]. Tyrosinase: (o-diphenol oxidase, EC1.14.18.1): The tyrosinase activity was measured with L-DOPA (3,4- dihydroxyphenylalanine) as the substrate [9]. Aryl alcohol oxidase: (AAO, EC1.1.3.7): AAO activity oxidizes primary aromatic alcohols like benzyl, cinnamyle, naphthyl and aliphatic unsaturated alcohols to corresponding aldehydes by transfer of two hydrogen from alcohol to molecular oxygen with the release of hydrogen peroxide. 4-methoxy benzyl alcohol (anis alcohol) was used as substrate. The enzyme activity was measured as per the method of Muheim *et al*. [10]. Lignin peroxidise: (LiP, EC1.11.1.14): The method of Tien and Kirk (11) was followed for the lignin peroxidase activity. Veratryl alcohol (3,4- dimethoxy benzyl alcohol) was used as substrate.Versatile peroxidise: (VP, EC 1.11.1.16): The VP activity was measured by decrease in absorbance of reactive Black-5 (Sigma) at acidic pH in sodium tartarat buffer. This enzyme has the capacity to oxidize $Mn^{2+}$ and different aromatic compounds and dyes, including compounds that are not efficiently oxidized by LiP and MnP.

## RESULTS AND DISCUSSION

The effect of temperature and pH on mycelial biomass with different culture media is shown in Table-1. There was less reduction in pH in Czapek dox medium and the pH remained almost neutral to slightly alkaline, while in Saborauds medium the medium pH becomes acidic in all the three temperature conditions. In wheat straw medium the pH slowly decreased upto 12 days and then again increased to neutral.

Maximum mycelial biomass was recorded in Saborauds medium at 30 and 35 $^0$C (17.36 and 17.03 mg/ml) followed by 25 $^0$C after 24 days. Least mycelial biomass (2.75 mg/ml) was recorded after 6 days in Czapek Dox medium. The effect of temperature on extra cellular protein is shown in Table 2. Wheat straw medium supported maximum protein (16.34 µg/ml) after 24 days at 30 $^0$C followed by 25 $^0$C and 35 $^0$C(14.50 and 14.43 mg/ml).. Saborauds medium gave 8.86 µg/ml protein after 24 Days. Least protein was found in Czapek dox medium.

The laccase activity in different culture medium and at different temperatures is shown in Table 3. There was vast difference in laccase activity in all three culture medium. Maximum laccase activity (183.38U/ml) was observed in wheat straw medium after 24 days at 30 $^0$C. While maximum activity per unit protein was in Czapek dox at all the three temperatures after 24 days. Although Saborauds medium has maximum biomass and reasonable amount of protein /ml. but it gave poorest laccase/unit ml and per unit protein concentration.

**Table 1.** Change in pH and mycelial biomass (mg/ml) in different culture media

| | pH | | | | Biomass(mg/ml) | | |
|---|---|---|---|---|---|---|---|
| **Day Interval** | **Temp.** | **Wheat st.** | **Sab.** | **Cza. Dox** | **Wheat st.** | **Sab.** | **Cza. Dox** |
| **6 Days** | 25°C | 5.65 | 5.35 | 6.74 | 3.4 | 5.7 | 2.76 |
| | 30°C | 6.47 | 5.68 | 6.87 | 4.5 | 6.9 | 2.46 |
| | 35°C | 6.54 | 5.8 | 6.97 | 5.8 | 6.06 | 2.66 |
| | 25°C | 7.1 | 5.6 | 7.7 | 3.76 | 6.96 | 3.5 |
| **12 Days** | 30°C | 7.0 | 4.4 | 7.8 | 5.2 | 13.16 | 3.6 |
| | 35°C | 6.4 | 4.5 | 7.5 | 6.16 | 14.26 | 3.43 |
| | 25°C | 7.3 | 5.1 | 7.4 | 4.06 | 14.86 | 3.3 |
| **18 Days** | 30°C | 7.1 | 3.5 | 7.45 | 4.26 | 13.5 | 3.5 |
| | 35°C | 6.0 | 3.3 | 7.3 | 6.23 | 16.46 | 3.93 |
| | 25°C | 7.09 | 3.8 | 7.7 | 4.76 | 14.53 | 5.2 |
| **24 Days** | 30°C | 6.9 | 3.4 | 7.7 | 9.1 | 17.36 | 4.96 |
| | 35°C | 6.2 | 3.1 | 7.1 | 7.76 | 17.03 | 5.46 |

**Table 2.** Extracellular proteins in different culture media

| | Protein(ug/ml) | | | |
|---|---|---|---|---|
| **Day Interval** | **Temperature** | **Wheat st µg/ml** | **Sab. µg/ml** | **Cza. Dox µg/ml** |
| **6 days** | 25°C | 10.31 | 0.132 | 0.331 |
| | 30°C | 11.2 | 0.761 | 0.143 |
| | 35°C | 8.84 | 0.397 | 0.165 |
| | 25°C | 12.70 | 2.20 | 0.48 |
| **12 Days** | 30°C | 13.86 | 2.69 | 1.92 |
| | 35°C | 12.39 | 3.36 | 1.26 |
| | 25°C | 13.60 | 3.14 | 0.40 |
| **18 Days** | 30°C | 15.19 | 4.92 | 0.187 |
| | 35°C | 15.36 | 6.14 | 0.264 |
| | 25°C | 14.50 | 5.13 | 0.23 |
| **24 Days** | 30°C | 16.34 | 6.31 | 0.34 |
| | 35°C | 14.43 | 8.86 | 0.35 |

**Table 3.** Laccase activity of *C.indica* in different culture media and temperatures

| | Laccase | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Day Interval** | | **Wheat st.** | | | **Sab.** | | | **Cza. Dox** | | |
| | **Temp.** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** |
| **6 days** | 25°C | 6.023 | 0.58 | 1.77 | 1.22 | 9.24 | 0.21 | 5.76 | 17.40 | 2.1 |
| | 30°C | 12.73 | 1.13 | 2.82 | 2.4 | 3.15 | 0.34 | 8.8 | 61.53 | 3.6 |
| | 35°C | 3.23 | 0.36 | 0.55 | 1.11 | 2.79 | 0.18 | 9.8 | 59.39 | 3.76 |
| | 25°C | 4.54 | 0.35 | 1.20 | 2.3 | 1.04 | 0.33 | 5.69 | 11.85 | 1.62 |
| **12 Days** | 30°C | 5.51 | 0.39 | 1.05 | 3.33 | 1.23 | 0.25 | 10.38 | 5.40 | 2.88 |
| | 35°C | 6.73 | 0.54 | 1.09 | 2.23 | 0.66 | 0.15 | 13.93 | 11.05 | 4.06 |
| | 25°C | 25.91 | 1.90 | 6.38 | 19.54 | 6.22 | 1.31 | 11.85 | 29.62 | 3.59 |
| **18 Days** | 30°C | 48.33 | 3.18 | 11.34 | 1.41 | 0.28 | 0.10 | 19.86 | 106.2 | 5.6 |
| | 35°C | 71.3 | 4.64 | 11.44 | 0.65 | 0.10 | 0.039 | 29.13 | 110.3 | 7.41 |
| | 25°C | 97.49 | 6.72 | 20.48 | 12.74 | 2.48 | 0.87 | 29.12 | 126.6 | 5.6 |
| **24 Days** | 30°C | 187.38 | 11.46 | 20.59 | 3.96 | 0.62 | 0.22 | 39.09 | 114.9 | 7.88 |
| | 35°C | 113.07 | 7.83 | 14.57 | 4.77 | 0.53 | 0.28 | 44.38 | 126.8 | 8.12 |

The Tyrosinase activity in different culture media and temperature is shown in Table 4. Tyrosinase activity was maximum in wheat straw followed by Czapek dox medium, while Sabourauds medium produced least activity. Czapek Dox medium gave more tyrosinase per unit mg of protein after 18 and 24 days at all the three temperature. Incubation temperature of 25 and 30 $^{0}$ cel gave more Tyrosinase then at 35 $^{0}$ cel.

The results of aryl alcohol peroxidase (AAO) are shown in Table 5. AAO is the main hydrogen peroxidase generating enzyme for the activity of lignin peroxidase (LiP), Manganese peroxoidase ( MnP) and versatile peroxidase (VP). The AAO activity started after 6 days in all the three culture media. Maximum activity was observed in Sabourauds medium and it continuously increased from 6th day to 24th day. It indicates that production of AAO requires more nutrient. Czapek dox medium gave more laccase per unit mg of protein than other media.

**Table 4. Tyrosinase activity in *C.indica* in different culture media and temperatures**

| | | | Tyrosinase activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Day Interval** | **Wheat st.** | | | | **Sab.** | | | **Cza. Dox** | | |
| | **Temp.** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** |
| **6 days** | 25°C | 9.69 | 0.93 | 2.85 | 3.8 | 28.78 | 0.66 | 2.36 | 7.12 | 0.85 |
| | 30°C | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.26 | 29.79 | 1.73 |
| | 35°C | 6.47 | 0.73 | 1.11 | 4.9 | 12.34 | 0.80 | 2.83 | 17.15 | 1.06 |
| | 25°C | 49.88 | 3.92 | 13.26 | 3.91 | 1.77 | 0.56 | 2.45 | 5.10 | 0.7 |
| **12 Days** | 30°C | 38.18 | 2.75 | 7.34 | 1.42 | 0.52 | 0.10 | 4.66 | 2.42 | 1.29 |
| | 35°C | 16.84 | 1.35 | 2.73 | 4.68 | 1.39 | 0.32 | 4.22 | 3.34 | 1.23 |
| | 25°C | 66.07 | 4.85 | 16.27 | 3.96 | 1.26 | 0.26 | 1.88 | 4.7 | 0.56 |
| **18 Days** | 30°C | 63.70 | 4.19 | 14.95 | 5.97 | 1.21 | 0.44 | 2.84 | 15.18 | 0.81 |
| | 35°C | 23.06 | 1.50 | 3.70 | 1.70 | 0.27 | 0.10 | 4.40 | 16.66 | 1.11 |
| | 25°C | 15.63 | 1.07 | 3.28 | 2.93 | 0.57 | 0.20 | 5.62 | 24.43 | 1.08 |
| **24 Days** | 30°C | 39.81 | 2.43 | 4.37 | 2.59 | 0.41 | 0.15 | 7.10 | 20.88 | 1.43 |
| | 35°C | 26.63 | 1.84 | 3.43 | 1.89 | 0.21 | 0.11 | 6.43 | 18.37 | 1.17 |

**Table 5.** Aryl alcohol peroxidase (AAO) activity of *C. indica* in different culture media and temperature

| | | | AAO | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Day Interval** | **Wheat st.** | | | | **Sab.** | | | **Cza. Dox** | | |
| | **Temp.** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** |
| **6 days** | 25°C | 9.95 | 0.96 | 2.92 | 2.91 | 22.04 | 0.51 | 0.48 | 1.45 | 0.17 |
| | 30°C | 7.76 | 0.69 | 1.72 | 1.35 | 1.77 | 0.19 | 0.88 | 6.15 | 0.35 |
| | 35°C | 9.56 | 1.08 | 1.64 | 0.91 | 2.29 | 0.15 | 1.41 | 8.54 | 0.53 |
| | 25°C | 0.92 | 0.072 | 0.24 | 3.94 | 1.79 | 0.56 | 1.44 | 3.0 | 0.41 |
| **12 Days** | 30°C | 2.35 | 0.16 | 0.45 | 5.79 | 2.15 | 0.43 | 1.69 | 0.88 | 0.46 |
| | 35°C | 1.76 | 0.14 | 0.28 | 5.69 | 1.69 | 0.39 | 1.29 | 1.02 | 0.37 |
| | 25°C | 8.2 | 0.60 | 2.01 | 10.54 | 3.35 | 0.70 | 2.66 | 6.65 | 0.80 |
| **18 Days** | 30°C | 4.0 | 0.26 | 0.62 | 15.33 | 3.11 | 0.13 | 2.0 | 10.69 | 0.57 |
| | 35°C | 7.03 | 0.45 | 1.08 | 6.2 | 1.00 | 0.37 | 3.42 | 12.95 | 0.87 |
| | 25°C | 12.02 | 0.82 | 2.52 | 21.51 | 4.19 | 1.48 | 3.95 | 17.17 | 0.75 |
| **24 Days** | 30°C | 2.99 | 0.18 | 0.32 | 23.55 | 3.73 | 1.35 | 3.18 | 9.35 | 0.64 |
| | 35°C | 6.68 | 0.46 | 0.86 | 19.29 | 2.17 | 1.13 | 4.10 | 11.71 | 0.75 |

The versatile peroxidase activity (U/ ml, U/mg protein and U/mg biomass ) in different culture media is shown in Table 6. VP was the main peroxidase activity which started just after 6th day in all the culture media. The VP activity increased from 12th day to 24th day in wheat straw medium and maximum activity was recorded at 35 $^0$ cel on 12th day. Temperature of 35 $^0$C is more suitable for VP. Activity of VP was much more in Czapek dox medium per unit of protein .

The effect of culture media on manganese peroxidase activity is shown in Table 7. The MnP activity was detected in all the three culture media after 6th day and recorded till 24th day. In wheat straw medium on 6th day at 30 $^0$C max MnP activity was recorded followed by at 35 $^0$C on 12th day. Similar to other enzymes Czapek dox medium gave highest enzyme /unit mg of protein.

**Table 6.** Versatile peroxidase (VP) activity of *C. indica* in different culture media and temperature

| Versatile peroxidase | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Day Interval** | **Wheat st.** | | | | **Sab.** | | | **Cza. Dox** | | |
| | **Temp.** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** |
| **6 days** | 25°C | 135.3 | 13.12 | 39.79 | 40.46 | 306.5 | 7.09 | 12.38 | 37.40 | 4.48 |
| | 30°C | 109.6 | 9.79 | 24.35 | 42.80 | 56.24 | 6.20 | 21.16 | 147.9 | 8.60 |
| | 35°C | 52.9 | 5.98 | 9.12 | 30.38 | 76.52 | 5.01 | 57.18 | 346.5 | 21.49 |
| | 25°C | 265.0 | 20.86 | 70.47 | 31.27 | 14.21 | 4.49 | 14.45 | 30.10 | 4.12 |
| **12 Days** | 30°C | 213.6 | 15.41 | 41.07 | 56.30 | 20.92 | 4.27 | 41.98 | 21.86 | 11.6 |
| | 35°C | 396.0 | 31.96 | 64.28 | 54.76 | 16.29 | 3.84 | 73.72 | 58.50 | 21.49 |
| | 25°C | 295.85 | 21.75 | 72.86 | 32.43 | 10.32 | 2.18 | 106.34 | 265.85 | 32.2 |
| **18 days** | 30°C | 161.66 | 10.64 | 37.94 | 85.53 | 17.38 | 6.33 | 56.36 | 301.39 | 16.10 |
| | 35°C | 266.57 | 17.35 | 42.78 | 93.2 | 15.17 | 5.66 | 110.0 | 416.66 | 27.88 |
| | 25°C | 137.74 | 9.49 | 28.93 | 165.24 | 32.21 | 11.37 | 9.66 | 42.0 | 1.85 |
| **24 Days** | 30°C | 140.39 | 8.59 | 15.42 | 68.12 | 10.79 | 3.92 | 106.10 | 312.0 | 21.39 |
| | 35°C | 193.25 | 13.3 | 24.90 | 91.07 | 10.27 | 5.34 | 101.48 | 289.9 | 18.58 |

**Table 7.** Manganese peroxidase (MnP) activity of *C. indica* in different culture media and temperature

| MnP | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Day Interval** | **Wheat st.** | | | | **Sab.** | | | **Cza. Dox** | | |
| | **Temp.** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** |
| **6 days** | 25°C | 62.59 | 6.07 | 18.4 | 19.33 | 146.4 | 3.39 | 20.77 | 62.74 | 7.52 |
| | 30°C | 124.55 | 11.13 | 27.6 | 15.15 | 19.9 | 2.19 | 2.76 | 19.30 | 1.12 |
| | 35°C | 39.95 | 4.51 | 6.88 | 20.29 | 51.1 | 3.34 | 10.4 | 63.03 | 3.90 |
| | 25°C | 23.2 | 1.82 | 6.17 | 45.65 | 20.75 | 6.55 | 5.27 | 10.97 | 1.50 |
| **12 Days** | 30°C | 37.2 | 2.68 | 7.15 | 12.12 | 4.50 | 0.92 | 27.9 | 14.53 | 7.75 |
| | 35°C | 118.6 | 9.57 | 19.25 | 18.14 | 5.39 | 1.27 | 10.5 | 8.33 | 3.06 |
| | 25°C | 39.1 | 2.875 | 9.63 | 9.66 | 3.07 | 0.65 | 2.38 | 5.95 | 0.72 |
| **18 days** | 30°C | 29.6 | 1.948 | 6.94 | 15.04 | 3.05 | 1.11 | 2.53 | 13.52 | 0.72 |
| | 35°C | 35.4 | 2.30 | 5.68 | 27.24 | 4.43 | 1.65 | 3.43 | 12.99 | 0.87 |
| | 25°C | 52.57 | 3.62 | 11.04 | 28.78 | 5.61 | 1.98 | 9.45 | 41.08 | 1.81 |
| **24 Days** | 30°C | 52.21 | 3.19 | 5.73 | 26.80 | 4.24 | 1.54 | 7.09 | 20.85 | 1.42 |
| | 35°C | 35.68 | 2.47 | 4.59 | 18.27 | 2.06 | 1.07 | 6.26 | 17.88 | 1.14 |

The results of LiP activity in different culture media and temperature is shown in table 8. LiP activity started after 6th day in all the three culture media. In wheat straw medium max LiP activity was recorded on 18th day followed by 24th day. Czapek dox medium gave maximum enzyme per unit mg of protein.

This is the 1st report of VP peroxidase in *Calocybe indica* after *Bjerkandera adust* and *Pleurotus* spp. We can use Czapek dox medium for enzyme production and purification, however sraw medium can be used for bulk production of VP for industrial uses.

**Table 8.** Lignin peroxidase (LiP) activity of *C. indica* in different culture media and temperature

| LiP | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Day Interval** | **Wheat st.** | | | | **Sab.** | | | **Cza. Dox** | | |
| | **Temp.** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** | **U/ml** | **U/mg protein** | **U/mg Biomass** |
| **6 days** | 25°C | 3.36 | 0.32 | 0.98 | 1.93 | 14.62 | 0.33 | 2.40 | 7.25 | 0.86 |
| | 30°C | 4.80 | 0.42 | 1.06 | 1.75 | 2.29 | 0.25 | 1.63 | 11.39 | 0.66 |
| | 35°C | 2.90 | 0.32 | 0.50 | 1.80 | 4.53 | 0.29 | 3.30 | 20.0 | 1.24 |
| | 25°C | 0.0 | 0.0 | 0.0 | 5.06 | 2.30 | 0.72 | 4.83 | 10.06 | 1.38 |
| **12 Days** | 30°C | 6.73 | 0.48 | 1.29 | 5.76 | 2.14 | 0.43 | 2.10 | 1.09 | 0.58 |
| | 35°C | 3.28 | 0.26 | 0.53 | 2.13 | 0.63 | 0.15 | 0.88 | 0.69 | 0.25 |
| | 25°C | 36.6 | 2.69 | 7.68 | 5.93 | 1.88 | 0.40 | 5.06 | 12.65 | 1.53 |
| **18 Days** | 30°C | 12.53 | 0.82 | 1.37 | 7.56 | 1.53 | 0.56 | 5.43 | 29.03 | 1.55 |
| | 35°C | 18.46 | 1.20 | 2.37 | 11.2 | 1.82 | 0.68 | 3.83 | 14.50 | 0.97 |
| | 25°C | 13.36 | 0.92 | 2.80 | 10.26 | 2.0 | 0.70 | 8.97 | 39.0 | 1.72 |
| **24 Days** | 30°C | 15.26 | 0.93 | 1.67 | 5.52 | 0.87 | 0.31 | 9.33 | 27.4 | 1.88 |
| | 35°C | 14.89 | 1.03 | 1.91 | 14.19 | 1.60 | 0.83 | 9.63 | 27.5 | 1.76 |

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# GROWTH OF THE EDIBLE MUSHROOM *PLEUROTUS OSTREATUS* ON DIFFERENT CONCENTRATIONS OF DI (2-ETHYL HEXYL) PHTHALATE IN SOLID AND IN LIQUID MEDIA

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## ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is one of the most widely used plasticizers, giving flexibility to the plastics. Specific growth rate (μ), maximum biomass ($X_{max}$), laccase and esterase activities, pH profiles and enzymatic kinetic parameters were evaluated in *Pleurotus ostreatus* grown in DEHP in flasks. Radial growth rate ($u_r$), laccase and esterase activities and mycelial biomass ($X$) of *P. ostreatus* grown in agar plates containing DEHP were also evaluated. Flasks of 125 ml and agar plates containing 0, 750, 1200 and 1500 mg of DEHP/l were used. All media were added with 10 g of glucose/l. Flasks containing 50 ml culture medium were inoculated and incubated at 25 °C for 16 days on a rotary shaker (120 rpm). Petri dishes were inoculated and incubated at 25 °C for 7 days. $X_{max}$, μ, and $u_r$ were evaluated using the logistic and lineal equations, respectively. $X$ was determined by dry weight method. Laccase and esterase activities were evaluated using 2, 6-dimethoxyphenol and *p*-nitrophenyl butyrate as substrates, respectively. The highest $X_{max}$ was observed in media containing 1500 mg of DEHP/l and the esterase activity was much higher than the laccase activity at the beginning of the stationary phase in medium containing 1000 mg of DEHP/l in flasks. In agar plates, the laccase activity was higher than the esterase activity in all the media containing DEHP. These results suggest that there was no catabolite repression (glucose effect) and that DEHP was used as carbon and energy source by this fungus.

**Keywords:** di (2-ethylhexyl) phthalate, esterase, laccase, liquid medium, *Pleurotus ostreatus*, solid medium

## INTRODUCTION

Di (2-ethylhexyl) phthalate (DEHP) belongs to the family of the phthalates or acid phthalic esters. More than 60 kinds of phthalates are produced nowadays [1]. These compounds are used each year as plasticizers in flexible polyvinyl chloride (PVC) products, resins, cellulosic, polyvinyl acetate and polyurethanes [2]. The annual worldwide production of phthalates exceeds 5 million tons [3]. DEHP is a high production volume chemical used in the manufacture of a wide variety of consumer food packaging, some children's products, and some polyvinyl chloride (PVC) medical devices.The European government banned the use of DEHP in toys and children's products that might be placed in the mouth (http://www.marchem.com/materials/plastisols/phthalatefree.html). It has been reported that phthalates are mutagenic, teratogenic and carcinogenic [4, 5]. In addition, phthalates are important environmental contaminants and are difficult to degrade easily. Elimination of DEHP by microorganisms is considered to be one of the major routes of environmental degradation. Hwang *et al*. [6] studied the degradation of 100 mg/l of butylbenzyl phthalate (BBP) by *P. ostreatus*. They found that the degradation of this compound was higher when the BBP was dispersed in an optimum liquid medium (yeast-malt extract-glucose) than in a minimal medium. They also reported that the esterases are more important than laccases in the degradation of this compound. On the other hand, *P. ostreatus* is the second most cultivated edible mushroom worldwide. This mushroom has a very important enzymatic machinery that is able to produce laccases and manganese peroxidases [7]. In this research, specific growth rate (μ), laccase and esterase activities, pH profiles and enzymatic kinetic parameters were evaluated in *P. ostreatus* grown in media containing 0, 750, 1200 and 1500 mg of DEHP/l in liquid media. Radial growth rate (μ), mycelial biomass and laccase and esterase activities were also determined in media added with 0, 750, 1200 and 1500 mg of DEHP/l in agar plates.

## MATERIALS AND METHODS

**Microorganism:** *P. ostreatus* from the American Type Culture Collection (ATCC 3526) (Manassas, Virginia, U.S.A.) was used. The strain was grown on malt extract agar (MEA) at 25 °C and stored at 4 °C until used.

**Culture media:** Four liquid and four solid media were used. The liquid media had; 1) 50 ml of glucose-yeast extract medium (GY) + 0 mg of DEHP/l, 2) 50 ml of GY + 750 mg of DEHP/l, 3) 50 ml of GY + 1200 mg of DEHP/l and 4) 50 ml of GY + 1500 mg of DEHP/l. The solid media had the same liquid media composition plus 20 g of agar/l (this amount of agar was required as a solidifying agent). The YG medium had (in g/l): glucose, 10; yeast extract, 5; $KH_2PO_4$, 0.6; $MgSO_4.7H_2O$, 0.5; $K_2HPO_4$, 0.4; $CuSO_4.5H_2O$, 0.25; $FeSO_4.7H_2O$, 0.05; $MnSO_4$, 0.05 and $ZnSO_4.7H_2O$, 0.001. The pH was adjusted at 6.5 using either 0.1 M HCl or 0.1 M NaOH. To prepare solid media, the agar was added to the culture media and then the media were autoclaved.  In solid and liquid media, the DEHP was added to the autoclaved media. The media containing the different concentrations of this compound were cool down at 50 °C approx. and sonicated for approximately 3 minutes using an ultrasonic processor (GEX 130) until the DEHP had been fully dispersed. The agar media were poured into Petri dishes [8]. We used these concentrations of DEHP, since in previous studies similar concentrations of this compound were used, and it was clearly observed the effect of dibutyl phthalate and DEHP on the growth of filamentous fungi [8].

**Specific growth rate:** Flasks of 125 ml containing 50 ml of the different culture media were inoculated with three mycelial plugs of 10 mm of diameter and incubated at 25 °C for 16 days on a rotary shaker at 120 rpm. The biomass (*X*) was obtained by filtration of the samples using filter paper (Whatman No. 4), and it was determined as difference of dry weight (g/l) [$X = X(t)$] using the Velhurst-Pearl or logistic equation (Equation 1). The *X* was measured daily until that the stationary phase of growth of the fungus started (16 days of incubation).

$dX/dt=\mu[1-X/X_{max}]X$ or $X[X_{max}/1+Ce^{-\mu t}]$ Equation 1

Where, $X = X_0$ (the initial biomass value), $C = (X_{max} - X_0)/X_0$, μ is the maximal specific growth rate and $X_{max}$ is the maximal (or equilibrium) biomass level achieved when $dX/dt = 0$ for $X > 0$. Thus, in logistic growth, the growth rate decreases as biomass increases. Evaluations of kinetic parameters of the logistic equation were carried out using a non-linear least square-fitting program (Solver; Excel, Microsoft) [9-11]. The value of μ was evaluated from the third to the 16 d of growth in order to avoid the variation adjustment problem.

**Laccase and esterase activities in liquid medium:** The supernatant obtained from the filtration of the samples corresponded to the enzymatic extract (EE). Laccase activity was determined in each EE by changes in the absorbance at 468 nm (using a Jenway 6405 UV/V is spectrophotometer), using 2, 6-dimethoxyphenol (DMP, SIGMA) as substrate. The assay mixture contained 900 µl of 2 mM DMP in 0.1 M acetate buffer pH 4.5 and 100 µl EE, which were incubated at 40 ºC for 1 min. Esterases activity was determined by changes in the absorbance at 405 nm (using a Jenway 6405UV/Vis spectrophotometer), using *p*-nitrophenyl butyrate (*p*NPB) as substrate. The reaction mixture contained 10 µl of a *p*NPB solution [1.76 % (v/v) of *p*NPB in acetonitrile], 790 µl of 50 mM acetates buffer pH 7.0, 0.04% Tritón X-100 and 100 µl of the EE, which were incubated at 37 °C for 5 min [12, 13]. One enzymatic unit of laccase activity or esterase activity (U) is defined as the amount of enzyme which gives an increase of 1 unit of absorbance per min in the reaction mixture. The enzymatic activities were expressed in U/l of EE.

**Enzymatic kinetic parameters:** The enzymatic kinetic parameters were evaluated in those cultures grown in liquid medium. Yield of laccases per unit of biomass produced ($Y_{E/X}$) was estimated as the relation between maximal enzymatic activity ($E_{max}$) and $X_{max}$ (see specific growth rate in the methodology section). Enzymatic productivity ($P$ = U/l h) was evaluated using the time of $E_{max}$. The specific rate of enzymatic production was calculated by the equation; $qP= (\mu)(Y_{E/X})$ [10, 11, 14]

**Radial growth rate, biomass and enzymatic assays in solid medium:** Petri dishes were inoculated in the center with a plug of 4 mm of diameter and incubated at 25 °C for 7 days. The radius of the mycelial growth was measured daily from

2 to 7 days of incubation using a vernier (digital mitutoyo). The radial growth rate ($u_r$) was calculated as the slope of the radius versus time plots, analyzed by lineal regression [8, 15]. The $X$ was evaluated in 7 days old colonies. The mycelium was separated from the culture medium using a boiling water bath and then placed in a pre-weighed watch glass. It was weighed, and then oven-dried at 60 °C for 24 h, then weighed again [8]. In solid media, the laccase and esterase activities were evaluated in 7 d old grown colonies. The colonies grown on agar were flooded with distilled water and left at room temperature for 24 h, after which the water was removed from the Petri dish and then centrifuged. The centrifuged water was considered the EE. Esterase and lacasse activities were determined as indicated above (see laccase and esterase activities in liquid medium in the methodology section). One enzymatic unit of esterase activity or laccase activity (U) is defined as the amount of enzyme which gives an increase of 1 unit of absorbance per min in the reaction mixture. The enzymatic activities were expressed in U/l of EE.

**Statistical analysis:** All the experiments were carried out by triplicated. Data were evaluated using one-way ANOVA and Tukey post-test using The Graph Pad Prism® program.

## RESULTS

In liquid medium, the highest μ was obtained in medium containing 750 mg of DEHP/l, followed by the media containing 1200 mg of DEHP/l, 1500 mg of DEHP/l and the medium lacking DEHP (Equation 1, Fig. 1, Table 1). The highest $X_{max}$ was obtained in the culture media with addition of 1500 and 1200 mg of DEHP/l, and the lowest $X_{max}$ was showed in the medium containing 750 mg of DEHP/l (equation 1, Table 1). In solid medium, the highest $u_r$ was observed in the medium lacking DEHP followed by the media containing 1500, 750 and 1200 mg of DEHP/l. The highest and lowest $X$ was obtained in media with addition of 1500 mg and without added DEHP, respectively (Table 1).

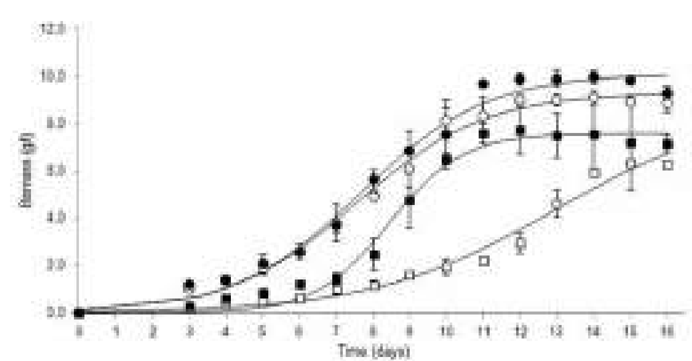


**Figure 1.** Specific growth rate of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (○) and 1500 (●) mg of DEHP/l in liquid medium. The experimental data of μ were adjusted using the equation 1

**Table 1.** Radial and specific growth rates, $X_{max}$ and $X$ of *P. ostreatus* grown in different concentrations of DEHP.

| Parameter | Culture system | Culture media DEHP (mg/l) | | | |
|---|---|---|---|---|---|
| | | 0 | 750 | 1200 | 1500 |
| $\mu$ (1/h) | Liquid | $0.016^{b}$ (0.001) | $0.041^{a}$ (0.007) | $0.024^{b}$ (0.003) | $0.024^{b}$ (0.003) |
| $X_{max}$ (g/l) | Liquid | $8.78^{d}$ (0.27) | $7.59^{c}$(0.64) | $9.31^{abc}$ (0.017) | $10.12^{ab}$ (0.17) |
| $u_r$ (mm/d ) | Solid | $0.49^{a}$(0.009) | $0.41^{c}$(0.003) | $0.40^{d}$(0.017) | $0.42^{b}$(0.001) |
| $X$ (mg/cm$^2$) | Solid | $0.116^{d}$(0.008) | $0.125^{c}$(0.010) | $0.13^{b}$(0.017) | $0.14^{a}$(0.006) |

Means with the same letter within a row are not significantly different. Numbers in parenthesis correspond to standard deviation of three separate experiments.

In liquid media, the highest activity of laccase was showed at the end of the exponential phase of growth (13 d of fermentation) in the media containing 1500 mg of DEHP/l (Fig. 2a). The highest activity of esterase was observed at the beginning of stationary phase (15 day of fermentation) in media containing 1500 mg of DEHP/l (Fig. 2b). In general, the lowest activities of laccase and esterase were observed in the medium lacking DEHP and in the medium containing 750 mg of DEHP/l (Figs. 2a, b). In general, the activity of esterase was observed in all the media containing DEHP, however, the activity of

laccase was only observed in media containing 1500 and 1200 mg of DEHP/l (Figs. 2a, b). In general, the medium lacking DEHP showed the lowest activities of laccase and esterase (Figs. 2a, b). The production of esterase at the beginning of the stationary phase of growth was similar in the media containing 0, 750, 1200 g of DEHP/l (Fig. 2a).

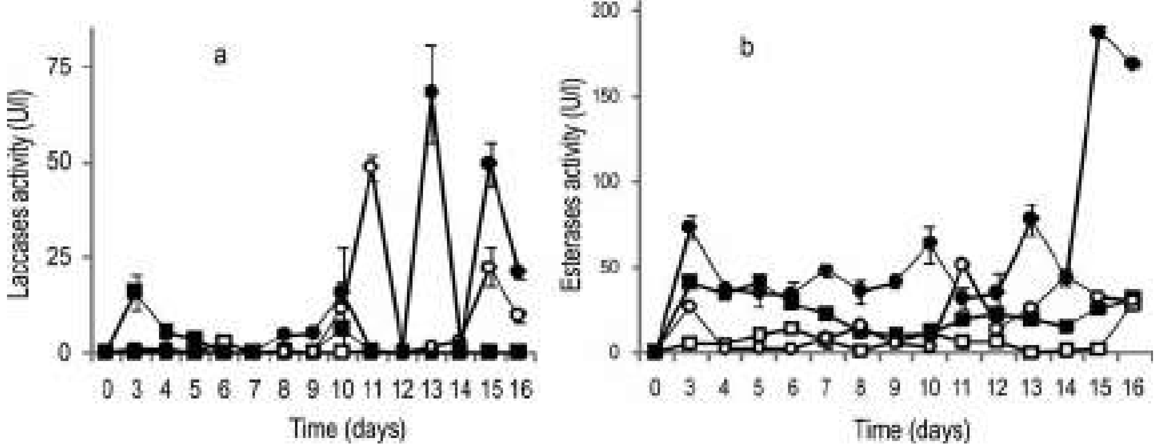


**Figure 2.** Laccases (a) and esterases (b) activities of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (○) and 1500 (●) mg of DEHP/l in liquid medium

The highest laccase and esterase kinetic parameters were observed in 1500 mg of DEHP/l, followed by the rest of the culture media DEHP/l (Tables 2, 3). From all the culture media, the medium containing 1500 mg of DEHP/l had the lowest pH value (during the 3 d of growth) (5.4 approx.).

**Table 2.** Laccase kinetic parameters of *P. ostreatus* grown in liquid medium in different concentrations of DEHP.

| Parameter | Culture media DEHP (mg/l) | | | |
|---|---|---|---|---|
| | 0 | 750 | 1200 | 1500 |
| $E_{max}$ (U/l) | 2.80$^{c}$(0.35) | 15.80$^{c}$(0.95) | 48.347$^{b}$(3.522) | 67.845$^{a}$(12.89) |
| $Y_{E/X}$ (U/g) | 0.3184$^{b}$(0.0317) | 2.0034$^{b}$(0.0519) | 5.1807$^{a}$(0.3843) | 6.6898$^{a}$(1.2993) |
| $qP$(U/g/h) | 0.0053$^{d}$(0.0002) | 0.0840$^{c}$(0.0150) | 0.1242$^{b}$(0.0051) | 0.1603$^{a}$(0.0161) |
| $P$ (U/l/h) | 0.0195$^{b}$(0.0024) | 0.02201$^{a}$(0.0132) | 0.1831$^{a}$(0.0133) | 0.2175$^{a}$(0.0413) |

Means with the same letter within a row are not significantly different. Numbers in parenthesis correspond to standard deviation of three separate experiments.

**Table 3.** Esterase kinetic parameters of *P. ostreatus* grown in liquid medium in different concentrations of DEHP.

| Parameter | Culture media DEHP (mg/l) | | | |
|---|---|---|---|---|
| | 0 | 750 | 1200 | 1500 |
| $E_{max}$ (U/l) | 27.8447$^{d}$(0.6219) | 41.2932$^{c}$(3.3415) | 51.4021$^{b}$(0.6411) | 188.4142$^{a}$(3.3126) |
| $Y_{E/X}$ (U/g) | 3.1687$^{c}$(0.1565) | 5.2432$^{b}$(0.6895) | 5.5078$^{b}$(0.0770) | 18.5692$^{a}$(0.4348) |
| $qP$(U/g/h) | 0.0529$^{c}$(0.0055) | 0.2228$^{b}$(0.0664) | 0.1326$^{cb}$(0.0129) | 0.4506$^{a}$(0.0385) |
| $P$ (U/l/h) | 0.0725$^{d}$(0.0016) | 0.3441$^{c}$(0.0278) | 0.1947$^{b}$(0.0024) | 0.5234$^{a}$(0.0092) |

Means with the same letter within a row are not significantly different. Numbers in parenthesis correspond to standard deviation of three separate experiments.

The media containing 1200 and 1500 g of DEHP /l showed a similar pH profile. The pH profiles of the medium added with 750 mg of DEHP/l and medium lacking DEHP were similar (Fig. 3).

The pH of the media containing 1200 and 1500 mg of DEHP/l showed higher pH than the rest of the culture media at the end of the fermentation. In solid medium, the laccases activity was much higher than the esterase activity in all the culture media. The highest laccases activity (Fig. 4a) and esterase activity (Fig. 4b) was observed in 1500 mg of DEHP/l, followed by that activity shown in the media containing 750 mg of DEHP/l, 1200 mg of DEHP/l and without added DEHP. The lowest activity of both enzymes was observed in media lacking DEHP.

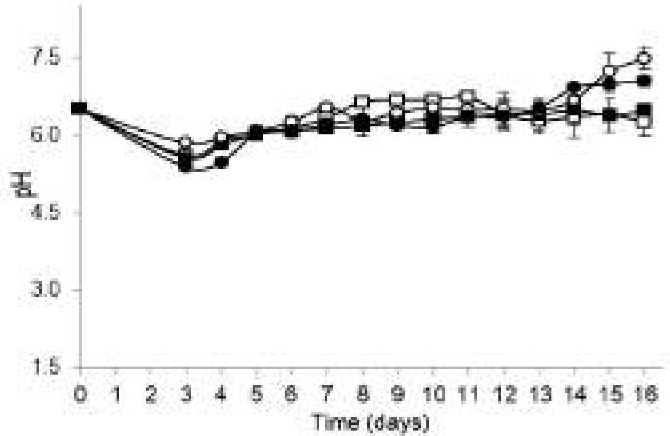


**Figure 3.** Profile of pH of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (○) and 1500 (●) mg of DEHP/l in liquid medium

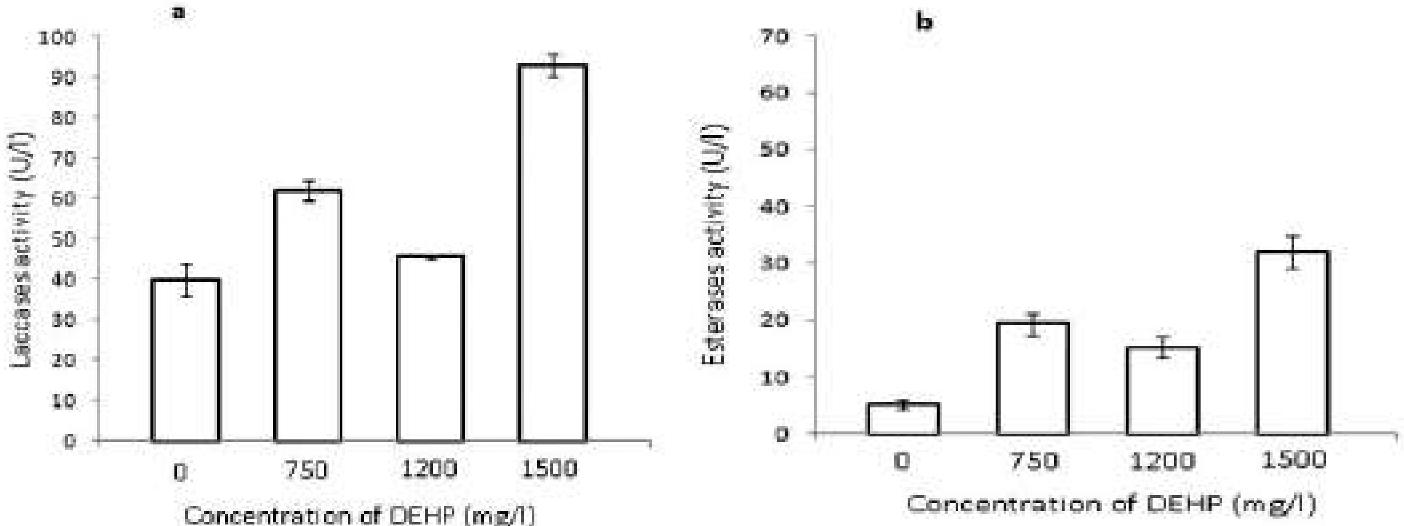


**Fig. 4.** Laccases (a) and esterases (b) activities of a 7 d old colony of *P. ostreatus* grown in solid medium in different concentrations of DEHP

## DISCUSSION

In liquid media, the μ and the highest $X_{max}$ were calculated using the logistic equation [14] (Eq. 1), fitting to an exponential growth model. In logistic growth, the growth rate decreases as biomass increases. These results showed that the DEHP is used by this fungus to grow, since the media containing the highest amount of DEHP showed the lowest μ and the highest $X_{max}$(Table 1). It is known that, from 100% of carbon source added to a culture media, 50% is used by microorganisms for biomass production and 50% for structure formation. In this study 10 g glucose/l were added to all the cultures (see materials and methods), since a diauxic growth might occurs in some microorganism that grow on complex compounds [6]. The $X_{max}$ produced in medium lacking DEHP was approx. 5 g/l (amount that corresponded to 50% of 10 g/l of glucose that was added to all the media (Table 1). These results showed that the DEHP was used as carbon and energy sources, since the biomass production was enhanced as the concentration of DEHP increased (Table 1). On the other hand, in solid medium, the highest $X$ was obtained in the media containing DEHP, however, the highest $u_r$ was observed in the medium lacking DEHP (Table 1). Suarez-Segundo *et al.* [8] reported that the growth (μ) of some strains of filamentous fungi on a minimal medium lacking DEHP could be due to the use of certain amount of nutrients that unavoidably remain in the inoculum and/or the use of nutrients produced by cellular lysis of the hyphae from the inoculum. The pH increased during the fermentation in the media containing 1200 and 1500 mg of DEHP/l. This could be due to the degradation of this

compound, releasing basic breakdown products of DEHP (http://umbdd.ethz.ch/index.html). The degradation of DEHP has been studied in liquid medium in shake flasks or laboratory scale fermenters using pure or mixed cultures or acclimatized activated sludge. In all cases, DEHP was observed to be readily degradable [16]. Chatterjee and Dutta [17] reported that *Gordonia* sp. and *Arthrobacter* sp. utilized butylbenzyl phthalate (BBP) individually as the sole source of carbon and energy. Hwang *et al.* [6] studied the addition of 100 mg/l of BBP to yeast-malt extract-glucose culture medium and reported that the esterases activity was induced by BBP itself and that these enzymes were more important than the laccases in the BBP degradation by *P. ostreatus* in liquid medium. Similarly, we found that *P. ostreatus* had higher activity of esterase than activity of laccase in liquid medium containing different concentrations of DEHP. It has been reported that the 16-hydroxihexadecanoic acid, a cutin monomer, was able to induce the esterase in the saprophytic fungus *Glomerella cingulate* [18]. Van der Vlught-Bergman *et al*. [19] studied the growth of the white rot fungus *Phlebia tremellosa*, in liquid medium containing benzylbutyl phtalate and diethyl phtalate in concentration of 30% and 80%, respectively, and found that the fungus increased the laccase production after 9 days of growth. On the other hand, the maximal enzymatic activity, enzymatic productivity and rate of the enzymatic production depend on the amount of phthalate added to the liquid medium (Tables 2 and 3). The kinetic parameters were higher in the medium containing 1500 mg of DEHP/l than in the rest of the media. It shows that high concentrations of DEHP increased the yield of enzyme and enhanced the metabolism of the strain. In solid medium, the laccases activity was higher than the activity of esterase in all the media (Figs. 4a, b). This might be due to the fact that laccases are very important in the mycelial invasion of solid substrates. These results show that the type of enzyme produced during the DEHP degradation depends, at least in part, on the culture system and on the DEHP concentration. The production of laccase and esterase, and the results of the growth of *P. ostreatus* in the medium containing 1500 mg of DEHP/l suggest that there was no catabolite repression (glucose effect) and that DEHP was used as carbon and energy source.

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# LACCASE GENE EXPRESSION OF *PLEUROTUS OSTREATUS* GROWN AT DIFFERENT pH OF THE LIQUID CULTURE MEDIUM

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## ABSTRACT

*Pleurotus ostreatus* is a white rot fungus capable of degrading many xenobiotic and recalcitrant compounds due to their ability to produce a nonspecific enzyme system able to catalyze the oxidation of many types of organic compounds including textile dyes. Expression profile of 5 genes of laccase of *P. ostreatus* developed in media at different pH in submerged fermentation was determined. The initial pH of the growing medium is an important factor for regulating the expression of laccase genes, having an effect on the activity and on the laccase isoenzymes number produced by *P. ostreatus* in SmF. Several studies reported that the number of isoenzymes depend on the conditions of development of *P. ostreatus*; expression profile also is conditioned to various dependent transcription factors of carbon source, nitrogen source and pH, etc.

**Key words:** *Pleurotus ostreatus*, laccases, expression, genes, submerged fermentation

## INTRODUCTION

Laccases (EC 1.10.3.2) are glycoproteins classified as multi-copper oxidases, that catalyze one electron oxidation of a wide range of inorganic and organic compounds with the reduction of oxygen to water [1]. The white rot fungi have been widely used for production of laccases production [2, 3]. It has been reported that in fungi the activity and laccase isoenzymes number depend on environmental conditions, temperature, pH, inductors, culture conditions and medium composition [4 - 7]. These fungi are easily adapted to different growth conditions including submerged cultures. It has been reported to degrade pollutants and phenolic compounds produced in different industries such as textiles and paper, among others. This degradation of xenobiotic compounds is attributed mainly to enzymes phenoloxidases [8]. *P. ostreatus* produces laccase isoenzymes both constitutive and inducible, depending on growth conditions [4, 9]. Palmieri *et al.*[10] studied the expression of two genes of laccases (*Lacc* 6 and *Lacc* 10) in *P. ostreatus* and observed that in the presence of copper, the *Lacc* 6 gene expression was increased. Castanera *et al.* [11] evaluated the expression profile of laccase genes from different strains of *Pleurotus ostreatus* and they observed that the activity and expression level of these enzymes were dependent on the strain and the growth medium either solid or submerged fermentation. Diaz *et al.* [9] evaluated the expression profile of five genes of *P. ostreatus* laccases in submerged fermentation developed different initial pH, where they observed that the expression of genes depends on the initial conditions of development, suggesting the presence of transcription factors activated by pH change in the culture medium. In this study, the expression profile of five genes of laccase (*Lacc*1, *Lacc*4, *Lacc*6, *Lacc*9 and *Lacc*10) of *P. ostreatus* developed in media of different pH (3.5, 4.5, 6.5 and 8.5) in SmF was determined.

## MATERIALS AND METHODS

### Microorganism

A strain of *Pleurotus ostreatus* from the American Type Culture Collection (ATCC 32783) (Manassas, Virginia, U.S.A.) was used.

### Culture conditions

Mycelial plugs (4 mm diameter) taken from the periphery of colonies of *P. ostreatus* grown for 7 days at 25ºC in Petri dishes containing potato dextrose agar were used as inoculum. A liquid medium, previously optimized for the production of laccases by this fungus in SmF, was prepared containing (in g/l): glucose, 10; yeast extract, 5; $KH_2PO4$, 0.6; MgSO4-

$7H_2O$, 0.5; $K_2HPO_4$, 0.4; $CuSO_4.5H_2O$, 0.25; $FeSO_4.7H_2O$, 0.05; $MnSO_4$, 0.05; $ZnSO_4.7H_2O$, 0.001 [4]. Flasks of 250 ml containing 50 ml of culture medium adjusted by separated at different pH values (3.5, 4.5, 6.5 and 8.5) using either 0.1 M HCl or NaOH were prepared. Flask were inoculated with three mycelial plugs and incubated at 25ºC for 23 days on a rotary shaker at 120 rpm [4]. Evaluations of biomass and laccases activity were carried out on samples taken every 24 h after the third day of growth. Four fermentations were performed in triplicate. The zymogram and expression patterns were obtained at 144, 168, 264, 312, 408, 504 and 528 h of fermentation.

**Biomass evaluation**

The biomass (X) was determined as difference of dry weight (g/l).

Assay of biomass X = X(t) was done using the Velhurst-Pearl or logistic equation,

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{\max}}\right]X$$

Where μ is the maximal specific growth rate and $X_{max}$ is the maximal (or equilibrium) biomass level achieved when dX/dt= 0 for *X*> 0. The solution of equation 1 is as follows;

$$X = \frac{X_{\max}}{1 + Ce^{-\mu t}}$$

Where, $C = (X_{max} - X_0)/X_0$, and $X = X_0$; the initial biomass value.

Estimation of kinetic parameters in the above equations was performed using a non-linear least square-fitting program “Solver” (Excel, Microsoft) [12].

**Total RNA isolation and RT-PCR**

A semi-quantitative RT-PCR was used to study genes expression of laccases of *P. ostreatus*. RNA was isolated from frozen mycelium produced at different fermentation times (see culture conditions section), using the TRIZOL (Invitrogen) extraction and was spectrophotometrically quantified by determining the optical density at $OD_{260/280}$. RNA was treated with DNAse I free of RNAse (Invitrogen) and then resuspended in 20 ìl of diethylpyrocarbonate-treated water,

**Table 1**. Specific primers of laccases genes of *P. ostreatus* grown at different pH initial in SmF.

| Access number | Gene (mRNA) | Sequence (5′-3′) |
|---|---|---|
| FM202673<br>FM202670.1 | Lacc1 | Fw ATGGACCAATCCCTACTCCC<br>Rv GGCATTGGGTGCTAAGATG |
| FM202672 | Lacc4 | Fw TGCGCCTGTACTCTCATTTG<br>Rv GGTAGAGACCGTGCCAATGT |
| AJ005018<br>AJ005017.2 | Lacc6 | Fw CGCTTGTTTCACTCGCATTA<br>Rv ATAGTGTCGAGTCGGGATGG |
| Z34847<br>Z22591.1 | Lacc9 | Fw GTCTCTCCTGACGGCTTCAC<br>Rv ATACTGGGTGGAAAGATGCG |
| Z34848 | Lacc10 | Fw ACGAGCTGGAGGCTAACAAA<br>Rv TCACGAAGCGAATAGTGACG |
| GU062704.1<br>AB690874.1 | gpd | Fw TCTGCGGTGTTAACCTTGAGTCGT<br>Rv TGGTAGCGTGGATGGTGCTCATTA |

http://www.ncbi.nlm.nih.gov/nuccore/?term=laccases%20*Pleurotus*%*ostreatus*

cDNA synthesis was performed using oligos dT and Moloney *Murine Leukaemia* virus reverse transcriptase (M-MuLV-RT; Fermentas) according to the manufacturer's instructions. The RT reaction mixture product (1 μg) and 10 ρmol of specific primers were mixed for specific PCR amplification using the Kit Accses Quick™ (PROMEGA^MR). Denaturation conditions were 94 °C for 3 min. The program included 35 cycles of 94°C for 40 s, 56°C for 40 s and 72°C for 50 s. Constitutive expression of glyceraldehyde-3-phosphate dehydrogenase (gpd) was tested as housekeeping. For the design of isoenzyme laccase specific primers (Table 1), the open *primer-blast* software was used (http://www.ncbi.nlm.nim.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The complete sequences of laccase mRNAs reported in the Gene bank were used (http://www.ncbi.nlm.nih.gov/nucleotide).

## RESULTS AND DISCUSSION

### Growth of *P. ostreatus* at different initial pH in submerged fermentation

Fig. 1 shows the growth of *P. ostreatus* at different initial pH. $X_{max}$ values of 5.2, 5.5, 9.6 and 8.3 g / l, and ì values of 0.006, 0.014, 0.018 and 0.02 $h^{-1}$ at initial pH of 3.5, 4.5, 6.5 and 8.5, respectively were observed. In this study, the ì values were around 0.02 $h^{-1}$ at initial pH values of 6.5 and 8.5. These values were similar to those reported by Téllez-Téllez *et al*. [4] for this same fungus at pH 6.0, and to those obtained when *P. ostreatus* grew in the presence of ammonium sulfate as nitrogen source at pH of 6.5 [13]. The ì values decreased 1.5-fold and 3-fold approx. at initial pH values of 4.5 and 3.5, respectively. The $X_{max}$ obtained at initial pH values of 6.5 and 8.5 was around 10% higher than that reported for *P. ostreatus* grown under similar conditions [13]. The $X_{max}$ obtained at pH values of 3.5 and 4.5 was 30% lower than that obtained at initial pH values of 6.5 and 8.5. It has been reported that the initial pH values of *P. ostreatus* growing media are between 6.0 and 6.5 [4, 13]. Díaz *et al*. [7] reported that *P. ostreatus* produced high yields of biomass in a short time, producing metabolites that modified the pH from 3.5 to 6.0-6.5 in a non buffered medium. In this research, this fungus grew between initial pH values of 3.5 to 8.5.

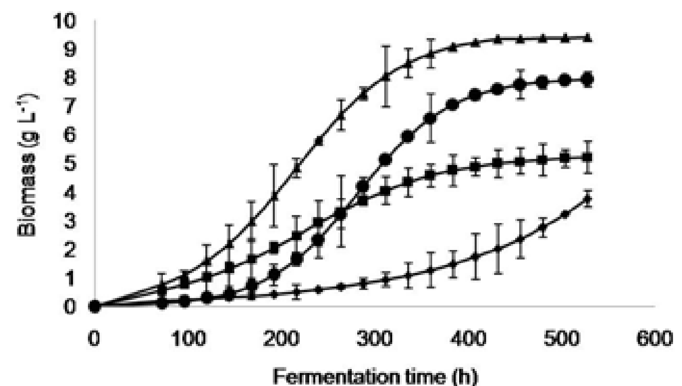


**Figure 1.** Growth of *P. ostreatus* at different initial pH; 3.5 (♦), 4.5(■), 6.5 (▲), and 8.5 (●) in SmF. Error bars show media ± SD of three replicate samples

### Laccase genes expression of *P. ostreatus*

Fig. 2 shows the PCR products obtained at pH 3.5 at different times of fermentation (144, 168, 264, 312, 408, 504 and 528 h). Five laccase genes were examined. Lacc10, Lacc4, Lacc1 and Lacc6 were expressed in nearly all the fermentation times, however, Lacc9 was not expressed at any time. Lacc10 and Lacc4 were not expressed at 312 and 264 h, respectively. Lacc1 was expressed at low level at 264 and 312 h. Lacc6 showed very little expression at 264 and 408 h of fermentation.

The expression profile of the laccase genes in culture to initial pH of 4.5 is shown in Fig. 3. Lacc9 was only expressed at 144 h of fermentation. Lacc10, Lacc4, Lacc1 and Lacc6 were expressed at different levels during the fermentation, however, Lacc6 showed more intensely-stained bands than the rest of the genes.

Fig. 4 shows the profile of laccase gene expression in culture at initial pH 6.5. In this case, Lacc9 was not expressed, however, Lacc10, Lacc4, Lacc1 and Lacc6 were expressed in all the fermentation times. The expression of Lacc10, Lacc4, Lacc1 and Lacc6 increased through the fermentation. The Fig 4 shows the profile of laccase gene expression in culture at initial pH 8.5. Lacc9 was not expressed. Lacc10 was expressed at low level during the fermentation. Lacc4, Lacc1 and Lacc6 were expressed during all the fermentation times. Lacc6 showed more intensely-stained bands than the rest of the genes.

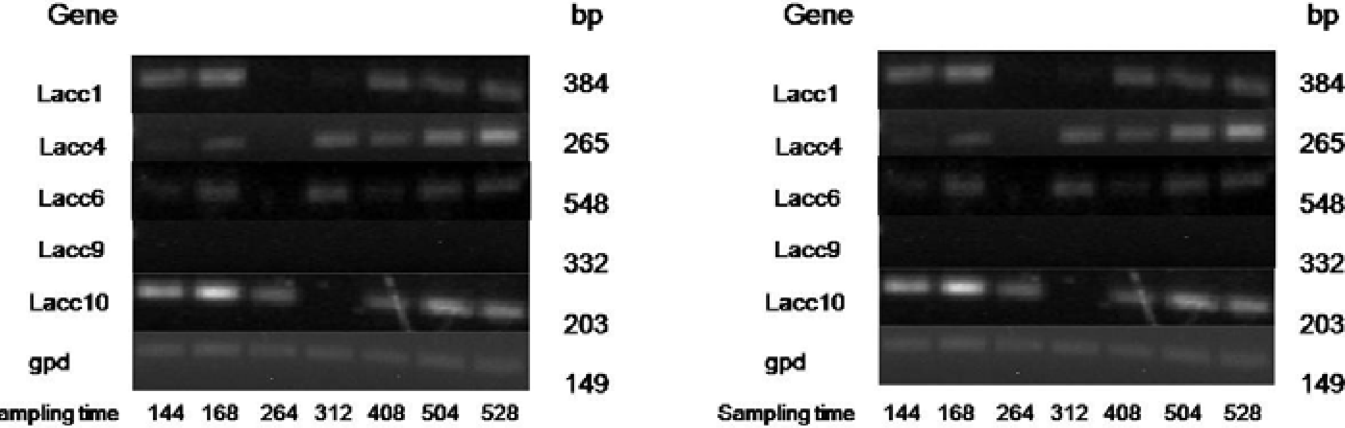


**Figure 2.** Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 3.5

**Figure 3.** Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 4.5

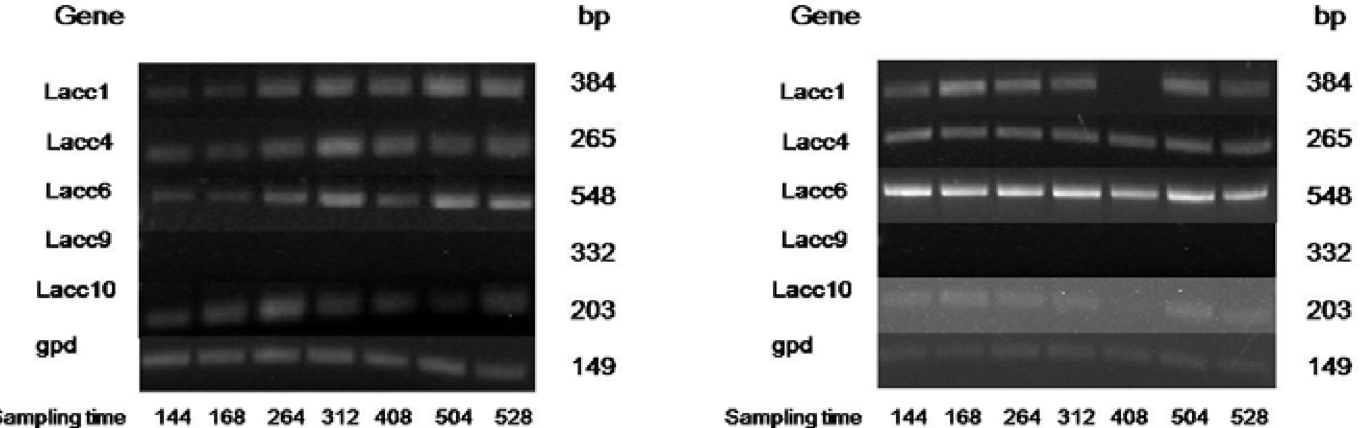


**Figure 4.** Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 4.5

**Figure 5.** Genes expression profile from laccases of *Pleurotus ostreatus* grown at initial pH of 4.5

## CONCLUSION

The initial pH of the culture medium is an important factor which regulates the expression of the laccase genes in addition to having an effect on the activity and number of isoenzymes produced. These results contribute to the understanding of the regulation of the expression of the laccase genes.

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# LACCASES AND MANGANESE PEROXIDASES OF *PLEUROTUS OSTREATUS* GROWN IN SOLID-STATE FERMENTATION

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## ABSTRACT

Ligninolytic system of white-rot fungi is directly involved in the degradation of various xenobiotic compounds and dyes. This system could show enzymes such as laccases, manganese peroxidases (MnP), veratryl alcohol oxidase (VAO) and versatile peroxidases (VP). In this work, laccase and manganese peroxidase activities of *Pleurotus ostreatus* grown in solid-state fermentation using polyurethane foam as inert support were evaluated. Maximal laccase activity was observed at the begin of stationary phase of growth and the manganese peroxidase showed the maximal activity at the end of fermentation.

**Keywords**: *Pleurotus*, fermentation, enzymes, laccase, manganese peroxidase

## INTRODUCTION

Over the years, have been generated products from chemicals that would be considered a necessity for daily life, but these products become a problem for the world population, because these could generate problem of pollution to the environment. These compounds are called xenobiotics, including pesticides and dyes present in waste of industrial processes such as in the effluent from the paper-producing industry and the textile industry. White rot-fungi are capable of degrading some xenobiotics compounds and different kinds of environmental pollutant due to its ligninolytic enzymatic system [1–2]. Lignin biodegradation is a key step of carbon recycling happening in almost all terrestrial ecosystems. In this process, all the white-rot basidiomycetes members (including *Pleurotus*) play an important role in the degradation of these recalcitrant woody polymers for the sustenance of microbial populations, allowing the proper utilization of the degraded cellulose [3]. Ligninolytic enzymes have potential applications in a large number of fields, including the chemical, fuel, food, agricultural, paper, textile, cosmetic industrial sectors and more. Species of *Pleurotus* genus, including *P. eryngii, P. sapidus, P. pulmonarius and P. ostreatus* produce ligninolytic enzymes such as laccases, manganese peroxidases (MnP), veratril alcohol oxidase (VAO) and versatile peroxidases (VP), in both, submerged (SF) or solid-state fermentation (SSF) [4–7].

## MATERIALS AND METHODS

**Microorganism:** A strain of *Pleurotus ostreatus* from the American Type Culture Collection (ATCC 32783) (Manassas, Virginia, U.S.A.) was used.

**Solid-state fermentation:** The solid-state fermentation (SSF) was carried out in a flask of 250 ml containing 0.5 g of polyurethane foam of low density (PUF; 17 kg/m3) cubes (0.5×0.5×0.5 cm) as an inert support [8] impregnated with 15 mL of sterile culture medium (pH 6.5) with composition (in g/L): glucose, 10; yeast extract, 5; $KH_2PO_4$, 0.6; $MgSO_4.7H_2O$, 0.5; $K_2HPO_4$, 0.4; $CuSO_4.5H_2O$, 0.25; $FeSO_4.7H_2O$, 0.05; $MnSO_4$, 0.05; $ZnSO_4.7H_2O$, 0.001 [9]. Three mycelia plugs (4 mm diameter) taken from the periphery of colonies of *Pleurotus ostreatus* grown for 7 d at 25 ºC in Petri dishes containing potato dextrose agar (DIFCO™) were used as inoculum for each flask. The cubes were washed twice with hot distilled water, oven-dried at 60 °C for 24 h, and then autoclaved at 120 °C for 15 min, before the culture. All inoculated flasks were incubated at 25 °C and samples were taken every 24 h after third day of growth.

**Enzymatic extract preparation and biomass evaluation.** The enzymatic extract (EE) was obtained by soft pressing the PUF cubes and the broth was filtrated using filter paper (Whatman No. 4), and the biomass (X) immobilized on PUF cubes was determined as difference of dry weight (g/L) [8].

The assay of biomass $X = X(t)$ was done using the Velhurst-Pearl logistic equation,

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{\max}}\right]X \qquad (1)$$

where $\mu$ is the maximal specific growth rate and $X_{max}$ is the maximal (or equilibrium) biomass level achieved when $dX/dt = 0$ for $X > 0$. The solution of equation 1 is as follows,

$$X = \frac{X_{\max}}{1 + Ce^{-\mu t}} \qquad (2),$$

where $C = (X_{max} - X_0)/X_0$ , and $X = X_0$ is the initial biomass value.

Estimation of kinetic parameters in the above equations was performed using the non-linear least square-fitting program "Solver" (Excel, Microsoft) [8, 9].

**Laccase activity assay.** Laccase activity was determined in each EE by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol (DMP) as substrate. The assay mixture contained 900 µL substrate (2 mM DMP in 0.1 M acetate buffer pH 4.5) and 100 µL EE, which were incubated at 40 ºC for 1 min [9]. The activity was expressed in International Units per liter (U/l) of EE ($å_{468}$= 35645 $M^{-1}$ $cm^{-1}$).

**Manganese peroxidase.** Manganese peroxidase (MnP) was determined by phenol red oxidation at 610 nm with extinction coefficient $å_{610}$=4460 $M^{-1}cm^{-1}$. The reaction mixture contained 20 mM succinc buffer, pH 4.5, 250 mM sodium lactate, 0.5 % bovine serum albumin, 2 mM $MnSO_4$, 0.01% phenol red, 2 mM $H_2O_2$ and 50 µl EE, which were incubated at 35 ºC for 1 h. The activity was expressed in U/l of EE.

## RESULTS AND DISCUSSION

Fig. 1 shows the growth of *Pleurotus ostreatus*, the $X_{max}$ was observed at the 120 h with of 5.5 g/l approximately, and a ì of 0.085 $h^{-1}$. The ì value was four times higher than the reported when the fungus grew in submerged culture, however, the $X_{max}$ decreased 42% approximately [10].

The maximum laccase activity was observed at 144-168 h of fermentation with a value of about 400 U/l, after that time, the laccase activity was constant of about 100 U/l (Fig. 2). On the other hand, the MnP activity was approximately 3 U/l from the beginning of the fermentation until 456 h, peaking at 500 h with 5 U/l (Fig. 3).

*Pleurotus ostreatus* HAI 595 and *Pleurotus eryngii* HAI 507 were distinguished from the other species by significantly higher laccase activities reaching maxima of 4808.7 ± 164.3 U $l^{-1}$ and 4531.0 ± 95.6 U $l^{-1}$, respectively, on day 14 of cultivation. The minimum MnP activity was found in *P. ostreatus* on day 7 of cultivation (12.6 ± 1.3 U $l^{-1}$), though with further cultivation it significantly increased to 1147.8 ± 162.5 U $l^{-1}$, during solid-state fermentation of wheat straw [11].

No peroxidase activity was detected during both *P. ostreatus* and *T. versicolor* fermentations. A maximum laccase activity production level of 15 U $g^{-1}$ of dry matter (U $g^{-1}$ d.m.) was achieved at the third to fourth day of *P. ostreatu*s fermentation, during *T. versicolor* SSF, a maximum laccase activity value of around 35 U $g^{-1}$ d.m. was produced at the 16th day, keeping constant at least until the 22nd day [12].

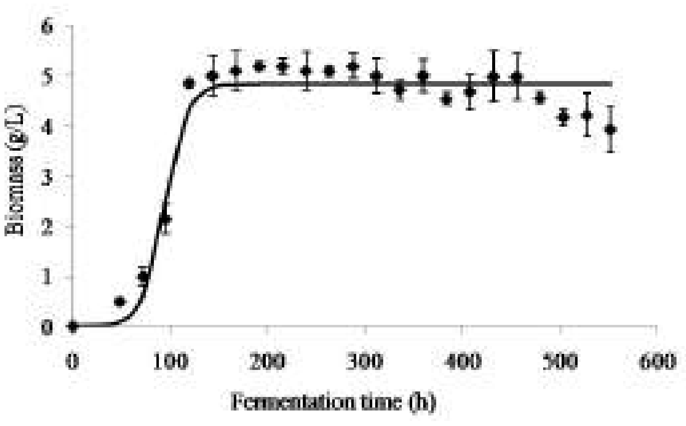


**Figure 1.** Biomass evolution of *Pleurotus ostreatus* grown in SSF on PUF

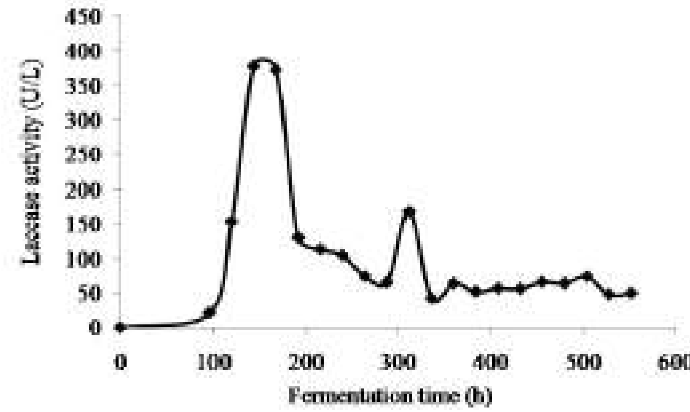


**Figure 2.** Laccase activity of *Pleurotus ostreatus* grown in SSF on PUF

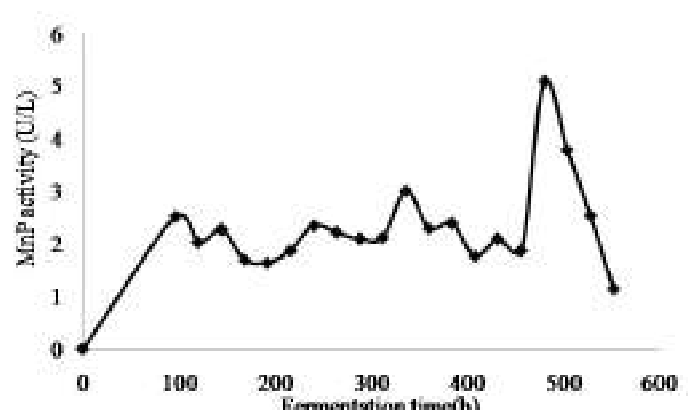


**Figure 3.** MnP activity of *Pleurotus ostreatus* grown in SSF on PUF

## CONCLUSIONS

The strain of *Pleurotus ostreatus* used in this study showed that it can produce ligninolytic enzymes by SSF on an inert support, so it is suggested further studies to establish the optimal conditions for increase production.

## ACKNOWLEDGEMENTS

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# IDENTIFICATION OF ERGOSTEROL IN MUSHROOMS

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## ABSTRACT

Mushroom has gained popularity as prized table delicacy with high nutritive and medicinal value. Ergosterol is a biological precursor (a provitamin) to vitamin D2. The contents of vitamin D2 and sterols in some wild and cultivated mushrooms were determined. Vitamin D2 was determined using an HPLC method, including saponification and semipreparative normal-phase HPLC purification Lv *et al.* [1]. Currently HPLC methods are regularly practiced for the estimation / determination of ergosterol [2]. The original procedure for extraction of ergosterol was worked out by Seitz *et al.* [3]. Four mushrooms *Calocybe indica*, *Ganoderma lucidum*, *Pleurotus florida* and *Volvariella volvacea* were grown on two synthetic (complete yeast extract agar and Lambert's agar) media and three semi- synthetic (malt extract agar, rice bran decoction and and wheat extract agar) media. The mycelial biomass of each mushroom was subjected to extraction of ergosterol and its identification using high performance liquid chromatography (HPLC). The ergosterol content ranged from 113 to 403µg/g with lowest retention peak was observed in *P. florida* showing 113 µg ergosterol per gram where as *C. indica* showed 243 µg ergosterol per gram and *V. volvacea* shows 159 µg ergosterol per gram and highest retention peak was observed in *G. lucidum* showing 403 µg ergosterol per gram of sample.

**Keywords**: ergosterol, high performance liquid chromatography (HPLC), *Ganoderma lucidum*

## INTRODUCTION

*Ganoderma lucidium* known as lingzhi in Chinese, reishi in Japanese, yeonghi in Korea is a mushroom in the group commonly known as polypore in the family *Ganoderma taceae* has a worldwide distribution in both tropical and temperate regions [4]. *G. lucidum* has been used widely in the treatment of hepatopathy, chronic hepatitis, nephritis, hypertension, arthritis, insomnia, bronchitis, asthma and gastric ulcer and also as antitumor, immunomodulatory, cardiovascular, antinociceptive (active against pain) effects [5]. The substances extracted from the mushroom can reduce blood pressure, blood cholesterol and blood sugar level as well as inhibition of platelet aggregation. The major compounds in *G. lucidum* which have pharmacological activities are ganoderic acid, triterpenes and polysaccharides. More than 150 triterpenes and more than 50 carcinostatic polysaccharides are isolated and are known to be unique compounds in this mushroom [6]. The extracts of *G. lucidium* contain bacteriolytic enzymes, lysozyme and acid protease and by these it show antimicrobial effect [7].

Ergosterol is an important membrane sterol in almost all eumycotic fungi and has been postulated to be strongly associated with living cytoplasmic fungi in the soil. However, ergosterol is not produced by all fungi and the ergosterol concentrations are known to vary between the same species depending on the physiological state of the fungus. Chiocchio and Matkoviæ [8] studied a modification to an ergosterol extraction technique which has the advantage of having a protocol with few steps of purification. This technique also includes an internal loss marker to evaluate the efficiency of extraction.

Ergosterol is a principal sterol of the cell membrane and is strongly bound to it in fungi [9] and is able to activate expression of a number of defense genes and increase the resistance of plants against the pathogens [10]. Ergosterol is a biological precursor (a provitamin) to vitamin D2. It is turned into viosterol by UV light, and is then converted into ergocalciferol, a form of vitamin D also known as D2 [11]. For this reason, when yeast (such as brewer's yeast) and fungi (such as mushrooms), are exposed to UV light, significant amounts of vitamin D2 are produced. Such vitamin D2 serves as the only available dietary source of vitamin D for those who eat no animal products, although such persons can obtain ample vitamin D through exposure to sunlight.

In HPLC, the analyte is forced through a column of stationary phase (usually a tube packed with small round particle with a certain surface chemistry) by pumping (mobile phase) at high pressure through the column. The sample to be analyzed was introduced in small volume to the stream of mobile phase and was retarded by specific chemical or physical interaction with the stationary phase it transverse the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of column) is called the rentention time and is considered a reasonably unique identifying characteristic [12]. Currently HPLC methods are regularly practiced for the estimation / determination of ergosterol, but a spectrophotometric method was also sensitive for semi micro-determination of ergosterol [2]. The original procedure for extraction of ergosterol was worked out by Seitz *et al*. [3].

## MATERIALS AND METHODS

### Preparation of mushroom extract

The mushroom mycelium was subjected to analytical assay for the extraction of biomedicinal components.

### Extraction of Ergosterol

The ergosterol extraction was carried out through saponification in the presence of alcoholic potassium hydroxide. The protocol was standardized with some modification in the method given by Brevik and Owades [13]. Mycelial biomass harvested from the broth was dried by pressing against filter paper to remove moisture and was further used for extraction of ergosterol. Dried sample each having 20-30% moisture was sponified with 25 ml of 25% alcoholic KOH (25 g of KOH, dissolved in 35 ml distilled water bath at 80-90 ºC for 2 to 3 hours. After saponification, samples were allowed to cool and the supernatant thus obtained was transferred to separatory funnel and 20 ml of methanol added over the residue to recover the residual ergosterol. Two aliquots were joined in the separatory funnel and 30-40 ml hexane (HPLC grade) was poured from top into the funnel. The contents were shaken and allowed to stand till the layers separate out. Hexane layer was collected, dried by evaporation in rotavapor and stored at 4 ºC till the HPLC analysis.

### Identification of ergosterol

The aliquots prepared were used for the identification of bioactive molecules using high performance liquid chromatography (HPLC) equipment DGU-20A5 at Punjab Agricultural University, Ludhiana. The operation condition of HPLC consisted a high rate flow of methanol and acetonitrile (80:20) at 1ml min > 1 through C=18 reverse phase column. Detection was carried out by a photodiode array detector at 280 nm as most of the compound present in the sample can be detected in this range. The column was first washed with water at least six times and then with solvent. The column clearance was done to remove the impurities and for base line stabilization.

After baseline stabilization, 20 µl of standard was first injected with the help of a sterile needle. The standard was allowed to run for 30 minutes. The photodiode array detector detects the retention time at which various compounds elute from the column and chromatogram was obtained. After 30 minutes the equipment automatically stops for the running of standard. The column was again cleaned by giving 2-3 injections of solvent. Samples were then run as above and after each injection of sample, column was thoroughly cleaned by washing it with solvent

The column was first calibrated with the standard. Standard of ergosterol was obtained from sigma aldrich (E6510) and stored at 4 ºC. The standard was dissolved in methanol with composition of 200 µg/ml and 1 mg/ml.

Quantity of egrosterol present in each sample could be quantified by using the formula given below:

$$\text{Quantity of ergosterol } (\mu g/g) = \frac{\eta g \text{ of std injected}}{\text{area of peck of std}} \times \frac{\text{area of sample}}{\mu l \text{ of sample injected}} \times \frac{\text{vol of sample}}{\text{wt of sample}}$$

## RESULTS

### Extraction and Identification of Ergosterol

Biomass of *Calocybe indica, Ganoderma lucidum, Pleurotus florida* and *Volvariella volvacea* was harvested after 15 days of inoculation on malt extract broth, complete yeast extract broth and rice bran decoction broth, respectively. The dried mycelial cake was used for the extraction of ergosterol. Samples were subjected to sponification using methanol and hexane. Final hexane layer containing ergosterol was dried to its minimum in rotavapor to the volume 1.6-2.5 ml. From each sample, 20 µl of solution was injected into C-18 reverse column (3.4.2). After 40 min of HPLC run, retention time graph was obtained for each sample (Plates I-III). The highest retention peak was observed in *G. lucidum* showing 403 µg ergosterol per gram of sample whereas lowest retention peak was observed in *P. florida* showing 113 µg ergosterol per gram (Table 1, Fig. 2)

**Table 1.** Ergosterol content from mycelium of mushroom cultures

| Culture | Retention peak of sample | Area under the peak | Ergosterol content(µg/g) |
|---|---|---|---|
| *Calocybe indica* (C3) | 32.862 | 426432 | 243 |
| *Ganoderma lucidum* (GL-I) | 32.993 | 1059612 | 403 |
| *Pleurotus florida* (PF) | 33.161 | 190640 | 113 |
| *Volvariella volvacea* (VV12) | 33.623 | 279254 | 159 |
| CD@ 5% | | 16 | |

Retenton time of standard – 32.639, Volume of sample injected -20µl, Weight of sample-1g.

**Figure 1.** Formation of Vitamin $D_2$ from Ergosterol
[ 9,10-Secoergosta-5,7,10 (19),22-tetraen-3-ol, (3â)- from Ergosta-5,7,22-trien-3-ol, (3â)- ]

## DISCUSSION

HPLC method for determining ergosterol was established for *G. lucidum* and various cultured tree species [14]. Samples from 17 different varieties and 14 different tree species were quantified by HPLC. The RP-HPLC was conducted on Diamonsil C18 column with acetonitrile as the mobile phase at 40 degrees C. The flow rate was 1.0 ml/min; and the detection wavelength was 282 nm. The fluctuations of ergosterol content was 0.093% - 0.243% among different varieties and 0.080%-0.227% among different tree species. Ergosterol was the most abundant sterol found in mushrooms, and its contents were higher in cultivated mushrooms (602.1–678.6) than in wild mushrooms (296–489 mg/100 g dry weight) [15].

The contents of vitamin D2 and sterols in some wild and cultivated mushrooms were determined. Vitamin D2 was determined using an HPLC method, including saponification and semi-preparative normal-phase HPLC purification before analytical reversed-phase quantification with an internal standard. Lv *et al.* [1] studied two species of *Ganoderma, G. sinense and G. lucidum*, used as Lingzhi in China. However, the content of triterpenoids and polysaccharides, main active compounds, were different, though the extracts of both *G. lucidum* and *G. sinense* have antitumoral proliferation effect. It was suspected that other compounds also contributed to their antitumoral activity. Sterols and fatty acids also have bioactivity. To elucidate the active components of Lingzhi, ergosterol, a specific component of fungal cell membrane, was rich in *G. lucidum* and *G. sinense*. But its content in *G. lucidum* (median content 705.0 ìg $g^{-1}$, range 189.1-1453.3 ìg $g^{-1}$, n = 19) was much higher than that in *G. sinense* (median content 80.1 ìg $g^{-1}$, range 16.0-409.8 ìg $g^{-1}$, n = 13). The mycochemical profiles and the antioxidant activities of the lipophilic extracts of the white and brown button mushrooms. Showed that only free ergosterol were present in both mushrooms at 2.04-4.82 mg/g dry matter (DM).

Ergosterol concentration was higher in early growth stages but decreased as the mushrooms grew, and it distributed evenly between the caps and stems during early developmental stages but accumulated more in the caps after maturation. The photochemiluminescence (PCL) values of the two mushrooms were 5.49-10.48 nmol trolox equivalent/mg DM, and the EC50 values of 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay ranged 20.19-41.49 mg DM/ìg DPPH. The ergosterol content positively correlated with the antioxidant activities ($r^2$>0.89) recorded by Shao *et al* [16].

## CONCULSION

*G. lucidum* had the highest ergosterol content (403 ì g/g). The observation made during the present study indicated that the mushrooms have medicinal properties like ergosterol, β-glucan which act against pathogenic bacteria.

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# BIOCHEMICAL FEATURES INFLUENCING MUSHROOM-SUBSTRATE COMPATIBILITY

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## ABSTRACT

The capacity of mushroom fungi to utilise the wide range of lignocellulosic substrates adopted for cultivation depends in large part on the production of hydrolytic and oxidative enzymes (cellulases, hemicellulases and ligninases) that degrade the major component macromolecules (cellulose, hemicellulose, lignin). The proportion of these macromolecules in different lignocellulosic materials, and the ability of a given mushroom species to degrade them, can vary significantly. In particular, although the cellulose and hemicellulose components are readily solubilised by the fungal hyphae to provide nutrients for growth, considerable variation exists in the ability of a given species to degrade the more complex and recalcitrant lignin element. This, in turn, is dependent on the different combinations of ligninases (e.g. lignin peroxidases, manganese peroxidases, laccases) produced by the fungus. Thus, while *Volvariella volvacea* is a prolific producer of key cellulolytic enzymes (endoglucanases, cellobiohydrolases, β-glucosidases), among the recognized ligninolytic enzymes it appears able to synthesize only laccases. This is reflected in the ability of the straw mushroom to grow well on cotton wastes consisting largely of cellulose but not on more "woody" materials such as sawdust. Conversely, mushroom species able to synthesize ligninolytic peroxidases (*Lentinula edodes*, *Pleurotus* spp.) can grow on a wider spectrum of lignocellulosic wastes. Also linked to mushroom-substrate compatibility is the enzymic capacity of the former to detoxify harmful compounds (e.g. phenolics) often present in the latter. Using specific examples, this presentation will detail key biochemical features involved in lignocellulose degradation and in the neutralisation of toxic constituents, and will correlate these features to mushroom-substrate compatibility.

**Keywords:** substrate compatibility, cellulases, hemicellulases, ligninolytic enzymes, *Volvariella volvacea, Lentinula edodes*, *Pleurotus*

## INTRODUCTION

Vast amounts of lignocellulosic waste which is the major structural component of woody and non-woody plants, are generated annually through the agricultural, forestry and food processing industries. Edible mushroom cultivation represents an economically-viable system for converting these wastes into an added-value commodity for use either as a nutritious food source or as a source of 'mushroom nutriceuticals' [1]. Consequently, a wide range of lignocellulosic residues have been adopted for this purpose (Table 1).

**Table 1.** Examples of lignocellulosic wastes used for mushroom cultivation

| | |
|---|---|
| Cereal straws | Sawdust |
| Bagasse | Corn cobs |
| Wood pulp | Oil palm waste |
| Cotton wastes | Coconut husks |
| Coffee grounds | Banana leaves |
| Water hyacinth | Tree bark |

Since mushrooms are unable to use solar energy, they synthesize a wide range of extracellular enzymes that allow them to degrade complex organic macromolecules present in the growth substrate into soluble compounds that can be absorbed by the fungus for nutrition ("nutritive absorption"). Both fungal and substrate-associated factors will determine the ability of a given mushroom species to grow and fruit on a particular lignocellulosic material.

Plant cell walls are composed of three major constituents: cellulose, hemicellulose and lignin. Lignin is particularly difficult to biodegrade, and reduces the bioavailability of the other cell wall constituents.Therefore, a key fungal-associated determinant will be the mushroom's capacity to synthesize the hydrolytic and oxidative enzymes required to degrade these macromolecules. Cellulose is a linear polymer of β-1,4-linked anhydrous glucose units, and the basic repeating unit is cellobiose. The chains are packed into arrays called microfibrils that contain both crystalline (ordered) and amorphous

(less-ordered) regions. The simplicity of the cellulosic structure, using repeated identical bonds, means that only a small number of enzymes are required to degrade this material. Hemicelluloses (xylans and mannans) are branched polymers of xylose, arabinose, galactose, mannose and glucose with various side-chain substituents (e.g. galactose, acetyl), attached by different glycosidic linkages. Hemicelluloses bind bundles of cellulose fibrils to form microfibrils, which enhance the stability of the cell wall. They also cross-link with lignin, creating a complex web of bonds which provide structural strength, but also challenge microbial degradation. Lignin is a complex polymer of phenylpropane units (*p*-coumaryl, coniferyl, sinapyl), which are cross-linked to each other with a variety of different chemical bonds (Fig. 1). Gymnosperm lignins are composed mainly of coniferyl alcohol units, angiosperm lignins of approximately equal amounts of coniferyl and sinapyl residues, and grass lignins of approximately equal amounts of all three cinnamyl alcohols [2].

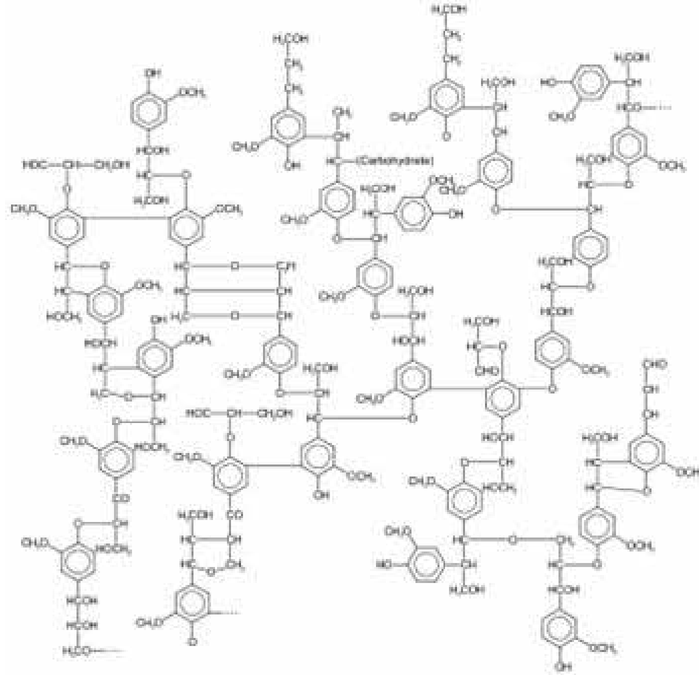

**Figure 1.** Representative section of the lignin macromolecule

Since lignin is the most recalcitrant component of plant cell walls, the higher the proportion of lignin the lower the bioavailability of the substrate. The effect of lignin on the bioavailability of other cell wall components is thought to be largely a physical restriction, with lignin molecules reducing the surface area available to enzymatic penetration and activity [3]. The distribution of cellulose, hemicellulose and lignin in some materials adopted for mushroom cultivation is shown in Table 2.

**Table 2.** Cellulose, hemicellulose and lignin content of some representative materials used for mushroom cultivation

| Residue | Lignin (%) | Cellulose (%) | Hemicellulose (%) |
|---|---|---|---|
| Wheat straw | 18.0 | 30.5 | 28.4 |
| Barley straw | 11.0 | 48.0 | 21.0 |
| Rice straw | 12.5 | 32.1 | 24.0 |
| Cotton straw | 15.0 | 42.0 | 12.0 |
| Cottonseed hulls | 24.0 | 49.0 | 17.0 |
| Bagasse | 18.9 | 33.4 | 30.0 |
| Peanut hulls | 23.0 | 42.0 | 9.0 |
| Ground corn cobs | 7.0 | 28.0 | 55.0 |
| Soy bean hulls | 2.0 | 48.0 | 17.0 |

## Enzymes involved in the degradation of lignocellulosic wastes

Three classes of enzymes catalyse the degradation of the major macromolecular components of lignocellulose, namely cellulases, hemicellulases and ligninases. Conversion of cellulose to glucose requires the activity of three hydrolytic enzymes: endoglucanases (EC 3.2.1.4) (EGs) that attack amorphous regions in the cellulose chain, cellobiohydrolases (EC 3.2.1.91) (CBHs) that release cellobiose units and cello-oligomers, and β-glucosidases (EC 3.2.1.21) (BGLs) that hydrolyse cellobiose and cello-oligomers to glucose. EGs and CBHs are located extracellularly, while three types of β-glucosidase have been reported, i.e. extracellular, intracellular and plasma membrane-bound. Complete breakdown of hemicellulose requires

endo-1,4- β-xylanases (EC 3.2.1.8) and endo-1,4- β-mannanases, which attack the backbone chains of xylans and mannans generating non-substituted or branched xylo- and manno-oligosaccharides, respectively. The main chain substituents are released by the corresponding glycosidases and esterases. Extracellular enzymes involved in lignin transformation are lignin peroxidases (EC 1.11.1.14) (LiPs), manganese peroxidases (EC 1.11.1.13) (MnPs), versatile peroxidases (EC 1.11.1.16) (VP, hybrid peroxidase, polyvalent peroxidase) [4] and laccases (EC 1.10.3.2). LiPs oxidizes nonphenolic lignin substructures by abstracting one electron and generating cation radicals that are then decomposed chemically [5]. MnPs oxidize Mn (II) to Mn (III) which then oxidizes phenolic rings to phenoxyl radicals which lead to decomposition [6]. VPs, although catalyzing the oxidation of $Mn^{2+}$ to $Mn^{3+}$ by $H_2O_2$, differ from the above enzymes by their manganese-independent activity enabling them to oxidize substituted phenols by a mechanism similar to that reported for LiP. Laccases oxidized phenols to phenoxyl radicals as well as catalyzing other reactions in the presence of mediators [7]. Based on the enzyme production patterns of a range of lignin-transforming fungi, three categories have been proposed: (i) lignin-manganese peroxidase group (ii) manganese peroxidase-laccase group and (iii) lignin peroxidase-laccase group [8]. The most efficient lignin degraders, based on the release of $^{14}CO_2$ from $^{14}C$-[Ring]-labelled synthetic lignin (DHP), fall into the first group. Many of the most selective lignin-degrading fungi belong to the second, although they have only moderate to good [$^{14}C$]DHP mineralizing ability. The lignin peroxidase-laccase fungi degrade [$^{14}C$]DHP relatively poorly [8].

## Lignocellulose degradation by cultivated mushroom species

Lignocellulose degradation by several edible fungi species and, in some cases, associated enzyme production and corresponding gene expression, has been well documented including *Pleurotus* species [4, 9, 10], *Agaricus bisporus* [11], *Lentinula* (*Lentinus*) *edodes* [12-14], *Grifola frondosa* [15-17] and *Volvariella volvacea* [18, 19]. Camarero *et al.* [4] reported the secretion of two peroxidases in lignocellulose cultures of *Pleurotus eryngii*, a manganese-dependent peroxidase similar to that first described in *Phanerochaete chrysosporium* [6], and a second protein (PS1) that catalyzed both the oxidation of Mn (II) to Mn (III) by $H_2O_2$ and oxidized substituted phenols by a manganese-independent activity similar to that reported for LiP. Addition of Mn (II) to cotton stalks enhanced the preferential degradation of lignin by *Pleurotus ostreatus* (oyster mushroom) [20]. Releasing Mn (II) suppression of a versatile peroxidase by over-expression of the MnP4 gene (one of the nine members of the MnP gene family that constitutes part of the ligninolytic system of *P. ostreatus*) improved its ligninolytic functionality [21]. Conversely, inactivation of the versatile peroxidase-encoding gene (*mnp2*) resulted in reduced lignin degradation by this fungus [22]. Recent sequencing of the *P. ostreatus*genome has revealed the absence of LiPs and the presence of three VPs and six MnPs [23].

Lignin peroxidase activity was not detected in the compost extracts used to cultivate *A. bisporus*. However, the correlation between manganese peroxidase and laccase activities and the decomposition of radio labelled lignin and synthetic lignins by *A. bisporus* suggested significant roles for these two enzymes in lignin degradation by this fungus [11].

Unlike the aforementioned mushrooms, *V. volvacea* appears to lack key enzymes required to degrade the lignin component of highly lignified substrates [18]. This may account in part for the relatively low biological efficiency (i.e. conversion of growth substrate into mushroom fruit bodies) of the straw mushroom, about 10% on rice straw but up to 40% following the introduction of high cellulose cotton waste 'composts' [24]. Of the recognized lignin-modifying enzymes, only laccase production is well-documented although the *V. volvacea* genome contains two putative MnP and two VP

**Table 3.** Key fungal enzymes catalyzing degradation of the main components of lignocellulose

| Lignocellulose component | Key enzymes |
|---|---|
| Cellulose | Endo-1,4-glucanases<br>Cellobiohydrolases<br>â-Glucosidases |
| Hemicellulose | Endo-1,4-â-xylanases<br>Endo-1,4-â-mannases<br>â-Xylosidases<br>â-Mannosidases<br>Side-chain-releasing glycosidases<br>p-Coumaryl/feruloylesterases |
| Lignin | Manganese-dependent peroxidases<br>Versatile peroxidases<br>Lignin peroxidases<br>Laccases |

encoding genes but no LiP gene sequences [25]. Interestingly, eleven genes (*vv-lac1* to *vv-lac11*) encoding laccase homologues were identified, five of which were identical to laccase genes reported in previous studies [26, 27]. However, the straw mushroom is equipped with the key enzymes necessary to hydrolyse cellulose; i.e. EG, CBH and BGL. Several different isoforms of these enzymes are synthesized when *V. volvacea* is grown in the presence of a cellulosic substrate in both submerged culture in the laboratory, and in solid-state cultivation systems representative of those used for industrial cultivation [19, 28-30]. The key fungal enzymes catalyzing degradation of the main components of lignocellulose are listed in Table 3.

**Figure 2 (A-C).** Cultivation of edible mushrooms on lignocellulose

A: *Lentinula edodes* on artificial sawdust logs, B: *Pleurotus eryngii* on a mixture of corncobs and sawdust, C: *Volvariella volvacea* on cotton waste.

### Detoxification of phenolic residues in lignocellulosic substrates used for mushroom cultivation

Lignocellulosic wastes of the type used for mushroom cultivation often contain phenolic monomers [31], some of which are reported to inhibit fungal growth [32] and the hydrolytic enzymes that catalyze the breakdown of cellulose and hemicellulose [33, 34]. In addition to a direct role in lignin transformation, laccase may also function in the polymerization of toxic phenols, and possibly other highly reactive species generated during lignin breakdown, from which the fungal mycelium must be protected [35]. This may explain the specific induction of laccase proteins by phenols and assign functionality to the numerous laccase isoforms identified in *V. volvacea* (Fig. 3) and many other fungi.

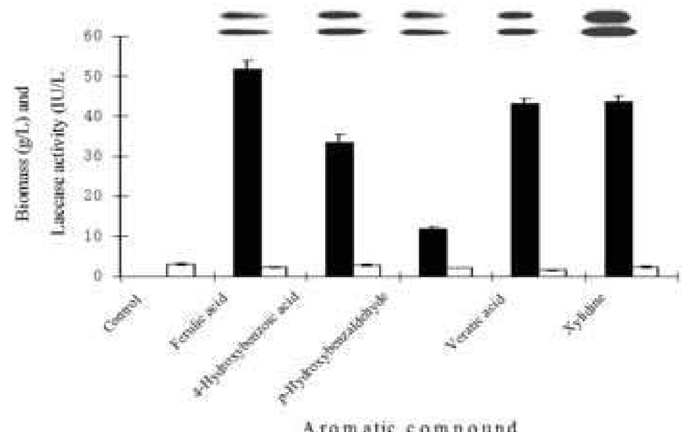


**Figure 3.** Induction of *Volvariella volvacea* laccase isoforms by aromatic compounds

A direct relationship has been established between lignocellulolytic enzyme profiles and the capacity of an individual mushroom species to grow and fruit on a particular lignocellulosic substrate. More information about the ligninolytic systems of other cultivated mushrooms, combined with the astute observations and fine-tuning of growers, will inevitably lead to greater mushroom-substrate compatibility and increased fruit body yields.

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# PRODUCTION OF SPAWN FOR LIGNICOLOUS MUSHROOMS IN A CLEAN ROOM- ENVIRONMENT: RISK ANALYSIS AND RULES OF CONDUCT

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## ABSTRACT

The aim of spawn producers is to deliver axenic mycelium with a constant quality, which is a prerequisite for a good mushroom yield. Very often, mycelium producers have a poor knowledge as how to estimate the microbiological quality of their working environment or how to implement their knowledge in a real-time situation. This leads to the building and organization of production facilities with an insufficient level of security. A lot of problems in mycelium production can be brought back to an incorrect product flow, as well as to wrong rules of conduct. The aim of this presentation is to analyze a number of problems, while offering a remedy for the latter, so as to prevent financial disasters.

**Keywords:** spawn production, mycelium, inoculum, axenic, sterilization

## INTRODUCTION

The production risks for mushroom spawn are often underestimated. It is a technologically highly advanced process, where scientific background knowledge is of utmost importance. There are many aspects to the production of mycelium, such as substrate components, the pretreatment and heat treatment of the substrate, hygiene before, during and after the time of inoculation, quality of the inoculum, climate and other conditions during incubation and maturation. Whenever problems arise, one must be able to analyze the causes correctly and find solutions within the shortest delay.

Spawn facilities involve considerable investment. As one seeks a proper return on investment, there is not much room for errors once production has started. As a rule, new facilities experience little problems, even if the infrastructure and working rules are not perfect. But after some time, pests and diseases can seek their way into the facilities, infection pressure builds up, resulting in a dangerous vicious circle of reinfection. Eventually, this results in loss of products, low yields or no output at all, the consequences of which are often disastrous. Such situations are encountered at large and small projects alike all over the world. The present discussion is to achieve two goals: (i) assist the spawn producer to find an affordable and efficient problem-solving method (ii) help new projects in preventing these problems.

## DISCUSSION

### Situation sketch

There are worldwide considerable differences in approach regarding mycelium production. Some of the larger facilities in the EU and the US use a bulk production system, but most spawn facilities handle individual portions, ranging from less than one liter to as much as 10 liters. The used recipients are mainly bottles and bags, which are provided with various closing and breathing systems, the one being more efficient than the other.

Ideally, the spawn production chain should be a strictly controlled activity, operated by highly skilled professionals. In most cases however, production methods are more hands-on, and for that reason more risky. As high competition makes financial margins very tight, mistakes are not affordable. But they do occur, not only with respect to the layout and infrastructure of the facility but also regarding mycelium production technology.

This article indicates that nearly each faulty analysis can be traced back to one single common denominator: lack of implemented scientific knowledge. Mycelium producers are not always scientifically trained and even if they are, that does not necessarily mean that they are familiar with the reality of industrial production, enabling them to make a proper problem analysis.

**What kind of knowledge is to be considered essential?**

In industrial mycelium production, one shall be aware that problems are not only situated at the macroscopic, but also at the microscopic and submicroscopic level, and that all three levels are equally important. A production unit can only be efficient when the layout and infrastructure of the building, as well as the working method are determined according to the understanding of this full spectrum.

One needs to have a good insight in both fungal and bacterial biology. Many bacteria, as well as so-called weed moulds, have the same specific requirements as the cultivable mushrooms. Competition is fierce, and when undesired microorganisms and mycelium of cultivable mushrooms are both present in the same substrate, the latter will mostly lose the battle. Therefore, mycelium must be protected during all phases of multiplication, where infection pressure comes into play.

Air is never empty, it is loaded with dust particles and propagules, which may float for an extended period of time. Dust is a major source of infections, especially if this dust is coming from an area where spores are produced. Examples are agricultural areas, harvest areas, animal production facilities, open-air fermenting installations and mushroom production areas. Dust is an assemblage of fine particles, and can contain anything fine enough to float, including spores and bacteria. Generally spoken, the infection pressure is proportional to the dust concentration.

But dust is not the only source of contamination. Every person and every object is by nature a producer and carrier of microorganisms, which are invisible to the eye but ever-present. They are stuck to the surface and are easily shed so as to become an airborne particle.

Whatever the source may be, when conditions are favourable, the unwanted microorganisms will start to grow and multiply. When reaching their sporulating phase, weed moulds will generate countless spores in a very short time frame, and these airborne spores will add to the contamination pressure. It is essential to understand that, no matter the location or surroundings, this infection risk is omnipresent in every production facility. For that reason, a number of safeguards are needed to keep the risk as low as possible.

A profitable mycelium production facility has control over the following safeguards at any time. If any one of them is neglected, contamination problems will arise, so as to lead to a vicious circle, in which there is no chance to succeed industrial spawn production.

**Sterilization of the raw materials**

An efficient sterilization procedure is the first and most important safeguard in the spawn production cycle. In its essence, it eliminates all living organisms, whilst limiting the qualitative degradation of the substrate.

Most spawn producers are fully aware of the risks of a bad sterilization. But what is often underestimated is the need to avoid re-infection of the hygienized substrate after the heat- treatment.

**Avoidance of re-infection from various origins**

- From airborne microorganisms: aids such as laminar air flows (LAF) and HEPA-filtered air through overpressure units minimize dust levels, and equally airborne microorganisms in the air.
- From various infection sources: a series of precautions shall be taken to isolate potential sources of infection from the hygienized substrates during cooling and inoculation. These include protective clothing for the production staff, manufacturing in clean room areas and product flow management.
- From non- suspended microorganisms: for example microorganisms stuck to machines, shoes, floors, walls, ceilings and people. A number of effective disinfection products are on the market, which can be used for surface disinfection.

But it is not all about preventing infections. To get the best return on investment, one should make his facility optimally profitable through having a full understanding of efficient mycelium production techniques, and this deserves an equal amount of attention.

First of all, the spawn recipes must be standardized, prepared and packed carefully, in the right type of breathable packaging. The sterilization cycle should 100% effective without over- sterilizing. Subsequently, the hygienized substrate shall be inoculated properly, the bag well sealed and placed in the most favourable climatic conditions so as to reach an optimal incubation. The final product shall be kept refrigerated at all times. And all the while, the end product must be traceable and controllable (Royse, 1997; Sanchez, 2004)

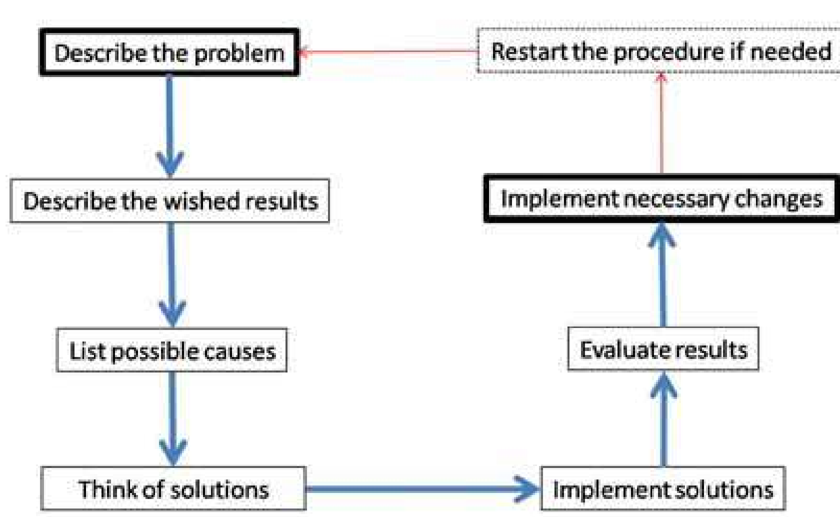
The ensemble of implication of safeguards and production methods make up a set of rules, which, four ease of understanding, we shall henceforth call 'rules of conduct'; (Willig, 2005).

**A correct problem analysis is essential in problem prevention**

It is not enough to establish that a certain percentage of products has been infected or that mushroom production has dwindled, one should also be able to detect the reasons behind it. We plead for a scientific problem solving method, as it is the most certain way to identifying solutions. Any problem can be related to a large number of causes and it is only through sharpness of analysis that one finds out the true nature of the problem.

The biggest obstacle to proper problem observation is the invisibility of spores and bacteria. Especially when upscaling a production unit, mycelium producers with insufficient understanding of the microscopic and submicroscopic level tend to make wrong conclusions. The susceptibility to infections of freshly sterilized substrates is often underestimated, and therefore ignored. One should realize that only regular maintenance of ultrafilter systems and strict hygiene measures will lead to success.

A true scientific problem solving analysis follows a standardized line of thought, which is depicted below. This method, which is self-evident for anyone with a scientific background, is based on a logic which follows a circular motion. In the case of problem solving, it starts with proper observation, then moves on to postulating a possible cause and their possible solutions, implying those solutions and verifying the outcome. If the outcome is good, those changes should be implemented and if the problem is still present, the procedure repeats itself.




**Transfer of know how**

The success of the analysis will depend on personal capabilities and on knowledge of Good Manufacturing Practices (GMP). But how does one achieve a good level of theoretical and practical knowledge? There are two solutions to this problem: (i) trial-and-error and (ii) transfer of know-how.

Trial-and-error is a process, which every company at some stage will have to face, and this is even more so for the mushroom sector. As compared to plant cultivation, it is a relatively 'new' type of horticulture, and the sector still faces many unanswered questions. As a result, there are not many databases or practical tutorials on mushroom cultivation, let alone spawn production. Anyone not having access to such a

database has only one choice of gaining experience: trial-and-error. Unfortunately, this is a time and money consuming system, and one should avoid it whenever possible.

The second solution is the transfer of know-how. There are a number of ways through which information is distributed, of which the most important are (i) internet, (ii) books, (iii) personal training and (iv) trouble shooting.

Information on the internet is scarce and often inaccurate. High-quality knowledge is mostly expensive, non-transparent or simply non-available. Making this information available represents one of today's largest challenges for the mycelium sector.

Books are a good source of knowledge, if they are written by scientifically trained and experienced professionals. However, for the starting mycelium producer it is difficult to estimate the quality of the provided information.

Personal training is probably the most effective way of learning the proper rules of conduct. Only a few experienced technologists are ready to distribute knowledge and provide training for an affordable price, whether through personal or group courses. The advantage is that for a limited investment, one gains access to a large amount of non-publicly accessible production data. If such training is followed before the production has started, the benefits are countless.

Trouble shooting also provides essential information, but unfortunately only after problems have arisen. In such cases, an external expert with extensive technical and practical knowledge is invited to the trouble site. In cooperation with the production responsible, he will start a proper problem analysis on the spot, and make proposals with respect to the improvement of infrastructure and rules of conduct.

All mentioned information channels have their own benefits, but the most efficient is a combination of all four.

## CONCLUSION

Mycelium producers face very specific challenges, as it is a technologically advanced, high-risk business. Financial margins are tight and for this reason, projects often save on important investments such as hygiene and know-how. Wrong assessment will most certainly lead to failures, irrespective the size of the production facility. Although some problems can be traced back to a lack of practical knowledge, most are due to wrong implementation of the safeguards that prevent substrates from being infected by so-called weed fungi and bacteria.

Magda Verfaillie, a biologist and mycologist, is the founding manager of Mycelia, a leading spawn production and technology training company from Belgium, Europe. Throughout the years, the company has been involved in numerous projects involving spawn production. Through professional trouble-shooting, and after having trained a large number of people in the sector, it has become clear that most problems can be traced back to a lack of basic knowledge.

Anyone who already is, or is planning to be involved in mycelium production should be aware of its challenges, and not underestimate the complexity of the process. Before starting, one should seriously consider to acquire the essential scientific and practical knowledge needed for continued success.

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# BIOTECHNOLOGICAL POTENTIAL OF TEN *PLEUROTUS DJAMOR* STRAINS

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## ABSTRACT

With the aim of knowing some capabilities of biotechnological interest of the edible mushroom *Pleurotus djamor*, ten strains of this species were characterized. Besides mycelial growth and sporophore production, laccase (on ABTS), Mn-peroxidase (on dimethoxyphenol), aryl alcohol oxidase (on veratryl alcohol) and phenol oxidase activities (on catechol) were determined. Also the antioxidant capacity was determined. Reported radial extension rate varied between 1.12 mm/d (*P. djamor* ECS-0150) and 3.9 mm/d (*P. djamor* ECS-0143). Biological efficiency, production rate, performance in two harvests varied between 36.4 and 71.4%, 1.14 and 2.1% and 0.033 and 0.069, respectively. All the strains tested have at a certain degree, laccase, Mn-Peroxidase and phenol oxidase, except for aryl alcohol oxidase. The strain ECS-0123 showed important ligninolytic activity, good antioxidant capacity, good growth and an average capacity to produce sporocarps (mushrooms).

**Keywords**: oyster mushroom, ligninolytic enzymes, antioxidants, edible mushrooms

## INTRODUCTION

Among the species in the genus *Pleurotus*, which includes edible mushroom species, *P. djamor* is one of the most commonly found mushroom species in the tropical areas of the world. *P. djamor* is reported more frequently in the wild in Mexico than any other species of this genus [1, 2]. Although *P. djamor* is recognised as edible, harvested for family consumption and sold in local markets, this species is not used in commercial cultivation, which is in contrast to the other commercially exploited species in the genus, such as *P. ostreatus* and *P. pulmonarius*. The cumulative production of these two mushroom species is the second highest in the country after *Agaricus bisporus* [3].

There is little information on tropical macrofungi, although the tropics are one of the most biologically diverse biomes. Therefore, studying strains that originate from the Soconusco region of southeastern Mexico is important. *P. djamor* specimens are commonly found in the wild in this under studied region, which has a high biodiversity [4].

White rot fungi are becoming more important because of their enzymatic potential. These mushrooms are known to degrade lignin and have recently been investigated for their antioxidant capacity. Fungi such as *P. sajor-caju* and *P. salmoneostramineus* have been reported to have a significant antioxidant capacity [5, 6]. These reports have increased the interest in the genus and in *P. djamor* in particular, which has a wide distribution, is able to degrade lignin and has the potential for use in the degradation of difficult compounds. The present work studies characteristics of ten *P. djamor* strains originating from Chiapas, Mexico that are of biotechnological interest. This characterisation includes the quantification of ligninolytic and antioxidant activities and production of sporocarp. These strains are maintained in the collection of macrofungi of Ecosur and have been deposited at the National Centre for Genetic Resources (CNRG in Spanish) of Mexico. The CNRG is an organisation that preserves microbial germplasm and native macrofungi of interest and has been in operation since 2012.

## MATERIALS AND METHODS

### Strains and preservation media

The *Pleurotus djamor* strains ECS-0122, ECS-0123, ECS-0127, ECS-0130, ECS-0139, ECS-0141, ECS-0143, ECS-0144, ECS-0150 and ECS-0151 originating from the state of Chiapas, Mexico were used in this study. Two *P. ostreatus* commercial strains (ECS-0152 and ECS-1123) from the collection of macrofungi of the College of the Southern Border (Ecosur) were used for comparison purposes. Strains that were preserved in distilled water at 4 °C were reactivated in malt extract liquid medium (Bioxon).

### Radial extension rate

The radial extension rate (RER) was measured in a Petri dish with malt extract agar (Bioxon) at pH 6. Agar fragments of 0.5 cm in diameter were colonised by each strain and seeded in the centre of a 15 x 100 mm Petri dish with a sterile medium. The dishes were then incubated for 10 days at 25 °C. The daily growth radius attained by the colony was marked under each dish in two perpendicular directions. The experiment was repeated five times for each strain, and the RER was calculated using the formula of Sinclair and Cantero [7] in which RER = [(ln X3 – ln X1)/2] [X2].

### Ligninolytic activity

The ligninolytic activity was determined for the mycelium and sporocarp. For the mycelium, 10 g of sorghum seeds were colonised by each strain, and an extract was prepared. This material was macerated in a mortar with 20 ml of a specific buffer for each enzymatic reaction and filtered through a cloth. The extract was then filtered through Whatman filter paper No. 6 and centrifuged at 5,000 rpm for 10 minutes. To measure the ligninolytic activity of the sporocarp of each strain, 5 g of pileus were freshly harvested, macerated in 10 ml of buffer and extracted as described above. The phenol oxidase activity was measured with catechol ($\delta_{420nm}$ = 3.45 $mM^{-1}cm^{-1}$) in a phosphate buffer at pH 7 [8]. The laccase activity was determined with an ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate ($\delta_{436}$ = 29.3 $mM^{-1}cm^{-1}$) in sodium acetate at pH 4.5 [9]. The manganese-peroxidase (MnP) activity was measured with dimethoxyphenol ($\delta_{470}$ = 49.6 $mM^{-1}cm^{-1}$) in the presence of $H_2O_2$ in a succinate buffer at pH 5 [10]. Finally, the aryl alcohol oxidase activity of veratryl alcohol ($\delta_{310}$ = 9.3 $mM^{-1}$ $cm^{-1}$) was determined in a sodium phosphate buffer at pH 6 [11]. A unit was defined as the amount of enzyme forming 1 ìmol of the product in one minute under these conditions.

### Antioxidant activity

The antioxidant activity was estimated in the harvested mycelium and sporocarp by determining the total phenols using the method described by Folin Ciocalteu. The activity was expressed as the equivalent of gallic acid [12]. The free radical scavenging activity was also determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described by Fukumoto and Mazza [13].

Finally, the taxonomic characterisation was performed on the harvested fruit bodies according to descriptions from the literature [1, 14-16].

### Production assays

Fruiting assays were performed by seeding an inoculum prepared on sorghum grain [17] (2.5 wt%) in batches of one kilogram of *Digitaria decumbens* Pangola grass with 2% lime and 70% humidity. This substrate was pasteurised at 90 °C for one hour using steam. Production (P) was determined as the weight in grams of mushrooms harvested in two waves, biological efficiency (BE) was calculated as BE = (fresh mushrooms harvested / dry substrate used) x 100 and the production rate (PR) was calculated as PR = (BE / days from planting to harvest) x 100 for the 10 strains studied. The yield or Performance (P) was calculated as the ratio of the grams of dry fungi obtained for the two crops to the dry weight of the substrate used.

## Statistical analysis

In all of the studies, a random model with five repetitions was used. Variance was analysed and the mean separation was calculated using Tukey's test at $\alpha = 0.05$. Analyses were performed using the JMP program version 4.0 from SAS Institute (Campus Drive Cary, NC 27513 USA 1998).

## RESULTS

## Radial extension rate

Figure 1 shows the rate of radial expansion of the strains studied. The values ranged between 1.12 and 3.9 mm/d for the strains ECS-0150 and ECS-0143, respectively. Group A was formed by the fastest strains and consisted of *P. ostreatus* strains ECS-0152 (control, 4.15 mm/d) and *P. djamor* strains ECS-0143 and ECS-0123. Radial extension values for ECS-0143 and ECS-0123 ranged between 3.69 and 4.15 mm/d, respectively.

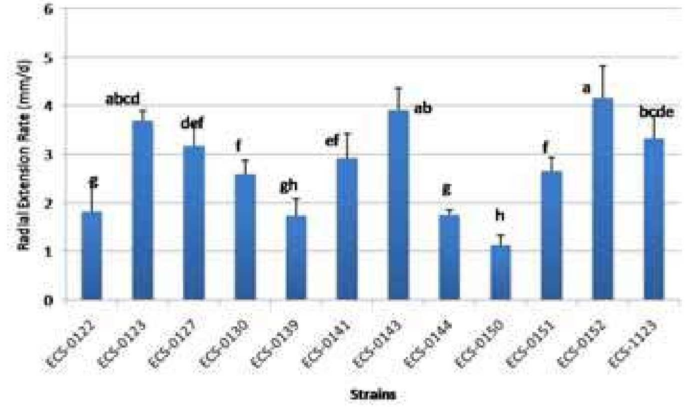


**Figure 1.** Radial extension rate (mm/d) of 10 *Pleurotus djamor* strains compared with two control strains (*P. ostreatus* ECS-0152 and ECS-1123) on MEA at 25 °C, pH 6. Mean of five repetitions. Different letters indicate significant statistical differences between strains (Tukey's test, $\alpha$=0.05).

## Ligninolytic activity

Table 1 shows the results of the ligninolytic activity tests performed on the extracts of the harvested mycelium and sporocarp. These tests determined the phenoloxidase, MnP, laccase and aryl alcohol oxidase activities for the two extracts of each strain.

**Table 1.** Ligninolytic activity (phenol oxidase, Mn-peroxidase, laccase and aryl alcohol oxidase) in mycelium and sporocarp extracts of 10 *Pleurotus djamor* strains and two control strains (*P. ostreatus* ECS-1123 and ECS-0152). Substrate (and pH) of each test: catechol (7), DMF (5), ABTS (4.5) and veratryl alcohol (6), respectively, at 25 ºC (U/ml). Mean of three repetitions

| Strain | Phenol oxidase | | Mn-Peroxidase | | Laccase | | AAO |
|---|---|---|---|---|---|---|---|
| | Mycelium | Sporocarp | Mycelium | Sporocarp | Mycelium | Sporocarp | Sporocarp |
| ECS-0122 | 6.42±0.27 CD | 70.92±0.61 D | 0.23±0.09 F | 0.16±0.03 D | 99.48±1.38 DE | 41.75±0.35 G | 1.63±0.02 DE |
| ECS-0123 | 7.08±0.79 BC | 7.78±0.16 F | 13.19±1.8 B | 0.67±0.01 D | 546.2±4.5 A | 91.30±2.25 B | 2.65±0.01 D |
| ECS-0127 | 0.65±0.07 G | 0.38±0.10 G | 8.23±1.04 C | 0.06±0.00 D | 105.49±0.83 D | 28.33±0.78 I | 1.16±0.02 DEF |
| ECS-0130 | 2.74±0.33 F | 1.01±0.37 G | 6.37±0.19 CD | 9.87±0.77 B | 79.54±17.6 FG | 47.10±3.11 F | 0.12±0.02 F |
| ECS-0139 | 4.76±0.50 DE | 130.18±2.89 B | 17.10±1.7 A | 4.89±0.16 C | 80.04±1.91 EF | 76.68±1.72 C | 4.44±0 C |
| ECS-0141 | 7.74±0.16 BC | 0.59±0.06 G | 6.81±1.3 C | 9.04±0.95 B | 60.19±1.73 G | 93.94±2.50 B | 0.01±0 F |
| ECS-0143 | 4.95±0.14 DE | 1.48±0.18 G | 2.81±0.31 EF | 11.23±0.13 B | 101.66±5.79 D | 33.14±0.84 H | 0.25±0 F |
| ECS-0144 | 22.69±1.43 A | 96.55±0.46 C | 14.79±0.79 AB | 1.53±0.09 D | 106.27±1.46 D | 56.84±0.27 E | 18.8±0.9 A |
| ECS-0150 | 8.88±1.28 B | 93.57±0.74 C | 3.58±0.06 DE | 0.28±0.05 D | 259.5±11.3 B | 55.08±0.43 E | 7.47±0.53 B |
| ECS-0151 | 4.95±0.71 DE | 183.31±0.67 A | 6.70±0.38 C | 4.52±0.09 C | 231.8±3.9 C | 107.92±0.39 A | 8.31±0.01 EB |
| ECS-0152 | 3.54±0.19 EF | 48.63±1.46 E | 1.91±0.65 EF | 15.60±2.52 A | 35.69±3.18 H | 28.62±0.85 I | 0.34±0.02 EF |
| ECS-01123 | 3.69±0.14 EF | 0.57±0.23 G | 2.87±0.41 EF | 1.12±1.02 D | 25.76±0.05 H | 8.25±0.43 J | 0.10±0.05 F |
| p | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |

Same letter in the same column indicate significant statistical differences in enzymatic activity between strains after Tukey's test ($\alpha$= 0.05).

The assessment of phenoloxidase activity in the extract from the fungal mycelium showed levels of activity between 0.65 and 22.6 U/ml. The statistical analysis showed significant differences between the strains ($p = 0.0001$) and separated the strains in seven groups. Strain ECS-0144 had the highest phenoloxidase activity (22.7U/ml) and formed group A. The two *P. ostreatus* strains used as the control were placed in the groups with the lowest activities (groups E and F with activities ranging from 3.54 to 3.69U/ml). *P. djamor* ECS-0127 was the strain with the lowest activity (0.65U/ml). The phenol oxidase activity measured in the harvested sporocarp varied between 0.38 U/ml (strain ECS-0127) and 183.3U/ml (strain ECS-0151). The statistical analysis showed significant differences between the strains ($p = 0.0001$) and separated the strains in seven groups. Six strains (ECS-0151, ECS-0139, ECS-0144, ECS-0150, ECS-0122 and ECS-0152) included in the statistical groups A to E had activity levels higher than 48.6 U/ml in the sporocarp extracts. These values were considerably higher in the sporocarp extract than in mycelium extract.

The MnP activity observed in the mycelium extract ranged from 0.23 U/ml (strain ECS-0122) to 17.1 U/ml (ECS-0139 strain). The statistical analysis found significant differences ($p = 0.0001$) and formed six groups. ECS-0139 was the most active strain and different from the other strains. The MnP activity in the pileus extracts ranged from 0.06 U/ml (strain ECS-0127) to 15.6 U/ml (control strain *P. ostreatus* ECS-0152). The statistical analysis found significant differences between the strains and formed four groups ($p = 0.0001$). In this case, the activity of the control strain *P. ostreatus* ECS-0152 was statistically superior to all of the *P. djamor* strains.

The laccase activity determined in the mycelium extracts ranged from 25.7 U/ml (control strain *P. ostreatus* ECS-1123) to 546.2 U/ml (strain ECS-0123, group A). The statistical analysis separated the strains into eight groups (p = 0.0001) in which groups B and C (strains *P. djamor* ECS-0150 and ECS-0151) showed activities ranging between 259.5 and 231.8 U/ml, respectively. In the sporocarp extract, differences between the strains were significant ($p = 0.0001$), but the values were lower and ranged from 8.25 U/ml (strain ECS-1123, group J) to 107.92 U/ml (strain ECS-0151).

The aryl alcohol oxidase activity detected in the mycelium extract was almost equal to zero, and the analysis found no significant differences between the different extracts ($p = 0.4238$). The aryl alcohol oxidase activity in the sporocarp extract showed values that ranged from 0.01 U/ml (strain ECS-0141, statistical group F) to 18.8 U/ml (ECS-0144 strain, group A) ($p = 0.0001$).

**Antioxidant activity**

The total phenolic content and antioxidant activity measured by the DPPH test are shown in Table 2. Strain ECS-0141 had the lowest total phenolic content in the mycelium extract (229 eq. gal. ac/g, group D). Four statistical groups were defined according to Tukey's test ($p = 0.0001$). Strain ECS-0123 had the highest value at 517.8 eq. gal. ac/g, which was significantly different from the other groups. Strain ECS-0143 had the lowest content of antioxidant in the sporocarp extract (54.1 eq. gal. ac/g). Group A had the highest antioxidant values, with contents ranging between 214.9 and 231 eq gal. ac/g. Group A was formed by *P. djamor* strains ECS-122, ECS-0139, ECS-0144, ECS-0151 and the *P. ostreatus* control strains ECS-0152 and ECS-1123.

The free radical scavenging activity of the mycelium extract was measured using DPPH and varied between 3.73 and 38.11%. The statistical analysis showed significant differences ($p = 0.0001$) and identified four groups. Group A had the highest values and was comprised solely of the extract of *P. ostreatus* strain ECS-1123 (38.1% activity). The other control strain (ECS-0152) formed the second group with the highest values (group B, 20.75%). The *P. djamor* strain ECS-0139 formed the third group (group C, 10.72%). The three groups were significantly different from each other, and the remaining strains were classified in group D with values between 3.73 and 5.82%. The free radical scavenging activity of the sporocarp extract varied between 8.85 and 41.4%. The statistical analysis identified three groups ($p = 0.0003$). Strains ECS-0151, ECS-0144, ECS-0143, ECS-0139, ECS-0123 and ECS-0122 formed group A and had the highest values. The two control strains had the lowest values and belonged to group C. All of the *P. djamor* strains had average activities that were substantially higher in the sporocarp extract than in the mycelium extract. However, *P. ostreatus* had similar or even lower activities in the sporocarp extract than in the mycelium extract.

**Table 2.** Antioxidant activity (measured as total phenols and by the DPPH reaction) of mycelium and sporocarp extracts of 10 *P. djamor* strains, compared with two commercial strains *P. ostreatus* ECS-0152 and ECS-1123. Mean of 3 repetitions

| **Strains** | **Total phenols (eq. gal. ac/g)** | | **DPPH(%)** | |
|---|---|---|---|---|
| | **Mycelium** | **Sporocarp** | **Mycelium** | **Sporocarp** |
| ECS-0122 | 341± 12.0 C | 223.1 ±1.0 AB | 3.84±1.15 D | 34.4±12.1 AB |
| ECS-0123 | 517.8± 15.3 A | 199.5±5.9 D | 4.54±0.16 D | 34.7±0.32 AB |
| ECS-0127 | 426.4± 31.4 B | 208.8±7.4 CD | 5.82± 0.33 D | 23.4±4.1 BC |
| ECS-0130 | 309.6± 24.0 CD | 73.0±2.0 E | 4.43± 0.32 D | 18.8±1.6 BC |
| ECS-0139 | 252±4.9 D | 219 ±2.0 ABC | 10.72± 0.98 C | 34.9±4.9 AB |
| ECS-0141 | 229.0±29.5 D | 56.5±0.7 F | 4.43±1.31 D | 19.3±1.6 BC |
| ECS-0143 | 351.6±32.3 BC | 54.1±3.1 F | 4.19±0.33 D | 25.8± 0 AB |
| ECS-0144 | 351.6±38.3 BC | 214.9±4.6 ABC | 3.96±1.97 D | 26.8± 0.32 AB |
| ECS-0150 | 348.3 ± 46.7 BC | 210.2±9.0 BCD | 5.59±0 D | 24.24± 1.9 BC |
| ECS-0151 | 273± 18.8 CD | 219.2±2.3 ABC | 3.73±0.66 D | 41.0± 3.2 A |
| ECS-0152 | ND | 231.3±4.2 A | 20.75±0.98 B | 18.41± 0 BC |
| ECS-1123 | ND | 219.8±1.3 ABC | 38.11±0.82 A | 8.85± 0.32 C |
| p | 0.0001 | 0.0001 | 0.0001 | 0.0003 |

ND: non determined
Different letters in the same column indicate significant statistical differences (Tukey's test, α= 0.05)

## Sporocarp production

Table 3 presents the production of sporocarp (S) in one kilogram of Pangola grass with 70% humidity for two crops as well as the biological efficiency (BE), production rate (PR) and performance (P) of the strains tested. The values varied between a minimum of S = 108 g, BE = 36.4%, PR = 1.14% and P = 0.0336 for the strain ECS-0151 and a maximum of S = 212.2 g, BE = 71.4%, PR = 2.06% and P = 0.069 for the strain ECS-0130. The statistical analysis defined three statistical groups for S, BE and P values. Strain ECS-0151 had the lowest values and was significantly different from the other

**Table 3.** Production variables after two flushes (Sporocarp production, Biological efficiency, Production rate and Performance) of 10 *Pleurotus djamor* strains, compared with two *P. ostreatus* strains ECS-0152 and ECS-1123, grown in 1 kg bags of Pangola grass with 70% moisture and 2% calcium hydroxide, at 22 ºC. Mean of five repetitions

| **Strain** | **Sg/bag** | **BE (%)** | **PR(%)** | **P($10^{-2}$)** |
|---|---|---|---|---|
| ECS-0122 | 125.0±38.9BC | 42.1±13.1 BC | 1.36±0.39 AB | 04.6±1.5 AB |
| ECS-0123 | 192.1±45.7ABC | 64.7±15.3 ABC | 1.94±0.32 A | 6.0±1.5 AB |
| ECS-0127 | 191±32.2ABC | 64.3±10.8 ABC | 1.62±0.44 AB | 6.02±0.8 AB |
| ECS-0130 | 212.2±65.9 A | 71.4±22.1 A | 2.06±0.18 A | 6.96±1.7 A |
| ECS-0139 | 149.2±41.6ABC | 50.2±14.0 ABC | 2.1±0.25 A | 6.5±0.17 A |
| ECS-0141 | 159.8±51.6ABC | 53.8±17.3 ABC | 1.64±0.55 AB | 5.16±1.6 AB |
| ECS-0143 | 174.6±24.8ABC | 58.8±8.3 ABC | 2.04±0.30 A | 6.6±1.1 A |
| ECS-0144 | 127.8±47.6ABC | 43.0±16.0 ABC | 1.48±0.56 AB | 4.76±1.1 AB |
| ECS-0150 | 188.6±15.8ABC | 63.5±5.3 ABC | 1.92±0.16 A | 5.9±0.7 Ab |
| ECS-0151 | 108.0±32.6 C | 36.4±11.0 C | 1.14±0.35 AB | 3.36±1.1 B |
| ECS-0152 | 208.4±22.8ABC | 69.4±7.6 AB | 1.74±0.20 AB | 6.52±0.9 A |
| ECS-1123 | 186.6±28.2ABC | 62.2±9.4 ABC | 1.38±0.22 AB | 6.06±0.4 AB |
| p | 0.0007 | 0.0008 | 0.0004 | 0.0018 |

Different letters in the same column indicate significant statistical differences between strains (Tukey's test, α= 0.05).

groups. Group A had the highest values and included the two control strains and strains ECS-0130, ECS-0123, ECS-0127, ECS-0139, ECS-0141, ECS-0143, ECS-0144 and ECS-0150. The PR analysis defined two groups, with group A formed by the previously mentioned strains and group B formed by the strains ECS-0122, ECS-0127, ECS-0141, ECS-0144, ECS-0151 and the two control strains.

Table 4 shows the morphological characteristics of the sporocarps cultivated on the Pangola grass at 25 °C. The colour varied between pink (strains ECS-0130 and ECS-0127) and white (remaining strains). A typical mushroom smell was detected in seven strains, and three of them had a floury smell (ECS-0130, ECS-0143 and ECS-0150). The taste varied from typical (ECS-0139 and ECS-0144) to sweet (ECS-0123, ECS-130 and ECS-141), floury (ECS-0150), oily (ECS-0144 and ECS-0151) or oily and sweet (ECS-0122 and ECS-0127). The texture was usually subcoriaceous to leathery except for strain ECS-0139, which had a smooth texture. The size of the sporocarps varied between 2 and 16 cm, and they had a firm and imbricated consistency. Overall, these variations are consistent with the description given for the species [14].

## DISCUSSION

In this work, we studied various characteristics of ten *P. djamor* strains that are of biotechnological interest, and compared them to two commercial *P. ostreatus* control strains. Strains showed significant variability in mycelium growth speed in the malt extract agar (between 1.12 and 4.15 mm/d). Strain ECS-0143 was the fastest and statistically similar to the *P. ostreatus* EC-0152 control strain. However, none of the tested strains had a mycelium growth speed superior to the two strains used as the control. Other studies have reported higher growth speeds for *P. djamor* strains, including that of Benitez Camilo *et al*. [28], who reported rates of 3.1 to 10.1 mm/d, and Hernández-Ibarra *et al*. [18], who documented rates of 5.9 to 7 mm/d. Moreover, in a study by Ahuatzi-Sastre *et al*. [19], five *P. ostreatus* strains showed values between 171 and 324 ìm/h (4.1 to 7.7 mm/d).

In the study of ligninolytic activity, we observed that the strains had high laccase and phenoloxidase activity, decreased MnP activity and little to no aril alcohol oxidase activity. The *P. djamor* strains had an overall higher oxidase and laccase activity than the *P. ostreatus* strains. The *P. ostreatus* strains belonged to the group with the lowest activity. These results are consistent with a study from Salmones and Mata [20] that compared the laccase production in liquid medium and determined that *P. djamor* strains had a higher activity than *P. ostreatus* and *P. pulmonarius* strains. These authors reported that the activities from strains grown in a liquid medium ranged between 3.5 and 4.9 U/ml, which were highly inferior to those found in a solid substrate in this study. A greater laccase activity was observed in the fungal mycelium extract compared to the sporocarps extract. However, a higher phenoloxidase activity was observed in the sporocarp extract than in the mycelium extract in seven of the strains tested. According to previous studies, this phenomenon is likely a result of the role of phenoloxidases and laccases, in particular, in macrofungi morphogenesis [21, 22].

The MnP activity was lower than the laccase and phenoloxidase activity but was detected with a higher intensity in the mycelium extract than in the pileus (in 8 of 12 strains). The MnP activity was statistically higher in the *P. ostreatus* control strain ECS-0152 than in the other strains. The highest laccase activity was shown by strain ECS-0123 ($546.2 \pm 4.5$ U / ml), which was particularly noteworthy. This strain was located in the second group with the highest mycelial extract MnP(13.19 U/ml) and phenoloxidase activities (7.08), and it was ranked among the highest for mycelial growth and production of intermediate fruiting bodies. Strains ECS-0150, ECS-0151 and ECS-0144 had higher enzymatic activities than the average and were considered outstanding and of high study interest. Ramirez *et al*. [23] obtained a laccase activity of 20 U/ml from a crude extract of a *P. ostreatus* strain grown on bran. Similarly, Salmones and Mata [20] found that *P. djamor* strains from different parts of the world usually had a higher laccase capacity when cultured in agar than *P. ostreatus* and *P. pulmonarius*. Because the activity was determined in a crude extract, an analysis with purified extracts must be performed to more accurately determine the potential and characteristics of the enzymes involved. Many enzymes have been shown to be involved in the degradation of toxic compounds, and this mode of bioremediation is considered inexpensive and environmentally friendly [24].

**Table 4.** Morphological characterization of *Pleurotusdjamor* strains studied and compared to control strains *P. ostreatus* ECS-0152 and ECS-1123

| Strain | Colour when ripe* | Odor | Flavor | Texture | Size (cm) | Form | Consistency | Habit | Stipe | Sporeprintcolour* |
|---|---|---|---|---|---|---|---|---|---|---|
| ECS-0122 | White (N00, M00, C00) | Fungus | Oily, Sweet | Subcoriaceous | 2-11.5 | Flabellate | Firm | Imbricate | NF | Gray (N10, M00, C00) |
| ECS-0123 | White (N00, M00, C00) | Fungus | Sweet | Coriaceous | 3.5-16.5 | Flabellate | Firm | Imbricate | NF | Gray (N10, M00, C00) |
| ECS-0127 | Rose (N00, M10, C00) | Fungus | Oily, Sweet | Coriaceous | 3.5-10.5 | Flabellate | Firm | Imbricate | NF | Rose (N00, Y20, M20) |
| ECS-0130 | Rose (N00, M10, C00) | Farinaceous | Sweet | Subcoriaceous | 2-9.5 | Flabellate | Firm | Imbricate | NF | Rose (N00, Y30, M20) |
| ECS-0139 | White (N00, M00, C00) | Fungus | Fungus | Soft | 2-7.5 | Flabellate-Recurved | Firm | Gregarious | 0.5 cm long | White (N00, M00, C00) |
| ECS-0141 | White (N00, M00, C00) | Fungus | Sweet | Coriaceous | 2-9.5 | Conchate - | Firm | Imbricate | NF | White (N00, M00, C00) |
| ECS-0143 | White (N00, M00, C00) | Farinaceous | Oily | Subcoriaceous | 2-8.5 | Conchate | Firm | Imbricate | NF | Gray (N10, M00, C00) |
| ECS-0144 | White (N00, M00, C00) | Fungus | Fungus | Coriaceous | 2-10.5 | Flabellate | Firm | Imbricate | NF | Gray (N10, M00, C00) |
| ECS-0150 | White (N00, M00, C00) | Farinaceous | Farinaceous | Subcoriaceous | 2-8.5 | Conchate | Firm | Imbricate | NF | Gray (N10, M00, C00) |
| ECS-0151 | White (N00, M00, C00) | Fungus | Oily | Coriaceous | 2-10.5 | Flabellate-Recurved | Firm | Gregarious | 0.5 cm long | White (N00, M00, C00) |
| ECS-0152 | White (N00, M00, C00) | Fungus | Fungus | Soft | 4-12.5 | Conchate | Firm | Imbricate | 0.5-4 cm long | Gray (N10, Y10, M0) |
| ECS-1123 | Sand (N50, Y10, M10) | Fungus | Fungus | Chalky | 4.5-16 | Conchate | Firm | Imbricate | 0.5-4 cm long | Gray (N10, Y10, M00) |

*Color determination based on Küppers 2002
NF: Without stipe

The determination of antioxidant activity using the Folin Ciocalteu test estimated that the total phenol content in the samples varied from 229 to 517.8 eq. gal. ac/g (strains ECS-0141 and ECS-123, respectively) in the mycelium extract and from 54.1 to 231.3 eq. gal. ac/g (strains ECS-0143 and ECS-0152, respectively) in the pileus extract. These values were higher than those reported by Kim *et al*. [6] for methanol extracts from three types of fungi of this genus that have grey, pink and yellow pileus; these authors reported values ranging from 21.2 to 39.3 mg/g. In all cases, the phenol content was higher in the extract from the mycelium than in the sporocarp. According to Gutfinger [12], a high polyphenol concentration was associated with a high resistance to oxidation, and there was a linear relationship between the polyphenol content and oxidative stability. DPPH is one of the most commonly used tests to determine antioxidant properties. The mycelium extracts had antioxidant values ranging between 3.7 and 38% for ECS-0151 and ECS-1123, respectively, and between 8.8% and 41% in the pileus. Thus far, *P. djamor* antioxidant activity has not been determined using DPPH. A high antioxidant activity has been reported for other species of this genus, such as *P. sajor-caju, P. ostreatus, P. salmoneostramineus* and *P. cornucopiae* [5, 6, 25]. Vamanu [26] found a 30 to 40% antioxidant capacity using the DPPH assay in samples containing 4 mg/ml of *P. ostreatus* exopolysaccharides and endopolysaccharides. A growing number of findings have evaluated the role of oxygen and free radicals on cell damage and their effects on disease and aging. For this reason, antioxidants are becoming more relevant not only as food supplements but also as food for direct consumption [27].

The strains evaluated in this study showed significant morphological and organoleptic (colour, flavour, odour and texture) variability. Variations in colour between pink and white were observed, and leathery and subcoriaceous textures were the most frequent textures. However, the strain ECS-0139 had a smooth texture. The production of fruiting bodies did not provide for a statistical differentiation of the strains because all of the strains had a BE value that ranged between 36.4 and 71.4%. These values were low compared to other results reported for this species, which were 52-92% [18] and 49-125% [28]. The authors of these two studies used a coffee pulp substrate, and they calculated the production using more than two crops. Huerta *et al*. [15] reported even higher BE values for *P. djamor* strains from other parts of Mexico that were grown with two crops of coffee pulp (138 to 178%). However, the *P. djamor* strain ECS-0150 studied here and tested by Huerta *et al*. [15] had a BE value of 63%, which was similar to what was produced in this study. It is likely that the substrate used in this study (Pangola grass) is not optimal for this species and does not allow the expression of their full productive potential. The production rate ranged from 1.36 to 2.1%, which was similar to the results obtained Hernández Ibarra *et al*. [18] (1.18 to 2.21%).

## ACKNOWLEDGEMENTS

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# *LENTINULA EDODES* CULTIVATION TECHNIQUES AND MODELS IN CHINA

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## ABSTRACT

*Lentinula edodes* (Xianggu) is a major cultivated mushroom in China, with the widest production area, highest total output and best economic returns. The mushroom was originally cultivated in China around 1100 AD using the 'hatchet-notching' method. Later, in the 1960s, the cultivation technique involved inoculating pure culture spawn into natural wood logs. During the following decade, the Institute of Edible Fungi, Shanghai Academy of Agricultural Science, developed the sawdust-based cultivation method whereby it became possible to cultivate the mushroom under natural environmental conditions using as mall-scale, family-oriented production model. After popularization of this technique, *L. edodes* cultivation areas expanded rapidly to all provinces in China southwards from the Yangtze River Basin. Since 1989, China has been the world's largest producer and exporter of *L. edodes*, and total output in 2012 reached 6.35 million tons. However, with China's fast economic growth in recent years, problems have arisen with the family-oriented *L.edodes* cultivation model. It is a heavily labour-intensive production process with a low level of mechanization, year-round production cannot be sustained due to the seasonal production cycle, product quality is often unstable, and there is a limited ability on the part of growers to resist fluctuations in market prices. Currently, an intensive cultivation model has been adopted in China's main *L. edodes* production areas which, compared with the traditional model, ensures the safety and security of the raw materials used for cultivation, and lends itself to increased mechanization. Standardized spawn production, inoculation procedures and management practices have helped to ensure higher yields, and integrated *L. edodes* product collection and distribution has served to reduce the risks associated with market price fluctuations. However, the intensive *L.edodes* cultivation model requires more careful supervision and stronger technical support, especiallyin the control of spawn quality. Furthermore, the intensive *L. edodes* cultivation model is still limited to seasonal production. Therefore, in order to ensure an all-year-round supply of fresh *L.edodes*, an industrialized cultivation model has emerged, and the development of a technological process with Chinese characteristics is in progress.

**Key words:** *Lentinula edodes* (Xianggu), cultivation techniques, cultivation models

*Lentinula edodes* has been a major contributor to the vigorous development of China's edible fungi industry, with the widest production area, highest total output and the most profitable economic returns. In 2012, the total output of *L. edodes* in China reached 6.35 million tons, accounting for approximately 80% of production worldwide. *L. edodes* is a traditional food in oriental countries, beloved especially by consumers in China, Japan and Korea.

Cultivation of *L. edodes* originated in China, dating back over 800 years, and its development can be divided into three important phases: 'hatchet-notching' cultivation, cut-log cultivation and artificial cultivation (Fig. 1). 'Hatchet-notching' cultivation is a semi-artificial method in which a hatchet was used to make cuts in felled broadleaf tree trunks and relied on wild *L. edodes* spores ('spawn') floating down with the wind to inoculate the cuts and ultimately lead to fruit body formation. A relatively primitive technique, *L. edodes* yields depended very much on the density and quality of the *L. edodes* spores in the natural environment and on the local climatic conditions.

**Figure 1.** 'Hatchet-notching' cultivation

When, during the last century, the Japanese scholar Hikosaburô Morimoto isolated a pure strain of *L. edodes*, production of this mushroom in China moved from the 'hatchet-notching' method to the artificially inoculated cut broadleaf-wood log cultivation technique. Introduction of this method into China necessitated adoption of a centralized manual management system in areas suited to *L. edodes* growth after pure *L. edodes* strains had been artificially inoculated into logs cut from fallen broadleaf trees. The cut-log procedure represented a technological revolution in *L. edodes* cultivation and unified both natural and artificial methodology. Not only did this technique shorten the length of the *L. edodes* cultivation cycle, it also led to substantially increased mushroom yields. A major drawback, however, was that it consumed huge amounts of forest resources and threatened ecological stabiliy. In 1978, a research team composed largely of scientific and technical personnel from the Shanghai Academy of Agricultural Sciences (SAAS) developed the sawdust brick system for cultivating *L. edodes* (Fig. 2). This system was focused on the use of an artificial substrate formulation based on sawdust waste, inoculated aseptically with a pure mushroom strain, and careful management of the growth facility. It reduced the demand for wood resources typified by the cut-log procedure, eliminated geographical restraints previously applicable to *L. edodes* cultivation, facilitated the expansion of *L. edodes* production from remote forest regions to more easily accessible lowland areas, greatly improved biological efficiency, and represented another significant technological revolution.

In 1983, inspired by the 'bag' cultivation of *Tremella fuciformis*, Zhaowang Peng and others from Gutian County, Fujian Province, modified the sawdust brick technique and developed the sawdust bag for cultivating *L. edodes* (Fig. 3). After popularization, this technique rapidly superceded the cut broad leaf-wood log cultivation technique and resulted in *L. edodes* cultivation expanding rapidly to all provinces in China southward from the Yangtze River Basin.

**Figure 2.** The sawdust brick system for cultivating *L. edodes*

**Figure 3.** The artificial sawdust log for cultivating *L. edodes*

As a consequence of this unceasing expansion, diverse cultivation models, and varieties suited to climatic conditions in different regions and to different cultivation traditions have emerged. Principal *L. edodes* artificial cultivation systems operating in the major producing areas include the 'shelf', 'bag-on-the ground' and 'soil-covering' models.

However, due to China's fast economic growth in recent years, problems have arisen with the small-scale, family-oriented *L. edodes* cultivation model. It is a heavily labour-intensive production process with only a low level of mechanization, year-round production cannot be sustained due to the seasonal production cycle, product quality is often unstable, and there is a limited capacity on the part of growers to resist fluctuations in market prices. Currently, an intensive cultivation model based on specialization and division of labour has been adopted in China's main *L. edodes* production areas which, compared with the traditional model, ensures the safety and security of the raw materials used for

cultivation, and lends itself to increased mechanization. Standardized spawn production, inoculation procedures, spawn-running and other management practices have helped to ensure higher yields, and integrated *L. edodes* product collection and distribution has served to reduce the risks associated with market price fluctuations. However, the intensive *L. edodes* cultivation model requires more careful supervision and stronger technical support, especially in the control of spawn quality.

Nevertheless, the intensive *L. edodes* cultivation model is still limited to seasonal production. Therefore, in order to ensure an all-year-round supply of fresh *L. edodes*, an industrialized cultivation model has emerged, and the development of a technological process with Chinese characteristics is in progress. After three years of exploratory work, researchers at the SAAS have proposed an 'industrial-scale production technology for *L. edodes* based on secondary culture'. Using special *L. edodes* strains, this model has adopted the concept of 'spawn running in the bottle, fruiting by means of briquetting'. The two-stage procedure combines the automated conversion of the colonized cultivation substrate from the culture bottles into briquettes for subsequent brown film formation and fruiting. While existing *L. edodes* industrial-scale production models operating in Japan and Korea require special equipment and materials, this new Chinese model allows for more automation to be introduced in the production line at lower cost, thereby greatly reducing the probability of contamination inherent in ventilated bag cultivation, and will make it easier to achieve a fully mechanized system for large-scale *L. edodes* production.

In the light of current developments, family-oriented production models for *L. edodes* will eventually be eliminated, and fully automated industrial systems involving intensification and division of labour based on specialization will become the main *L. edodes* cultivation model in China in the future.

**Figure 4.** The two-stage procedure for cultivation of *L.edodes*

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# BIOLOGICAL EFFICIENCY OF WILD TYPE AND COMMERCIAL *AGARICUS BISPORUS* STRAINS

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## ABSTRACT

In the cultivation of the button mushroom (*Agaricus bisporus*), the cost of substrate production (compost) represents a large part of the total production cost. In Europe, the button mushroom uses only 25% of the organic matter in the compost in two flushes. Most white button strains used worldwide today are closely related to the first hybrid strains developed in the Netherlands over 30 years ago. Wild strains may provide possibilities to improve the efficiency in utilization of the compost (biological efficiency). Our research group has a large collection of mushrooms strains, many of which are derived from the ARP collection. Analysis of this collection has shown a large genetic variation. A selection, representing the genetic variation of the collection, has been cultivated in the Dutch shelf-system on a small scale. A large variation in biological efficiency has been found. The present commercial strains are among the ones with the highest biological efficiency. Next to this we noticed a clear correlation between the dry weight of mushrooms produced and the drop in pH in the compost. Loss in dry matter in compost as a result of mushroom production can be attributed for 95% to loss in hemicellulose and cellulose. Total amounts of lignin and undefined organic matter do not decrease much during the cultivation of mushrooms. Furthermore, there is a clear correlation between mushroom production and consumption of hemicellulose from the substrate. This correlation is less clear for cellulose.

**Keywords:** *Agaricus bisporus*, compost, hemicellulose, genetic diversity

## INTRODUCTION

In the Netherlands, yields of button mushroom (*Agaricus bisporus*) range up to 350 kg of mushrooms per tonne of compost. Nevertheless, only 16% of the dry matter (25% of the organic matter) present in the compost is degraded to produce 2 flushes of mushrooms. Increasing the efficiency with which the substrate is converted into mushrooms will therefore have a considerable impact on the economics of production. It will also impact the environmental burden of the crop; a higher biological efficiency would result in less transport of compost and also in a smaller quantify of spent compost. Currently, mushrooms strains are only able to use a relatively small portion of the organic matter present in compost.Wild strains may provide possibilities to improve the biological efficiency with which compost can be converted into mushrooms but might also offer opportunities to use other ingredients to generate a good yielding substrate.

The Department of Plant Breeding of Wageningen University & Research Centre houses a large collection of button mushroom strains. This collection was started and expanded mainly by the Mushroom Experimental Station in Horst, the Netherlands, starting in 1954. It consist of old commercial strains (<1980), the currently available commercial strains and wild collected strains (mainly from the ARP collection) [1].

Research on biological efficiency of button mushroom may also improve our understanding of which nutrients may be limiting mushroom production. Basically, compost consists for a large part of an inert carrier material which provides a number of important physical characteristics such as water-holding capacity and structure / low bulk density allowing gas exchange. Cellulose and hemicellulose are the major source of carbon and represent only 32% of the dry matter in the compost. Sonnenberg and Blok [2] have shown that during production of flushes of mushrooms, strain Sylvan A15 only 16% of the dry matter in phase III compost is consumed, representing a loss of 25% of the total organic matter. While 83 % of the hemicellulose present is consumed, only 41% of the cellulose present is consumed. So, even though there is still quite a lot of cellulose present in the compost, a third flush of mushrooms usually has a much lower yield than the first two flushes.

Results described above were obtained, using *A. bisporus* strain Sylvan A15. In this article we present results of substrate consumption by a variety of genetically dissimilar button mushroom strains.

## MATERIALS AND METHODS

### Strains

Strains are maintained in liquid nitrogen according to the method published by Homolka and coworkers [3]. After revival from liquid nitrogen the strains were grown on malt extract agar, set at pH 7.0 using 10 mM MES/KOH. When needed, agar plates were covered with cellophane discs to facilitate collection of the mycelial colonies.

### Molecular techniques

DNA isolation was performed using the Promega magnetic particle Plant kit (Promega Corporation, Madison, WI, USA). DNA samples were subsequently used for SNP analysis. All publically available genomes of *A. bisporus* (H97 & JB137-S8 [4]) and 4 in house generated sequences were used to detect single nucleotide polymorphisms. This resulted in a list of 120 SNP markers. For each sequence we selected about 2 markers per scaffold. SNP analysis were performed by Van Haeringen Lab in Wageningen (https://www.vhlgenetics.com/en-us/home.aspx). After analysis, a dataset of 117 SNP markers was obtained. After modification towards an input file for the program NTSY Spc 2.1 [5] in which the UPGMA clustering method was used to analyse genetic relatedness of the strains.

### Cultivation techniques

For a selection of 66 *A. bisporus* strains, taken from the full width of genetic diversity, spawn was made. Strains were inoculated in triplicate into 8 kg portions of freshly prepared phase 2 compost (CNC Grondstoffen, Milsbeek, the Netherlands) and filled in plastic tubs with 0.1 $m^2$ growing surface (approx 80 kg compost/$m^2$). After 16 days of spawn run at 24 °C, a casing layer was applied. After 11 days of colonization, the casing soil was ruffled. After an additional 3-4 days of recovery growth, the trays were vented for a period of 4 days to reach an air temperature of 18 °C, 90% RH and 1000 ppm $CO_2$. Subsequently two flushes of mushrooms were harvested of quality I (closed mushrooms varying in size between 15 and 45 mm). Total yield per tray was recorded in grams wet weight. Dry weight of samples of the mushrooms was determined after drying at 105 °C.

### Compost analysis

Compost was sampled at spawning and after the second flush of mushrooms. Proximate composition of the compost samples was determined by Havens (Maashees, the Netherlands) using Near Infra-Red Spectroscopy (NIR). To determine substrate utilization during spawn-run, a duplicate experiment was performed. Trays in this experiment were sacrificed at venting and compost composition was determined using NIR Havens (Maashees, the Netherlands).

## RESULTS AND DISCUSSION

### Genomic analysis

In total, 235 strains of *A. bisporus* were analysed for genetic variability, using 120 SNP markers. Results are shown in Fig 1. Names of the different strains are lacking, as the format used makes them unreadable. Strains of *Pleurotus ostreatus*, *A. arvensis* and *A. blazei* have been used as an outgroup. Strains of the outgroup have been indicated in black. As can be seen two strains from the outgroup (*A. arvensis* and *A. blazei*) appear in the group of four-spored *A. bisporus* isolates from the Californian desert.

The analysis clearly discriminates large groups of strains. The traditional commercial white strains and the traditional off-white strains, both widely used in the pre-hybrid era, can easily be discriminated. Given the considerable variety of the traditional white strains, it seems unlikely that these varieties all originate from a single white strain isolated in 1926 on a bed of cream mushrooms as has been suggested in the past [6]. For comparison, the strains indicated in red represent the

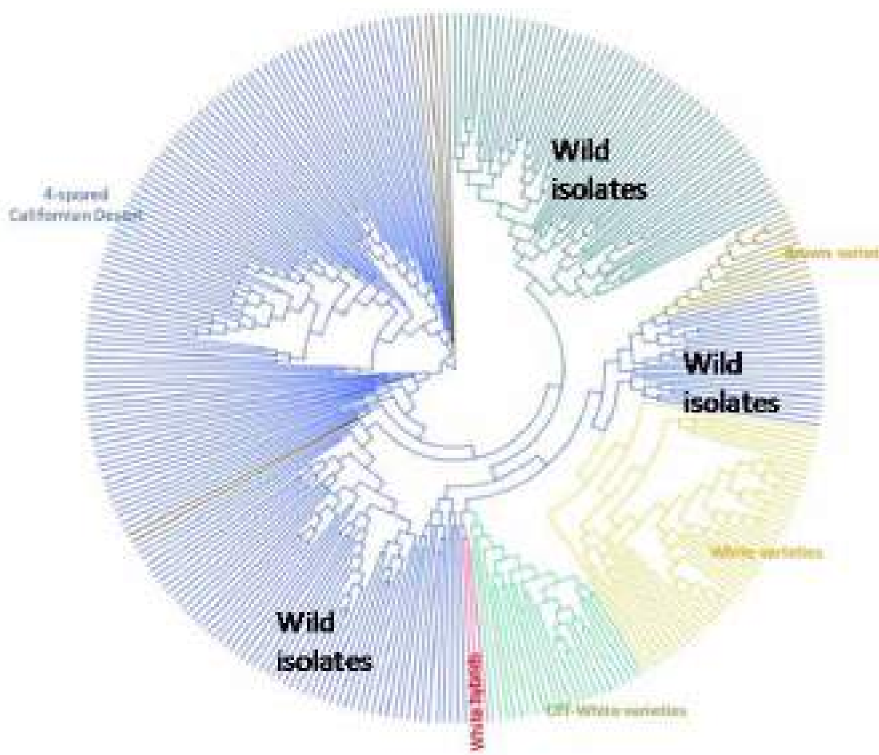


**Figure 1.** Analysis of genetic relatedness of 235 strains of *A. bisporus*. Analysis clearly identified the traditional commercial white and off-white varieties from the pre-hybrid era. It also shows the very limited genetic base of the currently commercially used white hybrids. Among the non-commercial strains, the large genetic diversity of the 4 spored *A. bisporus* var. *burnettii* strains from the Californian desert (blue) can be seen, as well as the genetic diversity of the wild type bisporic strains. The black lines represent the out-group.

currently used white hybrid strains. These strains are known to have all been derived directly or indirectly from the first white hybrids released to the market in 1980 and represent thus a very narrow genetic base. Also the traditional commercial brown varieties (used before the market introduction of Heirloom and Brawn by Amycel) can easily be identified as a group. Dispersed between these groups, wild collected bisporic *A. bisporus* strains are found (Fig. 1). The large blue group between the outliers represents four-spored varieties originating from the Californian desert. This genetic analysis clearly shows that only a very small part of the genetic diversity among *A. bisporus* strains is used for commercial cultivation and potential to generate new products for growers and consumers is left unused. As a result all currently used strains are susceptible to the same extent to pests and diseases.

## Substrate utilization

The trays used for the assessment of compost utilisation contain 8 kg of compost at spawning representing 2.66 kg of dry matter. This dry matter consists of 0.84 kg of ash and 1.83 kg of organic matter. The organic matter consists of 1.23 kg of lignocellulosic fibers (0.22 kg of hemicellulose, 0.66 kg of cellulose and 0.36 kg of lignin) and an undefined fraction (0.6 kg).

Yields of mushrooms ranged from 0 to 218 grams of dry matter per tray. As shown in Fig. 2 higher yields are accompanied by a larger drop in compost pH at the end of the second flush. Apparently mushroom production is accompanied by the production of acids. It is not clear however, which acid would be responsible for the pH drop. Oxalic acid is produced in large quantities by *A. bisporus* and may be a candidate. According to Gadd [7], oxalic acid plays an important role in degradation of lignocellulosics. Baldrian and Valášková [8] suggest a number of different roles for oxalate in degradation of cellulose, such as chelating metal-ions, as in Fig. 5. Relation between the amounts of consumed cellulose and hemicellulose. The coloured data points represent commercial strains. The red data point represents A15.

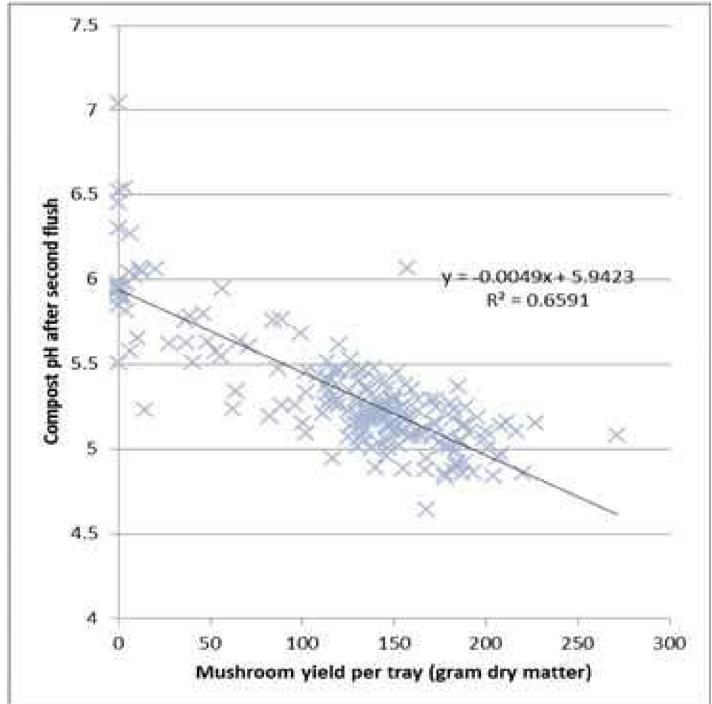


**Figure 2.** Relation between mushroom yield and drop in compost pH

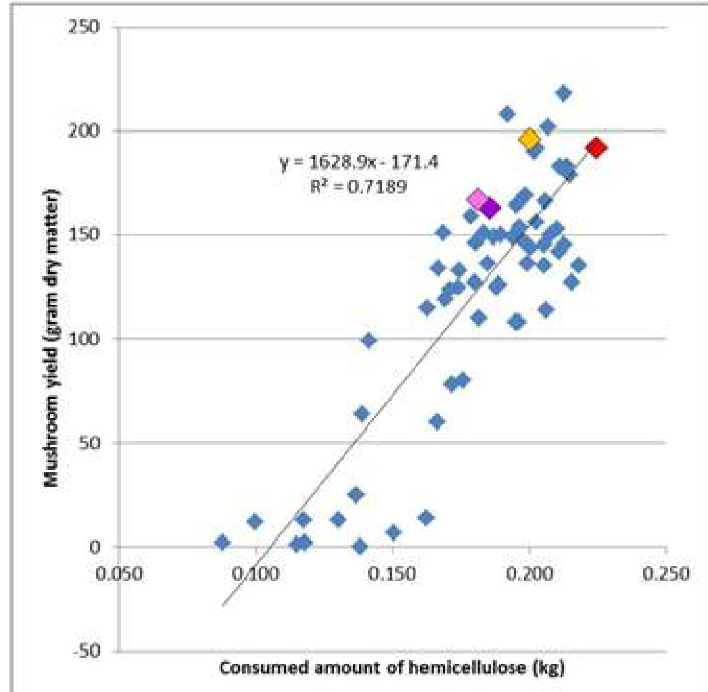


**Figure 3.** Relationship between mushroom yield and consumption of hemicellulose. The coloured data points represent commercial strains. The red data point represents A15

Mushroom yield (expressed in gram dry matter) correlates well with consumption of hemicellulose from the compost (Fig. 3). A less significant correlation is found for mushroom yield and consumption of cellulose (Fig. 4). Nevertheless, consumption of hemicellulose is closely linked to the consumption of cellulose (Fig. 5). High yielding strains consume cellulose and

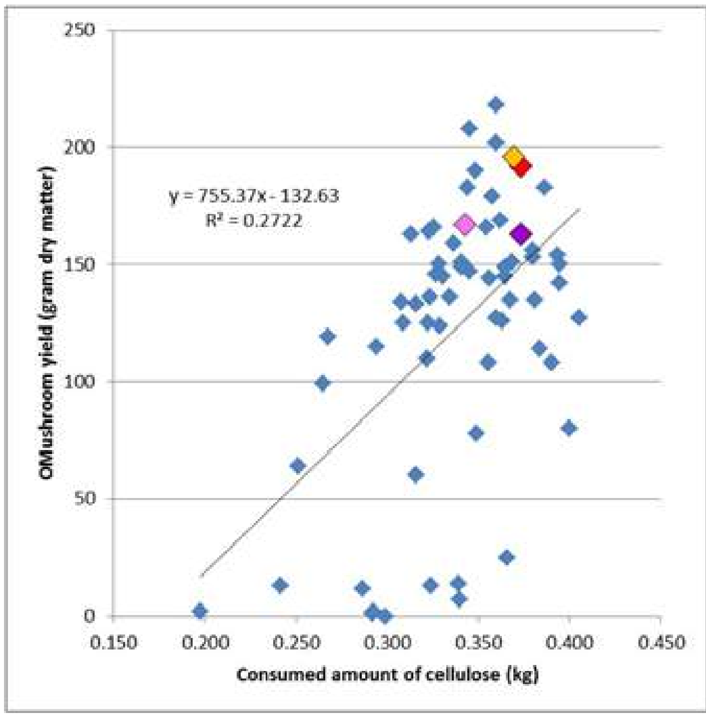


**Figure 4.** Relationship between mushroom yield and consumption of cellulose. The coloured data points represent commercial strains. The red data point represents A15

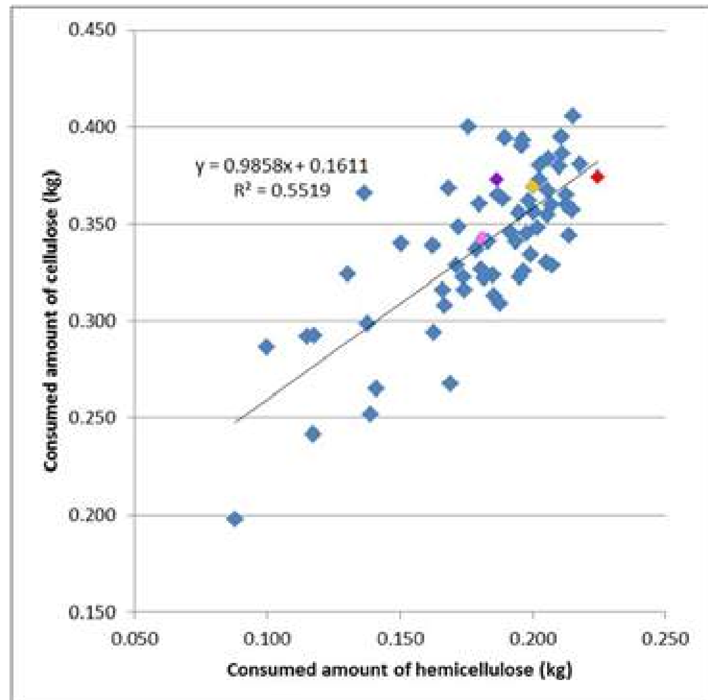


**Figure 5.** Relation between the amounts of consumed cellulose and hemicellulose. The coloured data points represent commercial strains. The red data point represents A15

hemicellulose in a fixed ratio (Fig. 6). Strains that yielded more than 100 gram of mushroom dry matter per tray, consumed cellulose and hemicellulose in a ratio of about 1.7. Strains that yielded between 50 and 100 gram of mushroom dry matter per tray, consumed cellulose and hemicellulose in a ratio close to 2. Strains that produces only low amounts of mushrooms (less than 50 gram of mushroom dry matter per tray) consumed cellulose and hemicellulose in a ratio between 2 and 3. As can be seen in the right graph in Fig. 3, the low yielding strains consumed up to 0.35 kg out of the 0.66 kg of cellulose present in a single tray, while producing only about 25 gram of mushroom dry matter.

**Biological efficiency**

Biological efficiency of mushroom production is here expressed as the amount of dry weight mushrooms produced per kilogram of organic matter consumed, with values ranging from 0 to 395 g DM/kg OM consumed. Analysis by ANOVA has shown differences to be statistically significant. At $p$=0.05 the least significant difference (l.s.d.) was 64.

Values for biological efficiency show a more or less normal distribution (Fig. 7). A number of strains produced no or barely any mushrooms, producing a rather large lowest bin. The 4 commercial strains tested showed biological efficiencies of 269, 280, 317 and 325 g DM/kg OM consumed, for a commercial brown strain (Somycel 856), two traditional white strains (Somycel 53 & Le Lion B80), and a hybrid strain (Sylvan A15), respectively. Given a l.s.d. of 64, the value of 317 g DM/kg OM consumed for Sylvan A15 is significantly lower than the highest biological efficiency recorded in this experiment (395 g DM/kg OM). Repeating the experiment will likely make the determination of the biological efficiency more accurate. The large variation in biological efficiency indicates that this collection has potentials to improve compost utilisation via breeding.

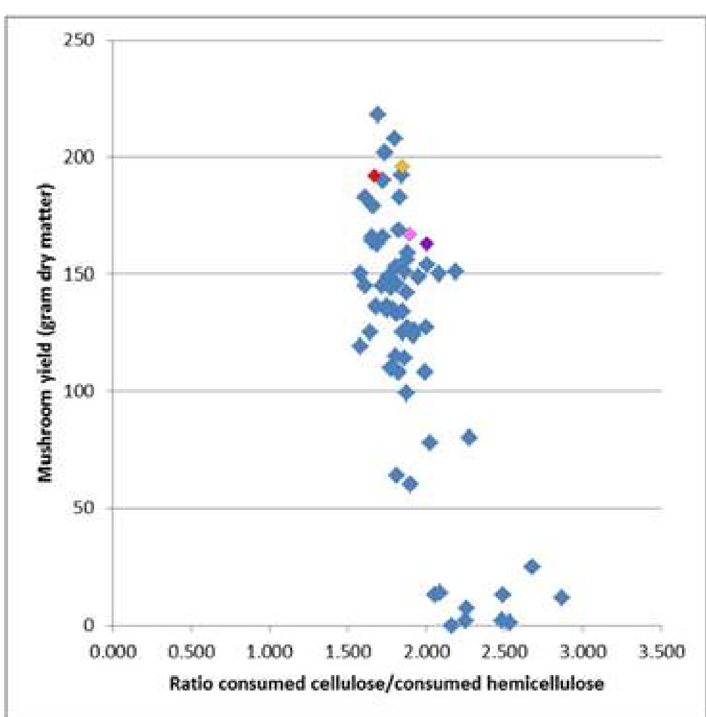


**Figure 6.** Relation between the ratio consumed cellulose/hemicellulose and yield. The coloured data points represent commercial strains. The red data point represents A15

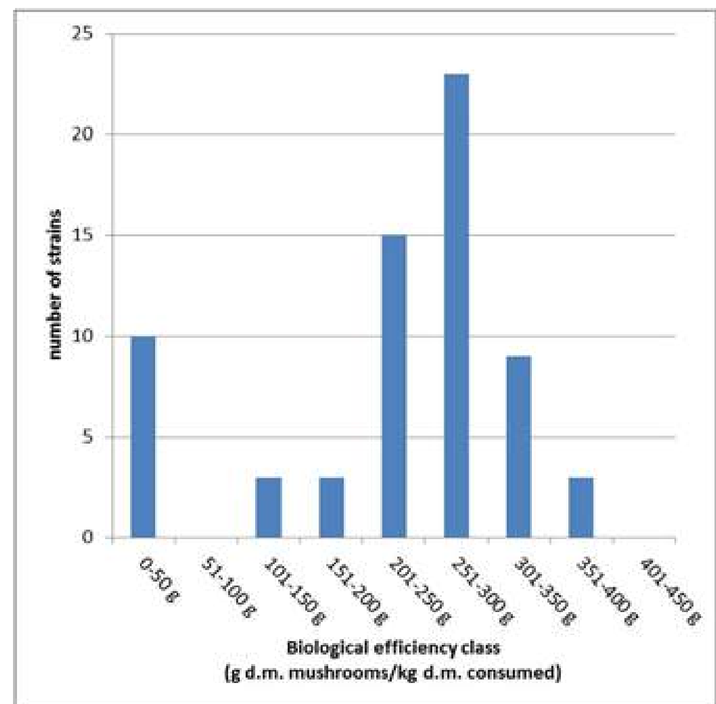


**Figure 7.** Distribution of biological efficiency among strains

## CONCLUSION

In conclusion, it can be said that the currently commercially used hybrid strains of *A. bisporus* represent a narrow genetic base. Traditional commercial strains (whites, off-whites and browns) represent a wider genetic diversity. Wild collected strains show an even wider genetic diversity.

Analysis of compost utilization by a range of 66 genetically dissimilar *A. bisporus* strains has shown that mushroom production in *A. bisporus* is strongly linked to the consumption of hemicellulose and to a lesser extend to cellulose with a remarkable fixed ratio in cellulose/hemicellulose consumption. The yield in mushroom production is also correlated with pH of the compost, i.e. the higher the yield, the lower the pH of the compost after two flushes.

Analysis of biological efficiency of 66 genetically dissimilar strains has shown that in this experiment mushroom production can reach a yield of 395 g of dry weight mushrooms per kg of organic matter consumed, which is higher than the production of the currently used hybrid strain A15 in the same experiment.

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# VEGETATIVE GROWTH OF DIFFERENT STRAINS OF *PLEUROTUS* AND *LENTINULA* SPECIES ON CASSAVA (*MANIHOT ESCULENTA*) AND YAM (*DIOSCOREA ROTUNDATA*) WASTES IN GHANA

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## ABSTRACT

Mushrooms are fast becoming important components of diets worldwide and it is necessary to find out appropriate substrates on which they can be grown. Eight strains of two mushrooms: *Pleurotus ostreatus* strain MES11797, 03416, 03772, 03364,03216 and *Lentinula edodes* strain MES 02008, 02052, 12060 were cultivated on substrate formulated from cassava and yam wastes such as Cassava peels, Cassava sticks and Yam peels. Sawdust of *Triplochitons cleroxylon,* which has been the traditional substrate for the cultivation of *Pleurotus* spp. in Ghana was used as the control substrate. Cassava peels agar supported the best growth for four *Pleurotus* spp. (MES 11797, MES 03772, MES 03216 and MES 03416) and one *Lentinula* strain (MES 12060). Yam peels however, support the best growth for *Pleurotus* strain MES 03364. Cassava sticks supported the best growth for *Lentinula* strain MES 02008. Sawdust supported the best growth for *Lentinula* strain MES 02052.

**Keywords**: substrates, mushroom, yield, *Pleurotus*, *Lentinula*

## INTRODUCTION

Ancient Egyptian empire considered mushrooms as a delicacy reserved for Pharaohs and the Romans and they ate mushrooms at feasts and believed that mushrooms provide strength for warriors during battle [1]. The Chinese on the other hand use mushrooms more because of the medicinal values they have [2]. Mushrooms are a delicacy in Ghana and are used in the preparation of many traditional and exotic dishes [3]. They are known to have anti cancerous, anti cholesterol and anti tumorous properties and are useful against ailments such as diabetes, high blood pressure, ulcer and lung diseases [4]. Edible Mushrooms are a good source of protein, vitamins and minerals [5]. Fresh mushrooms contain about 85-95% water, 3% Protein, 4% Carbohydrates, 0.1% fats,1% minerals and vitamins [6]. Mushrooms contain appreciable amounts of potassium, phosphorus, copper and iron but have low levels of calcium. Mushroom protein is intermediate between that of animals and vegetables [7, 8]. Mushrooms such as the genus *Pleurotus* are known to be among the largest of fungi composed of filaments and survive very well in a damp or moist condition [9].

Cassava (*Manihot esculenta* Crantz) is the third most important source of calories in the tropics, after rice and maize. Millions of people depend on cassava in Africa, Asia and Latin America [10]. Cassava processing, especially in areas where the industry is highly concentrated, is regarded as polluting and a burden on natural resources [11]. In many places where cassava is cultivated and processed, heaps of cassava peels are discarded along the roads and causes unpleasant odours and unhygienic conditions [12].

Cassava is cultivated in Ghana on a large scale and some of the waste is given to livestock as feed. The rest is left to the weather and eventually this creates a high pollution problem. Over 90 per cent of the peels are either burnt or left unattended to at dumping sites. A total of 3.6 million tonnes of cassava waste peels are discharged parts during the peeling process which are generated annually. Peels represent around two thirds of the waste, and about 200,000 tonnes of cassava can be saved through more efficient peeling which translates into potential savings of almost $37 million [13]. In the case of yams, waste is mostly generated at the consumption level through households, chop bars and food vendors. Since yam processing is very limited, it is done by a few small and medium enterprises. Yam (*Dioscorea rotundata*) peels constitute

about 14 per cent of the volume of yam consumed and approximately five per cent of volumes of the crop tend to go waste [13]. The cassava and yam peels can therefore be used as efficient substrate for mushroom cultivation.

This study seeks to evaluate the use of cassava and yam wastes as suitable substrates for the growth of five strains of *Pleurotus* spp. and three strains of *Lentinula* mushrooms within Ghanaian environmental conditions.

## MATERIALS AND METHODS

### Mushroom cultures used

The cultures used for this work were obtained from the Plant Research International of the University of Wageningen, Netherlands. These were: *P. ostreatus* strain MES 11797, MES 03416, MES 03772, MES 03364, MES 03216 and *L. edodes* strain MES 02008, MES 02052, MES 12060.

### Agar media preparations

Initial studies were carried out on agar media to ascertain the effectiveness of these media. The media used were: cassava peels agar -CPA, cassava sticks agar-CSA, yam peels agar-YPA and sawdust agar-SDA. Cassava and yam peels, cassava sticks were each solar dried for five days and milled mechanically using a hammer mill to a fine powder and used to prepare the agar media. This was prepared by soaking 20 g of each substrate in 200 ml of water and allowed to stand for four hours. Then strained and the supernatant topped with distilled water up to 1 liter. Fifty grams of select agar and 20 g of glucose were added and mixed. This mixture was put on a hot plate and stirred continuously till granules of the agar dissolved. Then it was sterilized for 1 hour at 121 $^{o}$C and poured into Petri dishes to set. Each substrate had two replicates. Sawdust of *Triplochiton scleroxylon* agar was prepared as above.

### Measurement of mycelia growth rates

The vegetative growth of mycelium of the mushroom on the different media were assessed by measuring growth of the fungus along two diameters drawn at right angles at the bottom of the Petri plates prior to inoculation. Measurements were made daily for five days.

## RESULTS AND DISCUSSION

### Mycelia growth on solid media

Mycelia extension of various strains varied on different solid media. *L. edodes* strain MES 02008 inoculated on YPA showed the lowest mycelia extension by the third (0.7 cm) and fifth days (1.0 cm) of incubation in comparison to all the other strains of mushrooms studied. Although the recorded low mycelia extension was not significantly different from values recorded for the same strain on the CPA, CSA, and SDA, the value was significantly lower than that recorded for *L. edodes* strain MES 12060 on all the solid media. Among the *P. ostreatus* strains, the lowest mycelia extension on the third day of incubation was 0.8 cm and was recorded for *P. ostreatus* strain MES 11797 inoculated on CSA (Table 1).

On the fourth day of incubation, the lowest mycelia extension (0.9 cm) was recorded for *L. edodes* strain MES 02052 inoculated on YPA. *P. ostreatus* strain MES 03364 recorded the highest mycelia extension of 1.9, 2.4 and 3.1 cm on days 3, 4, and 5 of incubation, respectively on YPA. With the exception of the value recorded for strain MES 12060 on CPA and YPA, the high mycelia extension recorded for strain MES 03364 on YPA on the third day of incubation (2.4 cm) was significantly higher ($P<0.05$) than values recorded for the same strain inoculated on all the other solid media (Table 1). Significantly lower mycelia extension of strain MES 03364 was also recorded when inoculated on CSA and SDA. Among the other MES strains (11797, 03416, 03772, 03216) on the various media, significantly lower mycelia extensions were recorded for strain MES 03216 on SDA and YPA and strains MES 03416 and MES 11797 on all the solid media.

The lowest, mean and the highest mycelia growth rates recorded were 0.1 cm/day for strain MES 02008 on YPA, 0.4 cm/ day for various strains and solid media, and 0.7 cm/day for strain MES 12060 as well as strain MES 03364 both incubated

on CPA (Table 1). Among the *L. edodes* strains studied, strain MES 12060 recorded the fastest mycelia growth on the solid media, whereas the other two strains showed comparable mycelia growth ratesfor the various solid media. On the other hand, among the *P. ostreatus* strains studied, strain 03364 showed the fastest mycelia growth ranging from 0.5-0.7 cm/day (Table 1), closely followed by strain MES 03216 with 0.5 and 0.6 cm/day on CSA and SDA, and CPA and YPA, respectively (Table 1).

In a study by Furlan *et al.* [14], the authors reported growth rates of 0.38 cm/day on potato dextrose agar (PDA) and 0.4 cm/day on both wheat dextrose agar (WDA) and malt extract agar (MEA) incubated in the dark at 30 ºC for *L. edodes* and growth rates of 0.75, 0.6 and 0.4 cm/day under the same condition on PDA, WDA and MEA, respectively for *P. ostreatus*. Although the reported mycelia growth rates by Furlan *et al*. [14] are comparable to those reported herein, some of the growth rates reported for *L. edodes* herein are lower. However, both studies indicate that mycelia growth rate of both mushroom species varies on the growth media on which the mycelium is incubated. Furlan *et al*. [14] showed that the difference in mycelia growth rate on the solid media was independent of the pH of the media and the incubation temperature. Inglet *et al.* [15] have recorded *L. edodes* mycelia growth rate of 0.6 cm/day using whey permeate at 40 g of lactose/L, temperature 23.6 °C, and pH 5.0. Strain-and growth media –dependent variations of *Lentinus squarrosulus* mycelia growth rate have also been reported [16]. As such, it appears reasonable to infer that variations in *L. edodes* and *P. ostreatus* mycelia growth rates are strain and growth media dependent and that this may be a general characteristic of mushroom mycelia growth regardless of the species or strain.

Knowledge about media requirements by strains of mushrooms is essential in tissue culturing for the further steps in mushroom studies or cultivation. Moreover, the information is required to assess the viability of the strain in question. For instance, knowledge of behavior of a particular mushroom species or strain at specific conditions (media and incubation parameters) would ensure that viable, vibrant cultures are not discarded with the assumption that they are not vibrant enough, especially when classical biotechnology techniques are being applied.

Across the strains, the slowest and fastest growth rates recorded on CPA were 0.4 and 0.7 cm/day, respectively, with a mean value of 0.45 cm/day (Fig. 1). Growth rates recorded for the strains on YPA showed wide variations, with values falling within the extreme ranges of 0.1-0.7 cm/day with a mean value of 0.38 cm/day (Fig. 1). Mean mycelia growth rates of 0.46 and 0.44 cm/day were recorded on CSA and SDA, respectively (Fig. 1). Mycelia densities recorded across the mushroom species and strains on CPA had various degrees of mycelia density (ranged from 2-4), those for CSA were quite dense (2 and 3), and YPA were generally highly dense (ranged from 3-5) (Fig. 2). The lowest mycelia densities (1 and 2) were observed on SDA (Fig. 2).

The mycelia densities of the strains studied across the solid media are shown in Fig. 3. The strains showed comparable mycelia densities on the various media, although strain MES 03216 showed relatively low mycelia densities across the

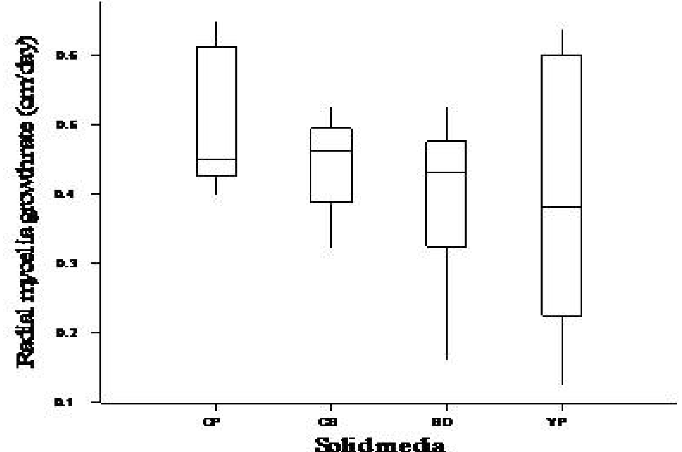


**Figure 1.** Box-and-whisker plots showing mycelia growth rate on the solid media

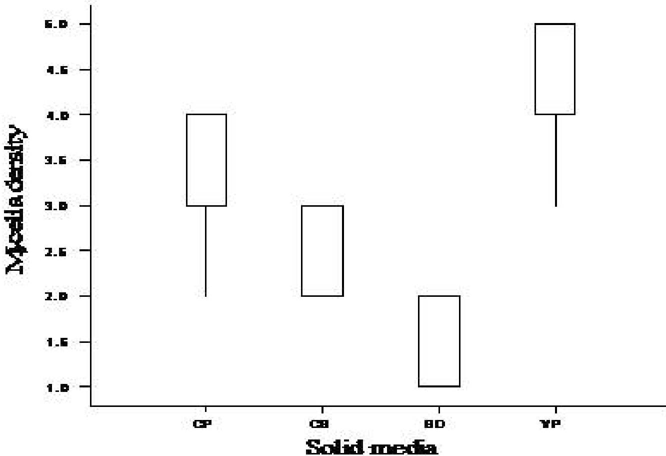


**Figure 2.** Box-and-whisker plots of MES strain mycelia density on the solid media

media. Mycelia densities of MES 03772 differed when incubated on the different solid media. These results indicate that mycelia growth rate does not directly correspond to the mycelia density. In fact, a weak negative correlation of -0.0635 was obtained between values recorded for mycelia growth rate and mycelia density for all the mushroom strains studied on the various solid media. Thus, there is a very low chance (less than 1%) that cultures with high mycelia growth rates would have low mycelia densities. As such, it is reasonable to infer that there is no correlation between the two parameters. This relationship between mycelia growth rate and mycelia density have been reported on *Lentinus squarrosulus* mycelia growth[16].

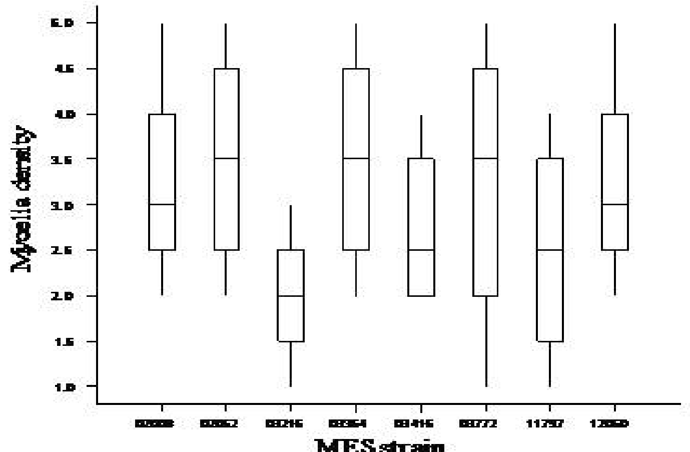


**Figure 3:** MES strain mycelia density on solid media

However, in selecting mushroom cultures for further work on mushroom cultivation, it is more reasonable to select mycelia with higher growth rates rather than higher mycelia densities as faster mycelia growth would enable the mushroom to rapidly fully colonize the substrate in order to outgrow contaminants in the substrate if there are any contaminants. Nevertheless, mycelia density is also an important factor as it has been observed that pinhead or primordial formation occurs only after the mycelia thickens or becomes dense on the substrate, indicating that basidiocarp formation and yield would be affected by the mycelia density on the substrate. Culture behavior of mushrooms have been indicated [17] to be directly linked to cultivation and pharmaceutical aspects of mushrooms.

**Morphological description of mycelial growth on different substrate media**

Various strains had different morphological characteristics during growth on the different media. MES 11797 strain showed clear zonation at the edge of the colony (Fig. 4a). In addition, longitudinal radial mycelia were observed on both sawdust and cassava sticks agar. Concentric longitudinally radial growth on YPA was quite prominent but not as prominent as on CPA. In the strain MES 03416 (Fig. 4b), the mycelial morphology on all media showed similar concentric longitudinally radial growth. Green mould contaminant partially restricted mycelia growth. Strain MES 03772 (Fig. 4c), growing on CPA and YPA had a concentric longitudinally radial mycelia growth while CSA and SDA showed a longitudinally radial mycelia growth. Growth of mycelia was partially inhibited by bacterial contaminant as well. Mycelia morphology of strain MES 03364 on all the substrates was either concentric, longitudinally or radial. The concentric morphology was prominent. Mycelial morphology of MES 02008 strain were similar on SDA, CSA and CPA (Longitudinally radial). The mycelial morphology of strain MES 02052 had similar morphology to that of MES 02008 on SDA, CSA and CPA (longitudinally radial). However, there was an uneven growth on YPA. For strain MES 03216 (Fig. 4d), the morphology was different on each different media: SDA, CSA and CPA had longitudinally radial mycelia growth, YPA had a concentric longitudinally radial growth and a dense mycelia but the strain was denser on CPA. The strain MES 12060 (Fig. 4e) had clear area (zone) formed at the periphery of the colony, this is possibly caused by an exudate from the mycelia. This was especially pronounced on YPA and is likely to be due to the presence of extracellular enzymes.The morphology of the strain MES 12060 on the medium SDA and CSA both showed a longitudinally radial growth.

## CONCLUSION

Different agar media supported the growth of the different strains of mushrooms with cassava peels agar supporting majority of the strains (*Pleurotus ostreatus* strain MES 11797, 03416, 03772, 03216 and *L. edodes* MES 12060).

## ACKNOWLEDGEMENTS

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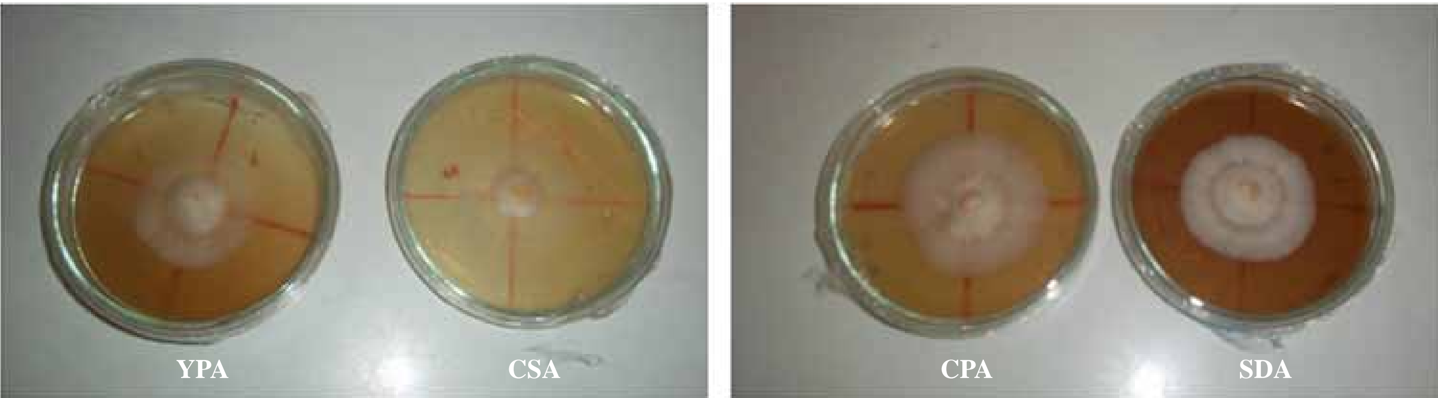
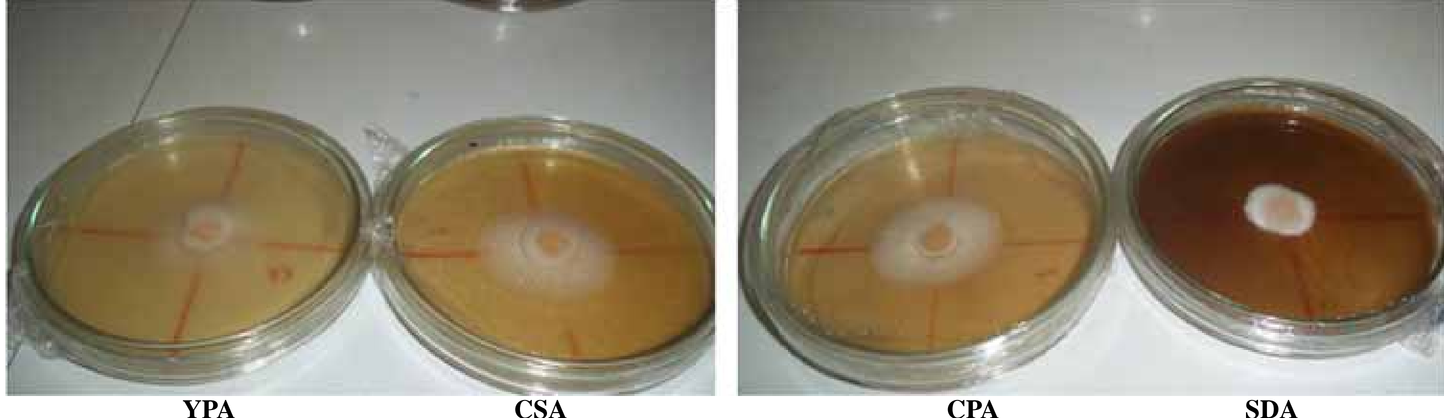
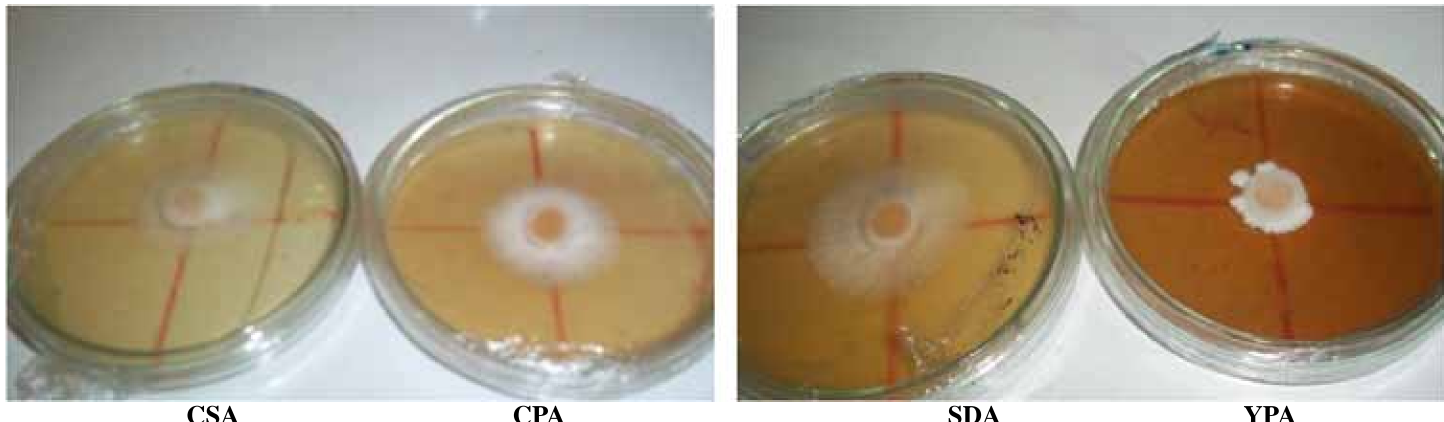



**Figure 4a.** Morphology of mycelial growth of *P. ostreatus* MES 11797 on various solid media on the second day of incubation



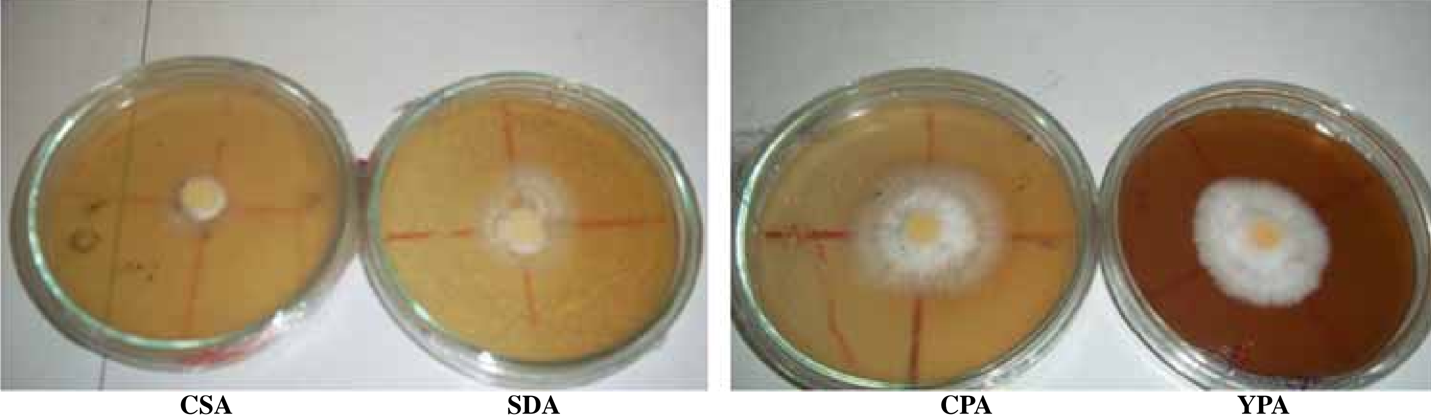
**YPA** **CSA** **CPA** **SDA**

**Figure 4b.** Morphology of mycelial growth of *P. ostreatus* MES 03416 on various solid media on the second day of incubation



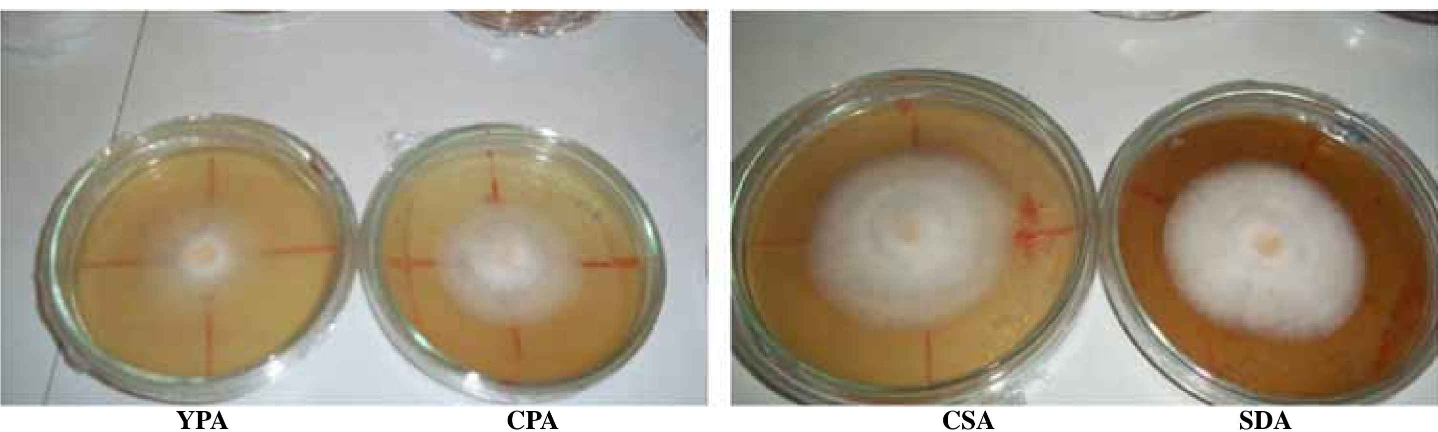
**CSA** **CPA** **SDA** **YPA**

**Figure 4c.** Morphology of mycelial growth of *P. ostreatus* MES 03772 on various solid media on the second day of incubation



**CSA** **SDA** **CPA** **YPA**

**Figure 4d.** Morphology of mycelial growth of *P. ostreatus* MES 03216 on various solid media on the second day of incubation



**YPA** **CPA** **CSA** **SDA**

**Figure 4e.** Morphology of mycelial growth of *L. edodes* MES 12060 on the various solid media on the fifth day of incubation

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# CULTIVATION OF OYSTER MUSHROOMS ON CASSAVA WASTE

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## ABSTRACT

Cassava is a major food crop for approximately 700 million people, especially in African countries. A large quantity of waste is produced after processing of cassava, mainly consisting of tuber peels. Although previous research has shown that these peels can be an ingredient for substrate to cultivate mushrooms, yields were usually inferior compared to traditional substrates such as saw dust. In a project funded by the European Union (http://www.fp7-gratitude.eu/) trials were done for the production of oyster mushrooms using fermented peels and stems from cassava crop produced in Ghana. Four mushroom strains representing two species (*Pleurotus ostreatus* and *P. pulmonarius*) were grown on fermented substrates made from cassava waste (peels & stems) without further heating/sterilisation. Peels and cassava stems were tested in different ratio's and supplemented with different amounts of rice or wheat bran. All the substrate samples colonized quickly (15-16 days) and time for pinning varied between 18 and 24 days. The *P. pulmonarius* strains produced three flushes within 47 days (starting from inoculation) and the *P. ostreatus* strains needed 57-63 days completing flush 3. Biological efficiencies after 3 flushes varied between 38% and 100%. The effect of bran supplementation on yields depended on the concentration (0, 1, 6 and 12% w/w), type of bran (rice or wheat) and strain used. The trials have shown that cassava waste (stems and peels) can be used well for the production of oyster mushrooms and that substrates containing up to 75% cassava peels have productions well comparable to yields obtained on the traditional saw dust based substrates.

**Keywords:** *Pleurotus* spp., cassava waste, biological efficiency, fermentation

## INTRODUCTION

During the processing of cassava, large quantities of peels and, to a lesser extent, plant stems are produced. At present, only part of this waste is used, mainly as feed for goats. In many places where cassava is cultivated and processed, heaps of cassava peels are discarded along the roads and cause unpleasant odours and unhygienic conditions. Previous reports on the utilisation of cassava waste for the cultivation of grey oyster (*Pleurotus ostreatus*) and lung oyster (*Pleurotus pulmonarius*) mushrooms have shown that yields were reasonably well but still lower than those obtained on traditional substrate prepared from saw dust [1, 2]. It is known that waste from cassava production is low in nitrogen, one of the ingredients known to enhance the productivity of mushrooms. In this paper trials are presented in which substrate was prepared from cassava waste (peels and stems) supplemented with rice or wheat bran. Cultivation of both oyster mushroom species for 3 flushes showed that the addition of these supplements increases yields considerable up to levels comparable or even better than on traditionally used sawdust based substrates. In addition, cassava waste based substrates are colonized in a shorter time than saw dust and thus more crop cycle per year increasing the productivity even more. The cassava peels and stems were fermented for six days and inoculated subsequently with spawn without sterilization of the substrate. No infections were seen indicating that, when done properly, cultivation on cassava waste can be done also at lower costs since sterilization or high temperature pasteurization costs a lot of energy.

## MATERIALS AND METHODS

### Substrate

Fermentation of substrate and cultivation of mushrooms were done at Unifarm (test facility of Wageningen University and Research Centre). Cassava peels and stems were obtained via the Food Research Institute, Accra, Ghana (Mary Obodai) and originate from a regular cassava production site in Ghana. Dried peels and stems were packed in nylon sacks and submerged in water for 3 days and subsequently drained for one day to remove excessive water. Mixtures of peels and

stems were made and subsequently chalk and bran were added and mixtures were homogenized well. For each type of substrate mixture (Table 1) 3 trays were filled, each containing ca. 27.8 kg of substrate. Trays were put in a room heated up by steam up to 45 °C. This was done to mimic fermentation in large heaps where a preheating to start fermentation is not needed. Trays were fermented for 6 days. Most substrates have been at least for 8 hours above 65 °C. All trays were weighed to measure weight loss (which was 3-6 kg/tray). After cooling to 22 °C, each substrate type was mixed with spawn (60 ml, i.e. 30 grams of spawn/kg) and distributed over bags (2 kg inoculated substrate per bag). Bags were obtained from Microsac (http://www.saco2.com/) and taped off after filling. Spawn run (mycelial growth) was done in a growing room at 25 °C for ca 15 days.

**Table 1.** List of substrate mixtures made for the cultivation of *Pleurotus* species

| Substrate mixtures | Cassave peels % (w/w) | Cassave sticks % (w/w) | $CaCO_3$ % (w/w) | Rice bran % (w/w) | Wheat bran % (w/w) |
|---|---|---|---|---|---|
| 1 | 73.5 | 24.5 | 2 | 0 | 0 |
| 2 | 72.75 | 24.25 | 2 | 1 | 0 |
| 3 | 69 | 23 | 2 | 6 | 0 |
| 4 | 64.5 | 21.5 | 2 | 12 | 0 |
| 5 | 72.75 | 24.25 | 2 | 0 | 1 |
| 6 | 69 | 23 | 2 | 0 | 6 |
| 7 | 64.5 | 21.5 | 2 | 0 | 12 |
| 8 | 48.5 | 48.5 | 2 | 1 | 0 |
| 9 | 49 | 49 | 2 | 0 | 0 |
| 10 | 46 | 46 | 2 | 6 | 0 |
| 11 | 43 | 43 | 2 | 12 | 0 |
| 12 | 48.5 | 48.5 | 2 | 0 | 1 |
| 13 | 46 | 46 | 2 | 0 | 6 |
| 14 | 43 | 43 | 2 | 0 | 12 |

## Varieties

Varieties tested were obtained from the Federal Institute of Industrial Research, Oshodi (FIIRO): One *P. ostreatus* (MES14030) and one *P. pulmonarius* strain (MES14029), gift from Agnes Asagbra at FIIRO, and 2 strains from the Plant Breeding collection of Wageningen UR: one commercial *P. ostreatus* (MES 03448) and one *P. pulmonarius* strain originating from Brazil (MES01997).

## Cultivation

After colonization, 4 slits were made in each bag (one in all four sides) with a sharp knife to allow the formation of mushrooms. The room was subsequently vented with fresh air and cooled to 20 °C with relative humidity of 88%. Light was on for 12 hours/day.

# RESULTS AND DISCUSSION

## Fermentation of substrates

Fermentation of each type of substrate was done in trays each containing 27.8 kg substrate. Degradation of starch in the peels and other easily degradable compounds by microorganisms will usually heat up the substrate resulting in killing insects and pathogens and making the substrate more selective for growth of white rot fungi. Since only a small amount of substrate is present in each tray, the heat was easily lost to the environment. For that reason the environment (the room) was heated up to 45 °C to mimic fermentation in large heaps (where temperature is easily built up). Especially addition of wheat bran

lead to a quick heating of the substrate up to temperatures above 70 °C, i.e. 25 °C above the room temperature indicating an active growth of thermophilic microorganisms. Not all trays reached this temperature (minimum temperature achieved 61°C) but we expect that this is due to the small volumes in each tray and will be higher in large heaps. A visual inspection of the substrates after fermentation showed that most substrates were well colonized by thermophilic microorganism (whitish appearance, fire fang). Especially trays with wheat bran showed a dense colonization by thermophiles. The addition of wheat bran up to 6 and 12% seems too much since the substrate smells a bit (ammonia). The weight loss of substrates during fermentation was on average 16% (based on wet weight).

## Production profile

Daily inspections by eye showed that fifteen days after spawning (inoculation), most substrates were colonized well. Four slits were then made on the bags (on each side one). Three days later the room temperature was decreased to 20 °C to initiate fruiting. Averaged over all types of substrates, pins were formed between 19 days (commercial *P. pulmonarius* strain) to 24 days (*P. pulmonarius* from Nigeria) after inoculation whereas the *P. ostreatus* strains produced pins 23 days after spawning. Twenty three days after inoculation the first mushrooms were picked from the fastest *P. pulmonarius* strains while the slowest *P. ostreatus* strains were ready to be picked 27 days after inoculation. The *P. pulmonarius* strains had produced a third flush by 43 days after inoculation while the *P. ostreatus* strains needed on average 12 days more. An overview of the production profile is given in Table 2. The statistical analysis (see appendix) showed that for time between inoculation (spawning) and pinning there is a significant difference between strains and the amounts of rice bran added. In addition, there is also an interaction between strains and amount of bran added, i.e. not all strains react in the same way. For wheat bran there is a significant difference in time to pinning between strains.

**Table 2.** Production profiles of varieties of 2 oyster mushroom species cultivated on substrate prepared from different mixtures of cassava waste (see Table 1). Significant differences were seen on time between spawning (inoculation) and pinning and between spawning and first flush between strains. This was significantly influenced by the amount of bran added (see appendix for statistical analysis).

| Strain | Amount of bran added % (w/w) | Time between spawning and pinning (days) | | Time between spawning and first flush (days) | |
|---|---|---|---|---|---|
| | | Rice Bran | Wheat Bran | Rice Bran | Wheat Bran |
| *P. ostreatus* (Brazil) | 0 | 23 | 22 | 27 | 28 |
| *P. ostreatus* (Brazil) | 1% | 22 | 21 | 27 | 25 |
| *P. ostreatus* (Brazil) | 6% | 23 | 23 | 25 | 27 |
| *P. ostreatus* (Brazil) | 12% | 21 | 26 | 27 | 29 |
| *P. ostreatus* (Brazil) | *average* | *23* | *23* | *27* | *27* |
| *P. ostreatus* (FIIRO) | 0 | 24 | 21 | 28 | 25 |
| *P. ostreatus* (FIIRO) | 1% | 21 | 22 | 24 | 25 |
| *P. ostreatus* (FIIRO) | 6% | 22 | 26 | 25 | 28 |
| *P. ostreatus* (FIIRO) | 12% | 18 | 27 | 24 | 34 |
| *P. ostreatus* (FIIRO) | *average* | *23* | *23* | *27* | *27* |
| *P. pulmonarius* (commercial) | 0 | 19 | 18 | 24 | 24 |
| *P. pulmonarius* (commercial) | 1% | 17 | 17 | 23 | 22 |
| *P. pulmonarius* (commercial) | 6% | 19 | 21 | 24 | 24 |
| *P. pulmonarius* (commercial) | 12% | 19 | 20 | 24 | 24 |
| *P. pulmonarius* (commercial) | *average* | *19* | *19* | *24* | *25* |
| *P. pulmonarius* (FIIRO) | 0 | 23 | 24 | 27 | 26 |
| *P. pulmonarius* (FIIRO) | 1% | 16 | 22 | 25 | 24 |
| *P. pulmonarius* (FIIRO) | 6% | 20 | 23 | 23 | 26 |
| *P. pulmonarius* (FIIRO) | 12% | 26 | 27 | 23 | 29 |
| *P. pulmonarius* (FIIRO) | *average* | *24* | *24* | *25* | *25* |

For time between spawning and first flush there are significant differences between strains and the amount of bran added. This indicates that for each variety there might be different optima of type and amount of supplements on the production profile.

## Yields on substrate fermented in trays

Averaged over all substrates, the total yields after 3 flushes varied between the different strains (Table 3). The *P. pulmonarius* variety from Nigeria yielded 319 grams of fresh mushrooms per bag of 2 kg whereas the *P. ostreatus* of Nigeria yielded 583 grams per bag of 2 kg. The yield in fresh weight mushrooms for each substrate type varied considerably. Best yields

were for the *P. ostreatus* strain from FIIRO on cassava waste supplemented with 1% rice bran, i.e. 632 grams per 2 kg of substrate in 3 flushes (fresh weight productions and biological efficiency for all treatments are presented in the appendix). Expressed as biological efficiency (BE; fresh mushrooms per kg dry substrate), there are significant differences in yields due to the amount of either rice or wheat bran added. BE varied from as low as 47% up to 99% with different rice bran amounts added and from 42% up to 98% with different amount of wheat bran added. The general trend is that for rice bran the optimal amount is around 6% and for wheat bran 1% (wet weight/wet weight). This might reflect the amount of protein present in these two types of bran. As expected, fruiting bodies of the *P. ostreatus* had a light grey color whereas those of *P. pulmonarius* were dark brown (Fig.1). It is important to mention that none of the bags were infected, despite the fact that the substrates were only fermented for 6 days and maximum temperatures reached varied between 60 and 72 °C.

**Table 3.** Yields of mushrooms produced on the different substrate mixtures. Yields were recorded for 3 flushes. Especially the *P. ostreatus* variety of FIIRO showed a high production well comparable to yields on the traditional sawdust substrates. Statistical analysis for biological efficiency (fresh mushrooms per dry weight substrate) showed significant differences in yield between varieties and for the amount of bran added (see appendix).

| | | Yields (grams/bag of 2 kg substrate) | | Biological efficiency | |
|---|---|---|---|---|---|
| Strain | Bran added | Rice bran | Wheat bran | Rice bran | Wheat bran |
| *P. ostreatus* (Brazil) | 0 | 482 | 508 | 77% | 79% |
| *P. ostreatus* (Brazil) | 1% | 542 | 513 | 85% | 80% |
| *P. ostreatus* (Brazil) | 6% | 559 | 551 | 87% | 86% |
| *P. ostreatus* (Brazil) | 12% | 511 | 440 | 78% | 74% |
| *P. ostreatus* (Brazil) | average | 524 | 503 | 80% | 80% |
| *P. ostreatus* (FIIRO) | 0 | 560 | 590 | 88% | 92% |
| *P. ostreatus* (FIIRO) | 1% | 615 | 632 | 97% | 98% |
| *P. ostreatus* (FIIRO) | 6% | 632 | 613 | 99% | 94% |
| *P. ostreatus* (FIIRO) | 12% | 608 | 483 | 93% | 80% |
| *P. ostreatus* (FIIRO) | average | 604 | 579 | 91% | 91% |
| *P. pulmonarius* (commercial) | 0 | 471 | 504 | 74% | 79% |
| *P. pulmonarius* (commercial) | 1% | 516 | 523 | 81% | 82% |
| *P. pulmonarius* (commercial) | 6% | 526 | 514 | 82% | 79% |
| *P. pulmonarius* (commercial) | 12% | 539 | 408 | 83% | 68% |
| *P. pulmonarius* (commercial) | average | 513 | 506 | 77% | 77% |
| *P. pulmonarius* (FIIRO) | 0 | 298 | 349 | 47% | 54% |
| *P. pulmonarius* (FIIRO) | 1% | 364 | 352 | 57% | 55% |
| *P. pulmonarius* (FIIRO) | 6% | 365 | 319 | 57% | 49% |
| *P. pulmonarius* (FIIRO) | 12% | 400 | 254 | 61% | 42% |
| *P. pulmonarius* (FIIRO) | average | 357 | 319 | 52% | 52% |

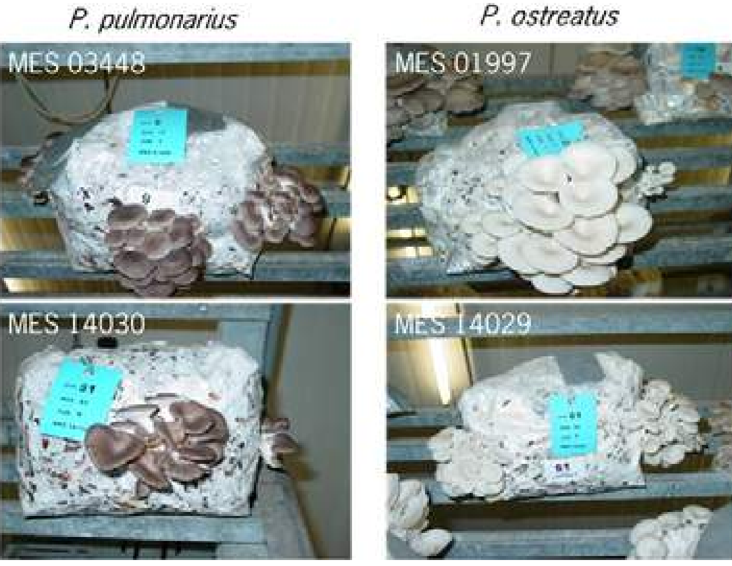


**Figure 1.** Representative photographs of fruiting bodies of flush 1 of the 4 varieties grown on substrate prepared from cassava waste

## CONCLUSION

The trials done with substrate prepared with cassava crops from Ghana showed clearly the potential of cassava waste as raw and safe (Obodai *et al.*) [3] materials for the production of oyster mushrooms. Although one has to keep in mind that cultivation conditions used here are much more optimal than on average in African countries the following conclusions can be drawn:

- Substrate based on cassava waste only can give yields of oyster mushrooms well comparable to traditional substrates such as saw dust. Yields close to 100% biological efficiency in only 3 flushes make this crop economically sustainable.
- Substrate based on cassava waste only (peels & stems) are more quickly colonized than the traditional saw dust based substrates.
- Fermentation of cassava waste can be done within 6 days. When done under proper conditions and inoculation under hygienic conditions, a sterilisation (or heating up to 100 °C) is not needed.

## ACKNOWLEDGEMENTS

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## APPENDIX

Statistical analysis.

Statistical analysis of time between spawning and pinning (days) on supplementation with rice bran. There is a significant difference between strains and between the amount of rice bran added. There is also a significant interaction between strains and the amount of rice bran added: not each strain reacts in the same way on the amount of bran added.

**Analysis of variance**

Variate: spawning_pinning

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| Herhaling stratum | | | | | | |
| rice_bran | 3 | | 3.71 | 1.24 | | |
| Residual | -2 | (1) | 2.27 | | | |
| Herhaling."Units" stratum | | | | | | |
| Strains | 3 | | 563.31 | 187.77 | 13.77 | <.001 |
| rice_bran | 3 | | 382.31 | 127.44 | 9.34 | <.001 |
| Strains.rice_bran | 9 | | 1164.54 | 129.39 | 9.49 | <.001 |
| Residual | 47 | (103) | 640.94 | 13.64 | | |
| Total | 63 | (104) | 1331.73 | | | |

Statistical analysis of time between spawning and first flush (days) on supplementation with rice bran. There is a significant difference between strains and between the amount of rice bran added but no significant interaction between strains and the amount of rice bran added.

**Analysis of variance**

Variate: spawning_pinning

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| Herhaling stratum | 1 | (1) | 0.08 | 0.08 | 0.00 | |
| Herhaling "Units" stratum | | | | | | |
| Strains | 3 | | 744.99 | 248.33 | 12.77 | <.001 |
| wheat_bran | 3 | | 345.02 | 115.01 | 5.91 | 0.002 |
| Strains.wheat_bran | 9 | | 140.10 | 15.57 | 0.80 | 0.618 |
| Residual | 47 | (103) | 914.18 | 19.45 | | |
| Total | 63 | (104) | 1331.73 | | | |

Statistical analysis of time between spawning and pinning (days) on supplementation with wheat bran. There is a significant difference between strains. There is no significant difference between the amount of wheat bran added and no interaction between strains and the amount of bran added.

**Analysis of variance**

Variate: Spawning_flush_1

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Herhaling stratum | 2 | 9.083 | 4.542 | 0.56 | |
| Herhaling.*Units* stratum | | | | | |
| Strains | 3 | 251.875 | 83.958 | 10.44 | <.001 |
| wheat_bran | 3 | 362.250 | 120.750 | 15.01 | <.001 |
| Strains.wheat_bran | 9 | 210.000 | 23.333 | 2.90 | 0.003 |
| Residual | 150 | 1206.500 | 8.043 | | |
| Total | 167 | 2039.708 | | | |

Statistical analysis of time between spawning and first flush (days) on supplementation with wheat bran. There is a significant difference between strains and between the amount of wheat bran added but no significant interaction between strains and the amount of rice bran added.*Volgensmij is eenwaarde van 0.003 wel significant.*

**Analysis of variance**

Variate: Spawning_flush_1

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Herhaling stratum | | | | | |
| rice_bran | 3 | 4.76 | 1.59 | | |
| Residual | -1 | 4.32 | | | |
| Herhaling.*Units* stratum | | | | | |
| Strains | 3 | 251.88 | 83.96 | 8.36 | <.001 |
| rice_bran | 3 | 169.00 | 56.33 | 5.61 | 0.001 |
| Strains.rice_bran | 9 | 102.60 | 11.40 | 1.13 | 0.342 |
| Residual | 150 | 1507.14 | 10.05 | | |
| Total | 167 | 2039.71 | | | |

# EXPLOITATION OF THERMOPHILIC FUNGI IN COMPOST PRODUCTION FOR WHITE BUTTON MUSHROOM (*AGARICUS BISPORUS*) CULTIVATION – A REVIEW

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## ABSTRACT

Compost production is the most important and integral part of button mushroom cultivation and its quality parameters mainly determine success or failure of a crop. It is a product of fermentation brought about by the activities of thermophilic organisms and among them fungi especially, *Scytalidium thermophilum, Humicola insolens* and *H. grisea* play a decisive role in bringing about the selectivity and productivity of the compost. In a pursuit to improve the present day composting procedures using thermophilic fungi and also to prepare compost in most environment friendly manner in shortest possible time, series of experiments were conducted at Directorate of Mushroom Research, Solan, India. Role of thermophilic fungi especially that of *H. insolens* was thoroughly investigated. Four different strains of *H. insolens* were isolated from different parts of India through molecular characterization. Based on many physiological, enzymatic and under *in vitro* studies, I-1 and X-21 strains of *H. insolens* and *S. thermophilum,* respectively were short listed as potent strains for their further exploitation in compost production. Artificial inoculation of these fungi on zero day under long method of composting (LMC) not only brought down the composting period to 20 days but also *S. thermophilum* significantly increased the button mushroom yield. Inoculation of these fungi under short method of composting period (phase-II) could be brought down from seven to five days. These two fungi and their combinations were further utilized in improving the environment friendly indoor composting (Anglo-Dutch method). High conversion of compounding mixture to compost was found in inoculated treatments with increased yield over control. Total composting operation lasted for 13 days. Highest degradation of cellulose, hemicellulose and carbon was observed in inoculated pile, which contributed in good spawn run and highest yield.

**Keywords:** compost, thermophilic fungi, indoor composting, *Humicola insolens, Scytalidium thermophilum*

## INTRODUCTION

Compost production is the most important and integral part of *Agaricus bisporus* (white button mushroom) cultivation. It is a product of fermentation brought about by the variety of organisms including bacteria, actinomycetes and fungi. These organisms convert and degrade the straw to form lignin humus complex and also convert soluble form of nitrogen into microbial cell substances [1-3]. This decomposed straw along with microbial biomass both become a source of organic and inorganic nutrition for the mushroom mycelium [4]. The process of composting is governed by a carefully ordered changing population of organisms [5]. Further, these mycoflora also play a key role towards selectivity and conditioning of the compost and make the growth of competitor microorganisms more difficult [6-8]. Compost if properly prepared, *A. bisporus* can only successfully grow in it at the practical exclusion of the competing organisms [9]. Various kinds of flora encountered during whole process of composting have different role to play. Mesophilc flora present in the initial process of composting bring about the biodegradation of the straw and other ingredients which results in heat energy resulting in establishment of thermophilic flora in the compost which later on govern the whole process of composting. Of the thermophilic fungi generated in the compost during the course of composting, *Scytalidium thermophilum*, *H. insolens*, *Humicola grisea* and *Chaetomium thermophile* are of utmost importance as they are responsible for nutrition of *A.bisporus* and faster composting process [10-12].

Work on isolation and role of thermophilic fungi in compost production started at the National Centre almost 2 decade back. Majority of those studies were based on isolation of different thermophilic flora across the country, from different compost piles having different N values. Molecular characterization of different thermophilc fungi, their role in composting

process, etc. Special emphasis was given to *S. thermophilum* in its role in composting process. All these studies were reported from time to time in different journals which were finally reviewed [12-15].

*H. insolens* is reported to be an ideal agent in compost preparation and hasten the composting process [16, 17]. It secretes various cellulolytic as well as lignolytic enzymes to degrade the compounding mixture of compost. Increased growth of *A. bisporus* mycelium in presence of this fungus is also reported [18]. Cellulolytic role of *H. insolens* is well defined in terms of Xylanase [19], â-glucosidase [20] but its lignolytic activity is only tangentially known. Stanek [6] indicated that *Humicola* spp. produce vitamins and amino acids which can be utilized by *A. bisporus* for its growth. No systematic studies on the role of this fungus on compost production for button mushroom however, have been reported so far in spite of the fact that this fungus remains a choice of interest due to its abundant occurrence in compost at various stages of its preparation.

In a pursuit to improve the present day composting procedures and also to prepare compost in most environment friendly manner in shortest possible time series of experiments were conducted at ICAR-Directorate of Mushroom Research, Solan, India from 2007 onwards. Role of thermophilic organisms and especially that of *H. insolens* was thoroughly investigated [21, 22, 23, 24, 25, 26, 27, 28, 29]. A thesis on *H. insolens* was also approved [30]. Significant findings there on and especially that of *H. insolens* are presented in this communication.

## Isolation of *Humicola insolens*

Primary objective of the study was to isolate varied diversity of *H. insolens* across the country. We could isolate *H. insolens* from 51 samples collected from Haryana, Himachal Pradesh, Jharkhand, Maharashtra, Meghalaya, Punjab, Rajasthan, Tamil Nadu and Uttar Pradesh. Based on morphological characters these were placed in twelve groups (I-1 to I-12). I-1 isolate was distributed in samples collected from Haryana, Himachal Pradesh, Punjab, Rajasthan and Uttar Pradesh states. I-2 isolate was isolated from Maharashtra and Tamil Nadu states. I-3 isolate from Haryana, Himachal Pradesh, Punjab and Uttar Pradesh. I-4 from Haryana, Jharkhand, and Uttar Pradesh. I-5 from Haryana and Himachal Pradesh. I-6 from Meghalaya only. I-7 was isolated from Haryana and Uttar Pradesh, I-8 from Himachal Pradesh and Uttar Pradesh, I-9 from Haryana and Uttar Pradesh. I-10 from Himachal Pradesh and Maharashtra and I-11 and I-12 Haryana and Punjab. Dominance of I-1 was maximum across the country followed by I-3. Frequency of occurrence of *H. insolens* was 51%. *S. thermophilum* was another fungus which was most dominantly isolated from all most all the samples (55% dominance) (Fig.-1). This fungus also showed lots of variations with respect to its morphology and was grouped on the basis of molecular characterization in seven groups (S-1 to X-21). Other thermophilic fungi isolated were *H. grisea*, *A. fumigatus*, *T. lanuginosus*, *H. fuscoatra*, *S. thermophile*, *S. chlorianum* and *T. duponti.* Most of them are known thermophilic taxa [31].

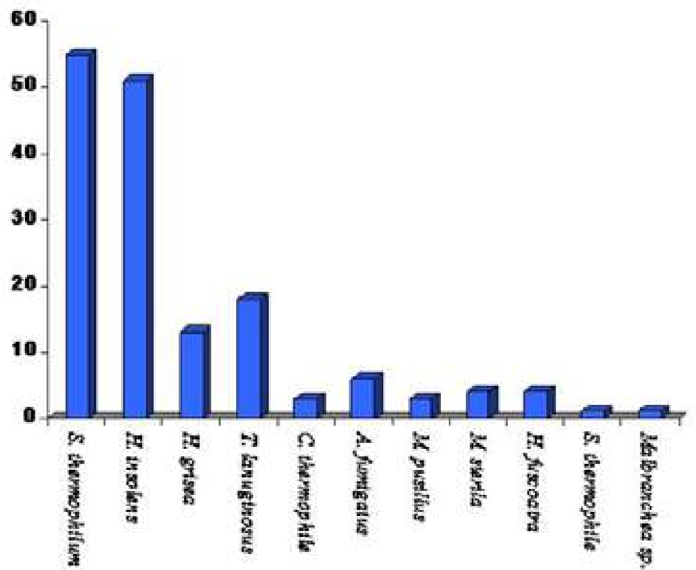

**Figure 1.** Relative dominance (% frequency) of *H. insolens* with respect to other thermophilic fungi

## Molecular identification and characterization of *H. insolens* isolates

To identify the 12 isolates at genus level PCR amplification of Internal Transcribed Spacer was done by using ITS 1 and ITS 4 primers. Amplified region of ITS of twelve isolates showed clear band between 500-600 bp (Fig. 2). No intra-species diversity could be detected as far as ITS lengths of 5.8S r-DNA region were concern. Since, 5.8S gene is highly conserved at genus level, it could be confirmed that all twelve isolates belonged to the same genus. The isolates were

sequenced using ITS-1 and ITS-4 universal primers and BLAST against website of NCBI confirmed the identity of the strains as *H. insolens* . Although the BLAST have shown more similarity of the isolates with *S. thermophilum,* but the consideration in BLAST such as expect value, differences in the initial sequences numbers between subject and query, and missing values helped the confirm the identity as *H. insolens*. The reason for giving more similarity might be due to the close taxonomic position of *S. thermophilum* with *H. insolens* and more n values in the *H. insolens* sequences. However, RAPD analysis of 12 strains showed the presence of 4 different strains of this fungus (Fig. 3) These strains were quite different from *H. insolens* strains reported earlier [32].

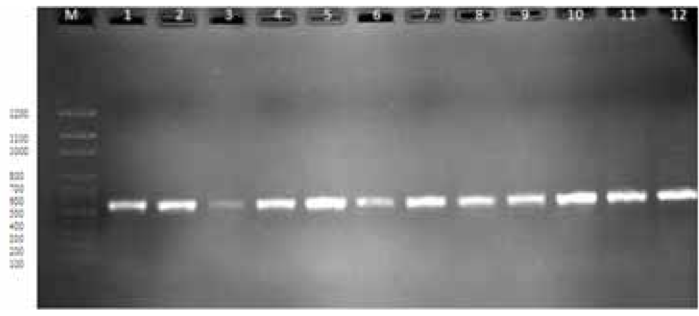

**Figure 2.** ITS profile of different isolates of *H. insolens*

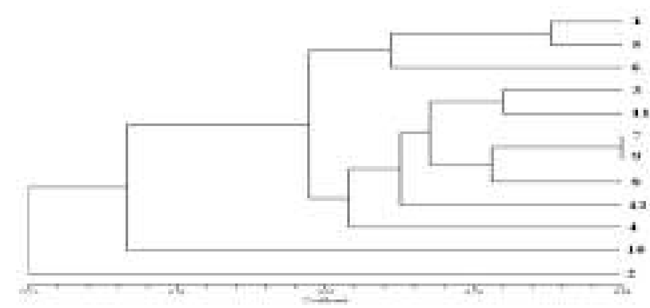

**Figure 3.** Phylogram of *H. insolens* isolates showing four different groups

## Physiological and interaction studies

In present investigation four *H. insolens* strains initially characterized by molecular techniques were taken for various physiological and interaction studies. Initially all the strains were grown on different media to screen suitable medium for these strains and also to shortlist the potent strain which shows best performance/growth as compared to others. Best growth and mycelial weight of all strains was observed in malt extract followed by yeast and compost extract (Fig. 4). PDA was not a preferred medium for *H. insolens*. Strain-1 showed best growth compared to all other strains. Growth of *H. insolens* on different pH was studied on MEA and on different agricultural residue (wheat straw, wheat bran, cotton seed cake, cotton seed meal) along with different compost formulations (Table-1) to shortlist the best agricultural residue and formulation used in compost production for *A. bisporus*. Under this study best growth of all the strains was found in cotton seed meal followed by wheat bran and wheat straw at 7.0-8.0 pH (Fig. 5). Strain-1 exhibited the highest growth among all the strains on all agricultural residues at different pH. All the four strains preferred most of the formulations for their growth. Highest growth was observed in strain-1 in formulation 1, 2, 5 and 6, followed by strain-2 in formulation- 2 at different pH ranges (5.0-8.0).After screening different strains on different media and pH, study was conducted to evaluate the cardinal temperature for different strains.

It was found that best growth of different strains was observed in the range of 45-55 $^{o}$C. Slow growth was recorded at 25-35$^{o}$C (Table 2). Present data obtained are very similar to what has been reported in the literature [31, 33-35].

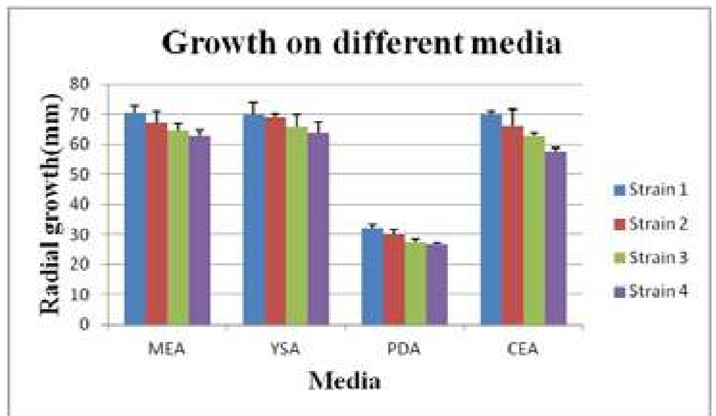


**Figure 4.** Showing growth of *H. insolens* strains on different media

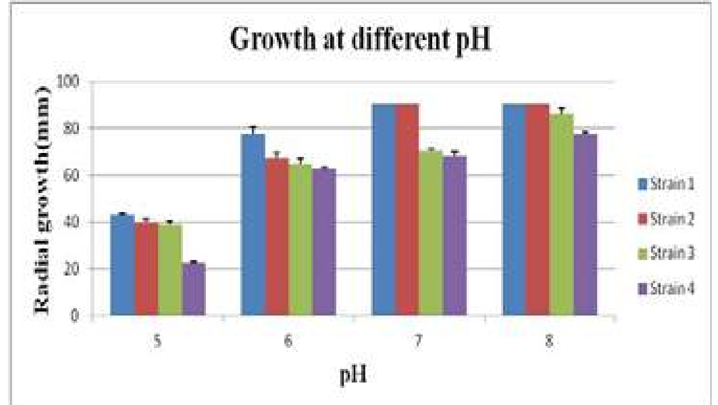


**Figure 5.** Showing growth of *H. insolens* strains on different pH levels in chicken manure based formulation

**Table 1.** Medium composition (compost formulations)

| | Formulations | | | | | |
|---|---|---|---|---|---|---|
| **Substrate** | **F-I (g)** | **F-II (g)** | **F-III (g)** | **F-IV (g)** | **F-V (g)** | **F-VI (g)** |
| Wheat Straw | 250.0 | 250.0 | 250.0 | 250.0 | 250.0 | 250.0 |
| Chicken manure | 125.0 | 125.0 | 125.0 | 125.0 | 62.5 | 100.0 |
| Wheat bran | _ | _ | _ | _ | 18.75 | 25.0 |
| Cotton seed meal | 50.0 | _ | _ | _ | _ | _ |
| Cotton seed cake + | | | | | | |
| Cotton seed meal | _ | 50.0 | | | 18.75 | _ |
| Soya bean meal | _ | _ | _ | 50.0 | | _ |
| Nutri | _ | _ | 50.0 | _ | _ | _ |
| Gypsum | 19.0 | 2.5 | 9.0 | 18.75 | 25.0 | 7.5 |
| Urea | 2.5 | 2.25 | 2.5 | 2.5 | _ | 3.75 |

**Table 2.** Growth of four strains of *H. insolens* at different temperature

| | Radial growth (mm) of *H. insolens* at different temperature (°C) | | | |
|---|---|---|---|---|
| **Strains** | **25** | **35** | **45** | **55** |
| I-1 | 22.33 | 43.66 | 70.33 | 89.16 |
| I-2 | 16.83 | 43.66 | 67.16 | 70.83 |
| I-3 | 16.83 | 33.50 | 64.83 | 68.33 |
| I-4 | 9.6 | 20.83 | 64.83 | 68.33 |
| $CD_{0.05}$ | 2.93 | 2.34 | 2.08 | 2.61 |

Dual culture studies between various strains and *A. bisporus* and other mesophilic fungi indicated that strain-1 increased the growth of *A. bisporus* while reduction in its growth was observed with rest three strains. When *A. bisporus* growth was again observed after 15 days it was found that it completely parasitized *H. insolens* and possibly utilized it as nutrient source. Increased growth of *A. bisporus* in dual culture with *H. insolens* could be due to the ability of *A. bisporus* to degrade the mycelium of this strain [36-38] or utilization of vitamins and amino acids produced by this strain of *H. insolens* [6]. Vijay [8, 39] also presented similar observations. Study therefore, gave indication that large population of this fungus if present in the compost may provide extra nutrition to the *A. bisporus* mycelium later, when such compost is spawned. Artificial inoculation of this fungus in the compost may also prove beneficial. Therefore, this strain was further chosen for field studies on improving the composting processes presently in vogue in the country and also to develop new composting techniques. Almost similar trend with respect to culture filtrates of four *H. insolens* strains was observed on *A. bisporus*. As regards to dual culture and culture filtrates studies on other mesophilic organisms, it was observed that strain-2 inhibited the growth of *Fusarium accuminatum* profoundly (Table 3).

This fungus is usually found in chicken manure, wheat bran and other stored materials commonly used in the compost. It is usually associated with damping off type symptoms on button mushroom pinheads. Strain-2 if present in the compost at spawn run and at cropping stage may inhibit the growth of this fungus thereby reducing the symptoms. Strain -1 inhibited the growth of *Chrysospermum luteum* and *Papulaspora bysinna* markedly both of which are strong competitors of button mushroom mycelium and are the causal agents of yellow mould disease and brown plaster mould respectively. Both these moulds are more prevalent in long method compost where thermophilic population is always at its low ebb. Such composts therefore, are more prone to attack by different competitors. Surprisingly other *H. insolens* strains increased the

**Table 3.** Growth (mm) and % inhibition of different mesophiles in culture filtrates of different strains of *Humicola insolens*

| | Growth( mm) (% inhibition) | | | |
|---|---|---|---|---|
| ***H. insolens* Strains** | ***Chaetomium luteum*** | ***Fusarium accuminatum*** | ***Papulaspora byssinia*** | ***Agaricus bisporus*** |
| I- 1 | 8.50 (-73.84) | 48.00 (+6.66) | 15.50 (-59.74) | 27.50 (+22.22) |
| I- 2 | 34.50 (+6.15) | 8.50 (-81.11) | 40.00 (+3.89) | 18.00 (-20.00) |
| I- 3 | 34.00 (+4.61) | 48.00 (+6.66) | 40.00 (+3.89) | 18.00 (-20.00) |
| I- 4 | 35.50 (+9.23) | 50.00 (+11.11) | 42.00 (+9.09) | 12.50 (-44.44) |
| Control (Fresh medium) | 32.50 (0.00) | 45.00 (0.00) | 38.50 (0.00) | 22.5 (0.00) |

growth of these competitors. Study indicated that strain-1 of *H. insolens* is very superior to other strains with many beneficial attributes.

## Production of extra-cellular enzymes by different strains of *Humicola insolens*

In the present investigation degradation of hemicellulose, cellulose and lignin at various pH levels was measured in terms on xylanase, exo-cellulase, endocellulase, β-glucosidase and laccase on different agricultural residues and on different compost formulations (Table-1). All these enzymes play an important role in degrading the polymeric components of agricultural residues in monomeric form or sugars which later can easily be utilized by different thermophilc fungi and *H. insolens* in particular. The highest activity of all the cellulolytic and lignolytic enzymes was recorded at pH 8.0 in all agricultural residues and formulations by all the strains.

Best xylanase, β-glucosidase, FPase, activity was observed in wheat bran followed by cotton seed meal and wheat straw when tried on different agriculture residues. Best CMCase activity was observed in cotton seed meal followed by cotton seed cake. Hence, high degradation of cellulose was observed in wheat bran as compared to other agricultural residues followed by cotton seed meal. Maheswari *et al.* [40] also reported the best cellulolytic activity of *H. insolens* in wheat bran. Other agricultural residues showed more or less similar trend. This indicated that *H. insolens* has high titer of xylanase enzyme in the medium and maximum activity was observed in neutral to alkaline medium in increasing order.

Next to the xylanase, high β-glucosidase activity was observed in different strains of *H. insolens* in wheat bran followed by wheat straw and the highest activity was observed at pH 8.0 by all the strains and best one was observed in strain-1. Flavio *et al*. [20] also reported best β-glucosidase activity of *H. insolens* at pH 8.0-9.0 which confirms our findings. Other celluloytic activities were less as compared to xylanase and β-glucosidase and highest of which was observed at pH 8.0 by all the strains in all the residues and formulations and best was observed in strain-1. Similar trend was found in different formulations, exhibiting the highest cellulolytic and lignolytic activity in formulation-6 (F-6) having wheat bran in medium followed by formulation-5 and 2 (F-5 and F-2) having cotton seed meal and cotton seed cake. This indicated that supplementation of medium with nitrogen compounds increases the laccase activity. The highest activity of cellulolytic and lignolytic enzymes was observed in strain-1 followed by strain-2 and 3. Least activity was observed in strain-4. Further wheat bran followed by wheat straw was best utilized by these strains and formulation (F-6) containing wheat bran supported maximum liberation of these enzymes. Therefore, strain-1 of this fungus along with formulation F-6 was chosen for further studies.

## Production of *Agaricus bisporus* compost using *Scytalidium thermophilum* and *H. insolens* under *in vitro* conditions

In the present studies seven different strains of *S. thermophilum* and four that of *H. insolens* were evaluated for their ability to convert agricultural residues to button mushroom compost under *in vitro* conditions in BOD incubators by providing exact conditions of composting. Main aim of the study was to select the suitable strains of both these fungi so that they could be inoculated in the compounding mixture for large scale compost production in the field for production of compost in shortest possible time without causing any environment pollution. All the strains were able to convert substrate

into compost both in unsterilized and sterilized substrates under *in vitro* conditions in seven days time. In both the sets mixture of *S. thermophilum* ( X-21 strain) and *H. insolens* (I-1 strain) performed exceedingly well as evident by various biological and physico-chemical parameters including spawn run and yield. Sterilized substrate performed better compared to unsterilized substrate (Table 4). Large number of representative strains which were inoculated in the compost were isolated in sterilized set as compared to unsterilized set. This was obvious as unsterilized substrate harbored other fungi like *A. fumigatus* and *M. pusillus* which reduced the extent of proliferation of representative strains in unsterilized treatments. Presence of these unwanted fungi later hampered the *A. bisporus* spawn run also. Several workers also suggested the role of these thermophilic fungi in making compost selective and nutritive for *A. bisporus* [3, 6, 10, 35, 41]. Less ammonia

**Table 4.** Physical and biological parameters of the composts prepared by different thermophilic fungi after 7 days of incubation (sterilized substrate)

| Strains | Temp. C | pH | Wt. Loss% | Colour | Ammonia ppm | cfu/g($10^4$) | Dominant Flora• (No of colonies) |
|---|---|---|---|---|---|---|---|
| | | | | ***S .thermophilum*** | | | |
| S-1 | 41.2 | 7.8 | 47.65 | +++ | 7.0 | 52.00 | 1(38.3)>7 (13.6) |
| S-2 | 41.9 | 7.6 | 47.62 | +++ | 7.0 | 53.66 | 1(36.0)>7 (15.6) |
| S-3 | 39.2 | 7.8 | 45.65 | ++ | 6.5 | 46.00 | 1(27.6)>7 (15.0) |
| S-4 | 39.1 | 7.8 | 47.78 | +++ | 6.0 | 37.76 | 1 (28.6)>5(7.3) |
| S-5 | 39.1 | 7.8 | 47.65 | +++ | 6.0 | 38.16 | 1(25.3)>9 (11.6) |
| S-6 | 39.2 | 7.6 | 47.82 | +++ | 6.0 | 30.46 | 1(21.0)>8 (6.6) |
| X-21 | 37.6 | 7.5 | 55.67 | ++++ | 5.5 | 65.00 | 1(55.6)>7 (8.3) |
| | | | | ***H. insolens*** | | | |
| I-1 | 37.1 | 7.6 | 40.72 | ++++ | 5.5 | 60.33 | 2(52.6)>8 (7.0) |
| I-2 | 44.9 | 7.8 | 32.67 | +++ | 6.0 | 40.66 | 2(32.0)>7(8.3) |
| I-3 | 43.5 | 7.8 | 33.19 | +++ | 6.0 | 42.33 | 2(32.3)>9 (8.3) |
| I-4 | 44.3 | 7.8 | 33.92 | ++ | 6.5 | 32.00 | 2(23.3)>7 (6.6) |
| | | | | **Control*** | | | |
| C-1 | 46.6 | 8.8 | 25.21 | ++ | 8.0 | 15.33 | 1(6.6)>12 (6.6) |
| C-2 | 43.8 | 7.6 | 43.62 | ++++ | 3.5 | 77.00 | 1(46.6)>2(30.3) |
| C-3 | 40.3 | 8.9 | 24.02 | +++ | 8.0 | 45.00 | 1 (23.3)> 2(15.6) |
| C-4 | 20.5 | 8.7 | 9.30 | + | 12.5 | 10.00 | 6 (8.3)>10 (1.6) |

*C-1 (uninoculated substrate incubated at 47±1C), C-2 (Substrate inoculated with X-21 and I-1 strains), C-3 (Substrate inoculated with pasteurized compost @ 20g/1.5Kg. compost) and C-4 (uninoculated substrate incubated at room temperature 25±1C)

*Dominant flora *1. S. thermophilum 2. H. insolens 5. C. thermophile 6. A. fumigatus, 7. S. thermophile 8, T. duponti 9. T. lanuginosus 10. T. viride 12. M. pusillus*

emission was observed in consortium and other inoculated treatments. C-3 treatment which was inoculated with pasteurized compost also performed well but it showed the presence of thermophilic fungi other then X-21 and I-1 strains which were not as effective as these two fungi and hence high ammonia levels were observed in this treatment coupled with slow and poor spawn run. Degradation of compost was measured in terms of NDF, ADF, hemicellulose, cellulose, carbon, nitrogen and ADL which defined the maturity of compost and are also known as compost maturity indices. It was found that best degradation in all of these parameters was found in C-2 and X-21 treatments followed by I-1treatment. More degradation of these components was found in sterilized set (Table 5).

**Table 5.** Physico-chemical analysis of compost prepared by different thermophilic fungi

| Strains | NDF% | ADF% | Hc % | ADL% | Cell. % | C% | N% | C/N |
|---|---|---|---|---|---|---|---|---|
| | | | | *Scytalidium thermophilum* | | | | |
| S-1 | 56.25 (14.46) | 50.18 (14.60) | 6.07 (13.28) | 20.15 (+9.39) | 20.65 (12.68) | 39.59 (2.60) | 1.73 (1.70) | 22.84 (1.08) |
| S-2 | 55.76 (15.20) | 49.11 (16.42) | 6.65 (5.00) | 22.16 (+20.30) | 20.54 (13.15) | 38.45 (5.41) | 1.72 (2.27) | 22.35 (3.20) |
| S-3 | 54.87 (16.56) | 48.11 (18.12) | 6.76 (3.42) | 20.67 (+12.21) | 20.66 (12.64) | 39.55 (2.70) | 1.75 (0.56) | 22.60 (2.12) |
| S-4 | 54.67 (16.86) | 48.65 (17.20) | 6.02 (14.00) | 21.65 (+17.53) | 19.65 (16.91) | 37.86 (6.86) | 1.75 (0.56) | 21.63 (6.32 |
| S-5 | 55.67 (15.34) | 50.65 (13.80) | 5.02 (28.28) | 20.67 (+12.21) | 19.78 (16.36) | 38.66 (4.89) | 1.76 (0.00) | 21.96 (4.89 |
| S-6 | 57.65 (12.33) | 50.70 (13.71) | 6.95 (0.71) | 20.65 (+12.10) | 19.65 (16.91) | 38.57 (5.11) | 1.72 (2.27) | 22.42 (10.52) |
| X-21 | 48.60 (26.09) | 43.84 (25.39) | 4.76 (32.00) | 18.21 (1.14) | 17.62 (25.49) | 36.78 (9.52) | 1.78 (+1.13) | 20.66 (10.52) |
| | | | | *Humicola insolens* | | | | |
| I-1 | 48.76 (25.85) | 45.76 (22.12) | 3.00 (57.14) | 18.67 (+1.35) | 20.12 (14.92) | 37.65 (7.38) | 1.78 (+1.13) | 21.71 (5.97) |
| I-2 | 56.75 (13.70) | 52.87 (10.02) | 3.88 (44.57) | 22.76 (+22.34) | 20.65 (12.68) | 38.67 (4.87) | 1.73 (1.70) | 21.77 (5.71) |
| I-3 | 54.76 (16.72) | 51.65 (12.10) | 3.11 (55.57) | 21.78 (+18.24) | 20.62 (12.81) | 40.28 (0.91) | 1.78 (+1.13) | 22.62 (2.03) |
| I-4 | 57.32 (12.83) | 52.65 (10.39) | 4.67 (33.28) | 23.76 (+28.99) | 21.65 (8.45) | 38.75 (4.67) | 1.78 (+1.13) | 22.89 (0.86) |
| | | | | **Control** | | | | |
| C-1 | 65.76 | 58.76 | 7.00 | 18.42 | 23.65 | 40.65 | 1.76 | 23.09 |
| C-2 | 38.65(41.22) | 36.52(37.84) | 2.13(69.57) | 20.76(+12.70) | 17.76(24.90) | 33.62(17.29) | 1.78(+1.13) | 18.88(18.23) |
| C-3 | 65.55(0.31) | 57.65(1.88) | 7.90(+12.85) | 25.75(+39.73) | 22.75(3.80) | 38.65(4.92) | 1.72(2.27) | 22.47(2.68) |
| C-4 | 70.66(+7.45) | 62.76(+6.80) | 7.90(+12.85) | 22.76(+23.56) | 24.76(+4.69) | 38.76(4.64) | 1.57(10.79) | 24.65(+6.75) |

Figures in parenthesis are % increase or decrease as compared to control (C-1)

Sterilization break the polymer of cellulose, hemicelluloses and lignin compounds which were easily accessible to the thermophilic fungi making compost favorable and selective for the growth of *A. bisporus* which resulted in higher yield (Table 6). Thus, from the study it was concluded that mixed inoculum of X-21, and I-1 was best as compared to other treatments. These strains were therefore, further evaluated in the field on large scale for improving the composting process with particular reference to environment controlled composting.

## Studies on improvements in *Agaricus bisporus* compost using thermophilic fungi

In India seasonal growers prepare their compost employing long method which gives low yields and also pollutes the atmosphere. Many environment controlled units still employ short method which gives satisfactory yields however, phase-I phase of such compost emits lots of malodorous gases and thus creates environment pollution. This method of composting has become unpopular with the commercial units mainly due to environment related issues and gradually is being replaced by almost environment friendly indoor composting process [42]. In this process compost is prepared totally under enclosed structures (bunkers+ tunnel) thus avoiding pollution to a greater extent. Further, compost by such method is prepared in 12 days time without much loss of the ingredients with higher productivity.

Main aim of the present studies was to improve the present day composting procedures (long, short and indoor methods) with the help of potent thermophilic fungi (I-1 & I-3 strains of *H. insolens* and X-21 strain of *S. thermophilum)* to reduce period of composting, obtained higher yields, more compost per unit weight of ingredients taken and environment friendliness. These two fungi and their strains were used as artificial inoculants in above three kinds of compost and findings there on are communicated.

**Table 6.** Spawn run and yield of unsterilized and sterilized set

| | Unsterilized set | | | | | Sterilized set | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatments** | **Days taken for spawn run** | **Condition of spawn run*** | **Yield g/kg compost** | **Incidence of competitor fungi** | **Treatments** | **Days taken for spawn run** | **Condition of spawn run*** | **Yield g/kg compost** | **Incidence of competitor fungi** |
| *Scytalidium thermophilum* strains | | | | | | | | | |
| S-1 | 8.0 | ++ | 68.3 | + | S-1 | 7.0 | +++ | 100.0 | - |
| S-2 | 8.0 | ++ | 74.3 | + | S-2 | 7.0 | +++ | 120.0 | - |
| S-3 | 8.0 | ++ | 67.0 | ++ | S-3 | 7.0 | +++ | 95.0 | - |
| S-4 | 8.0 | ++ | 40.0 | ++ | S-4 | 7.0 | +++ | 105.0 | - |
| S-5 | 8.0 | ++ | 85.0 | ++ | S-5 | 7.0 | +++ | 125.0 | - |
| S-6 | 8.0 | +++ | 100.0 | + | S-6 | 7.0 | +++ | 130.0 | - |
| X-21 | 7.0 | ++++ | 130.0 | - | X-21 | 6.0 | +++++ | 160.0 | - |
| *Humicola insolens* strains | | | | | | | | | |
| I-1 | 7.0 | ++++ | 105.0 | + | I-1 | 6.0 | +++++ | 155.0 | - |
| I-2 | 7.0 | +++ | 85.0 | ++ | I-2 | 6.0 | ++++ | 125.0 | - |
| I-3 | 8.0 | ++ | 45.0 | ++ | I-3 | 8.0 | ++++ | 100.0 | - |
| I-4 | 8.0 | + | 20.0 | +++ | I-4 | 8.0 | +++ | 85.0 | - |
| Control sets | | | | | | | | | |
| C-1 | 10.0 | + | 90.0 | +++ | C-1 | 9.0 | ++ | 120.0 | - |
| C-2 | 6.0 | +++++ | 155.0 | - | C-2 | 6.0 | +++++ | 170.0 | - |
| C-3 | 9.0 | +++ | 105.0 | + | C-3 | 8.0 | ++++ | 145.0 | - |
| C-4 | No spawn run | - | 0.0 | ++++ | C-4 | 10.0 | ++ | 70.0 | ++++ |
| CD(p=0.05) | | | 23.39 | | | | | | |

*+ poor, ++ Average, +++ good, ++++ very good, +++++ excellent

## Long method of composting (LMC)

Improvements in the yield and shortening the duration of LMC was attempted by inoculating potent strains of *S. thermophilium* (X-21), *H. insolens* (I-1 and I-3) and their consortium in compost at zero day. Data obtained indicated that composting period can be brought down from 28 to 20 days in inoculated treatments as compared to control. Significantly higher yield was obtained in compost inoculated with X-21 treatment (Table 7). Control treatment (uninoculated) harboured the heavy infestation of *Coprinius* sp. and *P. bysinna* resulting in lowest yield. Comparative results of each treatment were analyzed by measuring various physico-chemical factors such as temperature, pH, moisture %, C/N ratio, cellulose, hemicellulose, etc. at regular intervals of composting. Diversity of thermophilic mycoflora was also studied in each treatment at various stages of compost preparation. Heavy population of inoculated fungus/ fungi was isolated at spawning in respective treatments.

**Table 7.** Yield performance of *A. bisporus* in long method composts

| Pile No. | Treatments | Ammonic conc. (ppm) | Days taken for spawn run | Condition of spawn run | Total compost produced | Days taken for Ist harvest | Yield kg/q compost | Competitor fungi |
|---|---|---|---|---|---|---|---|---|
| 1 | *H.insolens* (I-1) | 8.0 | 13.0 | ++ | 550 | 35.0 | 11.92 | - |
| 2 | *H.insolens* (I-3) | 9.0 | 13.0 | ++ | 510 | 35.0 | 12.96 | - |
| 3 | *S.thermophilum*(X-21) | 7.0 | 12.0 | +++ | 580 | 35.0 | 16.16 | - |
| 4 | Consortium(I-1+I-3+X-21) | 8.0 | 12.0 | ++ | 630 | 34.0 | 12.34 | - |
| 5 | Control(uninoculated) | 14.0 | 15.0 | + | 540 | 36.0 | 10.44 | ** |
| $CD_{0.05}$ | | | | | | | 5.43 | |

**Occurrence of *Coprinus* sp. and *Papulaspora bysinna* in control treatment during spawn run
+good, ++ very good, +++ excellent

Composting period was reduced to 20 days compared to present 28 days normally taken when LMC is employed. Though control set also supported fairly good spawn run but many a bags showed heavy incidence of *Coprinus* sp. an indicator mould suggestive of free ammonia in such composts resulting in the lowest yield in the experiment. Incidence of *P. bysinna* was also observed in few bags in this treatment. No *Coprinus* growth was observed in thermophiles inoculated piles suggestive of the fact that no free ammonia was available in such composts indicating that it was fully converted into microbial proteins by inoculated fungi where their population was maximum in such piles compared to control pile. Above finding suggest that presence of specific fungi in large number is responsible for final selectivity of the compost. The potency of X-21 and I-1 strains was therefore, well established in the field conditions as well evidenced with increased yield obtained using these two fungi. Role of *S. thermophilum* in compost production and nutrition of button mushroom has earlier been outlined by several workers under short/indoor methods of composting [10-11, 40, 42]. Straatsma [10] hypothesized that *S. thermophilum* increases the growth of *A. bisporus* by unknown mechanism. In the present investigation we also found early spawn run and highest yield in this treatment. This finding can go a long way to the seasonal growers who prepare compost by such method as they will be saving time, labour, energy and money by reducing the composting period. Growers will also get more selective compost with higher yields as compared to 28 days of composting.

## Short method of composting

Here efforts were made to prepare compost by short method by artificially inoculating the compounding mixture with potent strains (X-21 & I-1) on day zero. Main aim of the study was to shorten the duration of composting and also to increase in yield in such compost. Here we kept the compost in the tunnel for five days only against the seven days normally kept as a standard protocol. We could complete the phase –II operation in five days particularly in inoculated composts as evidenced by low ammonia level (< 5.00 ppm) detected in such composts at spawning. On the contrary control treatment showed higher ammonia (9.00 ppm). This treatment though showed good spawn run but incidence of *Coprinus* sp. was observed in many bags. This treatment therefore offered lowest yield. Quick disappearance of ammonia from inoculated piles was attributed to the presence large population of S -7 and I-1 strains in respective treatments at spawning. Above finding suggest that these two strains can convert ammonia into microbial proteins at a rapid pace and can make the compost selective for the growth of *A. bisporus* in shortest possible time causing little environment pollution [17, 35]. Though phase–II period in inoculated treatments was reduced to five days but these treatments could not increased the yield significantly (Table 8).

Degradation of compost was also measured. More degradation of cellulose and hemicelluloses was observed in inoculated piles which was almost same in I-1 and X-21 treatments and also in consortium of these fungi. Least degradation was observed in control treatment. Higher degradation of these organic matter in the treated sets resulted in high quality compost

**Table 8.** Yield performance of *A. bisporus* on short method composts

| Pile No. | Treatments | Colour of compost | Ammonia (ppm) at spawning | Days taken for spawan run | Condition of spawn run | Conversion ratio | Total compost produced | Total yield (kg/q compost) |
|---|---|---|---|---|---|---|---|---|
| 1 | *H.insolens* (I-1) | Dark brown | 3.00 | 15 | +++ | 2.87 | 420 | 15.00 |
| 2 | *S.thermophilum* (X-21) | Dark brown | 5.00 | 14 | ++++ | 2.99 | 430 | 15.50 |
| 3 | Consortium (I-1+X-21) | Dark brown | 5.00 | 15 | +++ | 2.03 | 500 | 13.89 |
| 4 | Control(uninoculated) | Brown | 9.00 | 16 | ++ | 2.02 | 470 | 12.90 |
| $CD_{0.05}$ | | | | | | | | 3.46 |

++ good, +++ very good, ++++ excellent

with high yield as compared to control**.** Although increase in lignin content was exhibited in all inoculated and control treatments during spawning.

## Indoor composting

Laws governing pollution are becoming stringent day by day in the country and many of the units producing compost by long and short methods are facing environmental issues, as such composting procedures pollute the atmosphere and create nuisances to the residents residing nearby. In a pursuit to lessen the environment pollution, Vijay [42] standardized a technology for express composting using a combination of INRA and Anglo Dutch methods. In this technology author completed phase-I in specially built phase-I tunnels (bunkers) where compost was fermented under aerobic conditions by providing artificial aeration through blowers. Temperature was manipulated inside the bunkers in such a fashion which was congenial for the growth of thermophilic fungi. Natural thermophilic population present in the compost was utilized in completing the process in as less in 12-14 days time. This technology is being adopted by several units across the country successfully, however, it could not mitigate environment pollution completely. We have successfully reported the use of X-21 and I-1 strains in LMC and SMC in shortening the duration and also increasing the yield. Therefore, it was thought prudent to utilize these two fungal strains in this technology also for its further refinement with an ultimate objective to lessen the environment pollution, period of composting, raw materials losses, ease in material handling and higher productivity. Series of experiments were conducted to refine this technology results obtained in brief are presented below.

## Anglo Dutch method

The first experiment conducted in this series was on the improvement in the already standardized Anglo Dutch technique (cold process). Here we inoculated the compost with X-21 and I-1 strains and found that in this technique these inoculants were not much effective and marginally higher yield in treated compost was observed. Population dynamics of inoculated piles revealed the progressive increase of inoculated fungi from 3rd day to spawning (maximum population). Low count of other fungi was noticed (Table 9)

**Table 9.** Population dynamics of thermophilic fungi in inoculated compost at various intervals

| | Population dynamics of thermophillic fungi on | | |
|---|---|---|---|
| **Organisms** | **+3 day** | **+ 6 day** | **At spawning** |
| | **cfu/g of compost** | | |
| *H. insolens* (I-1) | 3.6 | 5.6 | 8.0 |
| *S. thermophilum* (X-21) | 8.3 | 7.30 | 11.6 |
| *S. thermophilum* | 3.3 | 4.6 | 6.0 |
| *A. fumigates* | 4.3 | 3.2 | 2.6 |
| *T. lanuginosus* | 0.0 | 0.66 | 0.0 |
| *H. fuscoatra* | 0.33 | 0./0 | 0.0 |

In control set four fungi were isolated and similar trend as reported above was observed here too, however, their population was low compared to inoculated treatments (Table 10). Further strains of *S. thermophilum* and *H. insolens* isolated were different than I-1 and X-21.

**Table 10.** Population dynamics of thermophilic fungi in uninoculated (control) compost

| Organisms | Population dynamics of thermophillic fungi on | | |
|---|---|---|---|
| | +3 day | + 6 day | At spawning |
| | cfu/g of compost | | |
| *H. insolens* | 2.6 | 4.6 | 8.00 |
| *S. thermophilum* | 6.5 | 9.33 | 12.3 |
| *A. fumigates* | 7.3 | 5.6 | 0.0 |
| *H. fuscoatra* | 0.33 | 0.33 | 0.0 |

Both the composts showed almost similar trend with respect to cfu and different thermophilic fungi. This fact can be attributed to the conditions that here compost was fermented at 45-52 $^{o}$C in the phase–I tunnel which was most congenial for the growth of thermophilic flora. Given these conditions test fungi present in the treated lots and ingredients habouring thermophilic fungi in control set grew rapidly in equal proportions and almost similar trend was observed in both sets. Marginal increase in yield in treated set can be attributed to the presence of X-21 & I-1 in inoculated compost which are known to increase the yield as evidenced in experiments conducted on LMC and SMC (not significant increase).

## INRA method

Second experiment conducted was on improvement in the INRA technology [43]. Contrary to Anglo Dutch method, compost is fermented at 75-80 $^{o}$C in bunkers in this technology. Since most of the thermophilic fungi are eliminated at higher temperature artificial inoculation of compost with thermophilic fungi becomes imperative. In the present investigation we inoculated the compost with the potent strains (X-21 & I-1) at beginning of phase-II. Population dynamics indicated almost complete dominance of inoculated fungi in treated set (Table 11) .

**Table 11.** Population dynamics of thermophilic fungi in inoculated compost

| Organisms | cfu/g of compost | | |
|---|---|---|---|
| | +3 day | + 6 day | At spawning |
| | **Inoculated** | | |
| *H. insolens*(I-1) | 0.0 | 3.3 | 6.0 |
| *S. thermophilum* (X-21) | 0.0 | 4.6 | 15.3 |
| *S. thermophilum* | 0.66 | 1.3 | 0.33 |
| *A. fumigates* | 3.3 | 2.3 | 0.66 |
| *T. lanuginosus* | 1.0 | 0.66 | 0.0 |
| | **Control (ininoculated)** | | |
| *S. thermophilum* | 1.33 | 0.66 | 6.66 |
| *A. fumigatus* | 2.3 | 2.6 | 1.3 |

Treated compost gave very high productivity compared to control (Table 12). Low yield in control set was the function of absence of test fungi and very low population of *S. thermophilum* in particular which is responsible for conditioning of compost and nutrition of button mushroom [35].

**Table 12.** Yield obtained with indoor compost (hot process)

| Treatments | Moisture % | pH | N% at spawning | Days taken for spawn run | Condition of spawn run | Conversion ratio | Total yield (kg) | Yield kg/q. compost |
|---|---|---|---|---|---|---|---|---|
| Consortium | 65.3 | 7.45 | 2.01 | 12 | ++++ | 2.90 | 237.8 | 16.40 |
| Control | 64.2 | 7.50 | 2.14 | 15 | ++ | 3.10 | 190.65 | 12.30 |

## Total Indoor Compost Technology (TICT)

In both the experiments discussed above, composting process was completed in 12-13 days time and phase–I was completed in bunkers where slight environmental pollution was noticed. Main aim of the present studies was to prepare the compost in most environment friendly manner without any pollution so that a perfect composting technology is passed on to the commercial units which are presently reeling under the environment related issues and many are at the verge of closing. In the third experiment we completely bypassed the phase-I in bunkers and entire composting process was completed in phase-II tunnel itself except for the slight period of wetting and flipping in composting yard. Strains X-21 & I-1 were used as the inoculants (3 % on fresh weight of the straw). Entire composting period lasted for 10 days only.

## Methodology followed

- 0 day: Wheat straw was properly wetted (75% moisture). Later all the ingredients were thoroughly mixed into it along with half of inoculum (1.5% on dry wt. basis). Heap was made thereafter
- +1 day: break opened the heap, water added if required, mixed the ingredients properly, again made a heap ( temp around 58-60 °C)
- +2 day: Again turned the compost ingredients and made a heap ( temp 62-65 °C)
- +3 day: -do- (temp around 70 °C)
- +4 day: Transferred the entire mass to the phase-II tunnel after adding rest of inoculum (1.5%) and equalized at 45-48°C
- +5 day: Maintained the compost temp at 45-52 °C ( PPC)
- +6 day: - do –
- +7day: Kill at 59 °C for 6 hours through self-heat generation of heat. later (POPC) at 48-52 °C
- +8 day: Post pasteurization conditioning (POPC) continue
- +9 day: POPC
- +10 day: POPC and cooled down at night
- +11day: Spawn

Minimum of 10 % fresh air during entire operation, even during kill was maintained.

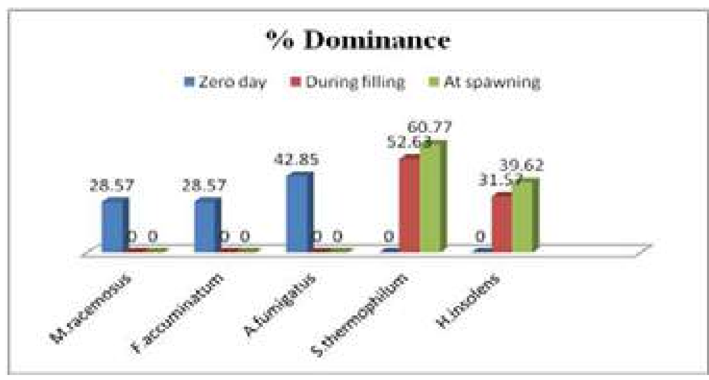


**Figure 4.** % dominance of thermophilic fungi

During filling most dominating fungus was *S. thermophilum* having dominance of 52.63 followed by *H.insolens* (31.57%). Not a single colony of mesophilic fungi isolated at zero day was noticed at filling. During spawning or after phase-II operation similar results were observed with high dominance of *S. thermophilum* followed by *H. insolens* (Fig. 4).

Satisfactory yields were obtained with such technology especially in the treated set (Table 13). We termed this technique as Total Indoor Compost Technology (TICT). Here degradation of ingredients by thermophiles leads to final selectivity of compost and making compost more nutritive for *A. bisporus*. Further in this technology degradation of organic compounds was more as compared to bunker + tunnel technology and less emission of ammonia was observed causing minimal pollution and more conversion of raw material to valuable compost. Duration of composting reduced from 14-16 days in bunker technology to 10-11 days with more output of compost and satisfactorily yields. Steam requirement during phase-II was nil. This technique has since been validated several times at DMR and also at selected All India Co ordinate Research Projects on Mushrooms and also at farmer's field giving satisfactory yields.

**Table 13.** Yield data in TICT compost

| Trial Name | Treatment | Compost colour | Days taken for spawn | Condition of spawn run | Days taken for Ist harvest | Convertion ratio | Total compost produced (kg) | Total yield (kg) | Yield kg/ 100 kg compost |
|---|---|---|---|---|---|---|---|---|---|
| TICT | Consortium (I-1+X-21) | Dark brown | 13 | ++++ | 31 | 2.96 | 1480 | 226.44 | 15.30 |
| | Control | brown | 15 | +++ | 33 | 3.10 | 1550 | 204.60 | 13.20 |

## Modified Indoor Composting Technology

The fourth and last experiment conducted in the series was on further improvement in the TICT. We termed this technique as Modified Total Indoor Composting Technology (MICT). Compost prepared above using various techniques (three experiments) was successful and inoculation of compost with X-21 and I-1 strains always gave superior product with higher yield. In the last experiment we have developed a total indoor composting technique (TICT), however, since in this technique substrate was fermented out doors for couple of days, slight pollution was observed. We wanted to avoid this even so that a technology is perfected with zero pollution. We further wanted to cut down the time of composting from 11-12 days to 8 days so that commercial farms may take more crop of button mushroom.

Further in the above technologies substrate was inoculated with test fungi as such (unsterilized) and hence, recovery of the inoculated fungi from the respective inoculated lots was only to the tune of 50-60 per cent and besides these other thermophilic fungi also came into play and they along with I-1 and X-21 strains also participated in the composting process. Role of test fungi (I-1 and X-21) in compost production could not therefore be fully justified. To nullify such effect wetted substrate in the present investigation was first sterilized/ pasteurized by live steam for 6-8 hours at 75 °C in the pasteurization tunnel so that colonies of other thermophilic fungi being harboured by substrate are almost killed. Then inoculations were done in such substrate justifying the full potential of X-21 and I-1 strains [29]. *In vitro* experiments reported in the present review also gave an indication that if sterilized substrate is inoculated with test fungi then they degrade it at faster pace due to softening of the straw and liberation of cellulosic components in the medium. Such treatment also gave quick spawn run and higher yields.

In this technique required conditioning and pasteurization temperature was achieved through self generation of heat due to rapid multiplication of test fungi in the inoculated piles as evidenced by their huge count in respective piles at spawning (Table 15) ( tunnels at DMR usually require steam).

**Table 14.** Population dynamics of thermophilic microorganisms

| Population Dynamics | Av. Colony count ( X $10^4$) | Dominant fungi |
|---|---|---|
| Mixing (Phase 0) | 4.0 | *A. fumigatus* |
| After sterilization (Phase I) | 2.6 | *S. thermophilum* (1.0), *A. fumigatus* (1.6) |
| During spawning ( after Phase II) T-1 *H. insolens*(I-1) | 12.33 | *H. insolens* |
| T-2 *S. thermophilum*(X-21) | 12.66 | *S. thermophilum* |
| T-3 Consortium(I-1+X-21) | 14.66 | *S. thermophilum* (10.0), *H. insolens*,(4.66) |
| T-4 Pasteurized compost | 13.33 | *S. thermophilum* ( 9.0), *H. insolens* (3.33) |
| T-5 Control(uninoculated) | 8.33 | *S. thermophilum* |

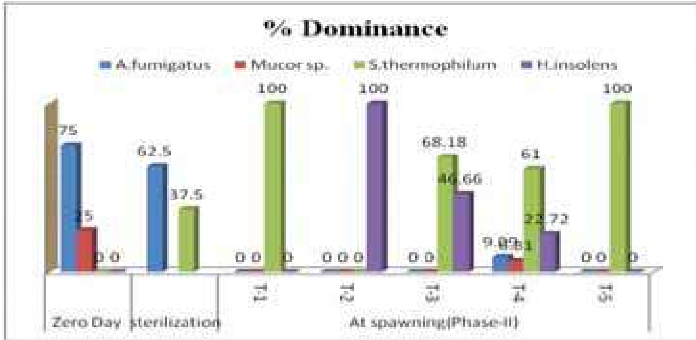


**Fig. 5.** % Dominance of thermophilic fungi

Population dynamics data showed that only inoculated fungus/fungi were isolated in the respective treatments which indicated that they only participated in the composting process (Table 14 and Fig.5). Thermophilic fungi particularly *S. thermophilum* and *H. insolens* are responsible of faster composting process [12]. Artificial inoculation of phase-I compost with *S. thermophilum* is reported to enhance the composting operation and further higher button mushroom yield has been reported by such treatment [44]

Similarily ability of *H. insolens* in faster composting process and early disappearance of ammonia from compost has been reported [45]. In the present investigation compost was free from ammonia inside the tunnel in as less in seven days time presumably with the combined effect of the thermophilic flora without being subjected to phase-I. Compost was ready in 6 days time as evidenced by sweet smell. Since the compost was prepared solely in phase-II tunnel under fully aerobic conditions, no environment pollution was observed in this technique. Only little of the ammonia generated which disbursed in the atmosphere making compost in most eco friendly manner which is the need of the hour.

Comparative results of each treatment were also analyzed by measuring various physio-chemical factors (Table 15). Highest reduction in C:N ratio, hemicellulose and cellulose were observed in consortium treatment which corroborated well with highest yield obtained in this treatment ( Table 16). Least reduction of cellulose, hemicellulose and C:N ratio was observed in control which resulted in lowest yield.

Excellent compost was obtained after termination of the composting phase. There was no smell of ammonia in any of the treatments. It was brown to dark brown in colour with full growth of inoculated fungus/ fungi. Spawn run was completed in 12 days time in all the treatments including control and it was rated as excellent ++++. No barren patch of compost was observed in any of the 652 bags spawned for the trial and it was milky white in colour. Very heavy first flush was obtained in treated compost (around 10 % conversion). Highest yield of 19.96 kg mushrooms / 100 kg compost was achieved in consortium treatment followed by 19.06 kg in *H. insolens* treatment. Control yielded 16.10 kg mushrooms mainly due to poor second and third flush compared to other treatments (Table 16.).

**Table 15.** Physico-chemical characteristics of compost prepared with thermophilic fungi

| Treatment | Moisture% | pH | C% | N% | C/N | Cell % | NDF% | ADF% | Hc.% | ADL% | Colour |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Before filling (Phase- 0) | 73 | 7.8 | 51.62 | 1.68 | 30.74 | 36.00 | 69.64 | 48.00 | 21.64 | 7.87 | Pale Yellow |
| After sterilization (Phase- I) | 65 | 7.8 | 47.71 | 1.64 | 29.28 | 28.00 | 67.00 | 50.00 | 17.00 | 8.50 | Brown |
| During spawning (Phase- II) T-1 *H. insolens* (I-1) | 60 | 7.3 | 37.12 | 1.72 | 21.56 | 22.66 | 50.33 | 47.33 | 2.78 | 20.15 | Dark brown |
| T-2 *S. thermophilum* (X-21) | 60 | 7.5 | 37.12 | 1.70 | 21.83 | 23.66 | 55.66 | 50.66 | 4.98 | 25.33 | Dark brown |
| T-3 Consortium (I-1+X-21) | 57 | 7.3 | 33.12 | 1.68 | 19.70 | 20.33 | 50.66 | 47.66 | 2.73 | 18.11 | Dark brown |
| T-4 Pasteurized compost | 60 | 7.3 | 38.86 | 1.90 | 20.44 | 23.33 | 55.66 | 51.66 | 6.66 | 26.66 | Dark brown |
| T5 Control (uninoculated) | 60 | 7.3 | 39.44 | 1.68 | 23.48 | 23.33 | 57.66 | 51.66 | 6.00 | 29.16 | Brown |

**Table 16.** Button mushroom yield in compost prepared with indoor compost method

| S.No. | Treatment | Days taken for spawn | Condition of spawn run | Days taken for Ist harvest | Convention ratio | Total yield kg/ q compost |
|---|---|---|---|---|---|---|
| 1 | *H. insolens* (I-1) | 12.0 | ++++ | 36.0 | 3.36 | 19.06 |
| 2 | *S. thermophilum* (X-21) | 12 .0 | ++++ | 36.0 | 2.84 | 18.60 |
| 3 | Consortium (I-1+X-21) | 12 .0 | ++++ | 36.0 | 3.01 | 19.96 |
| 4 | Pasteurized compost | 12 .0 | ++++ | 36.0 | 2.89 | 16.42 |
| 5 | Control (uninoculated) | 12 .0 | ++++ | 36.0 | 3.12 | 16.10 |
| CD0.05 | | | | | | 2.50 |

**++++ excellent**

New technique improved the consistency of compost quality, more compost per unit wt. of ingredients taken, higher yields with no or minimum environment pollution. Such technique will be a boon for those who are facing environment related issues and they will be able to produce productive compost in as less in eight days time with the help of thermophilic fungi short listed and reported in this communication. Cultures of these potent strains have been deposited at NBAIM, Mau (UP) for their preservation and also at DMR, Solan, India.

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# INVESTIGATION INTO THE MICROBIAL COMMUNITY CHANGES THAT OCCUR IN THE CASING LAYER DURING CROPPING OF THE WHITE BUTTON MUSHROOM, *AGARICUS BISPORUS*

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## ABSTRACT

Decrease in yield occurs after each subsequent flush, or break, of the commercially produced white button mushroom, *Agaricus bisporus*. The exact cause behind the reduced yield is unknown, though there are several theories. One theory is that chemical byproducts (e.g. salt crystals) form on the mycelium thereby reducing nutrient uptake and subsequent fruiting. A second theory is that the nutrition of the substrate is reduced throughout the cropping process thereby limiting yield potential and subsequently reducing the yield of each break. Another explanation for the reduced yield may be related to the diverse microbial communities present in either the casing layer or the compost substrate itself. It is believed that certain microbial communities are needed and are responsible for promoting primordial formation and maturity. This study was an investigation into the differences that occurred in the major bacterial groups present in the casing layer during cropping. The casing layer consisted of a sphagnum peat moss buffered with calcium carbonate that was added to the compost after a 17 day spawn run. Samples of the casing layer from an *A. bisporus* crop were collected on days 6, 13, 22 and 29 (days post-casing) during the production period. DNA was extracted from the replicate casing samples and bacterial DNA was amplified using PCR and then isolated from each sample for metagenomic analysis. Statistically significant changes in populations of Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria were observed, with minor changes also seen in Chloroflexi, Gammatimonadetes, and Planctomycetes. A better understanding of the microbial communities in the compost and casing will hopefully allow us to increase the bioefficiency during production as well as possibly help us to better understand microbial community-pathogen relationships as they relate to disease development.

**Keywords:** casing, phylogenetics, microbial population, *Agaricus bisporus*

## INTRODUCTION

In 2013, the US produced approximately 882 million pounds of *Agaricus bisporus*, the white button mushroom, valued at $1.05 billion USD (USDA) [1]. Profitability continues to be a challenge to US mushroom growers because of increased cost of production due to increase in raw material, energy and labour expenses. Most US producers harvest mushrooms for three breaks, (flushes) with the highest yield occurring during the first break and declining yields during subsequent beaks. In an attempt to explain this phenomenon three major hypotheses have emerged. Beyer and Mutherbaugh [2], proposed that depletion of compost nutrients over a cropping period may limit future yields. Beyer also supports Schisler's hypothesis that an accumulation of toxic metabolites may inhibit mushroom growth [2]. Zarenejad *et al*. [3], assert that *A. bisporus* yields are affected by changes in the casing microflora that may change during cropping and consequently inhibit fruiting.

The aforementioned hypotheses are not mutually exclusive; therefore, it is likely that the yield decrease as a product of many complex interactions. Although previous studies, including those conducted by Hayes *et al.* [4], have shown that the bacteria in the casing layer exhibit growth-stimulating activity, the majority of research into *A. bisporus* cultivation has focused on compost preparation. For example, Gerrits produced a detailed list of thermophilic fungi and actinomycetes that he suggests are important organisms, along with temperature ranges for optimal activity of each species [5]. Schisler also described specific thermophiles required for normal compost preparation, which includes ammonifying bacteria from the genera *Proteus*, *Micrococcus* and *Aerobacter* [6].

To address the dearth of research, this study will serve as an initial survey of the major bacterial phyla present in the casing layer. Significant changes in the sizes of their populations over a typical harvesting period will also be assessed. Future research investigating these changes may lead to solutions for more efficient mushroom cultivation, higher yields, decreased disease pressures and subsequent increased profitability for growers of *A. bisporus*.

Before the cause of reduced yields can be investigated, it is important to understand the cropping process and the role of casing microbiota in commercial *A. bisporus* production. First, compost consisting largely of horse manure is prepared to serve as the growth medium [7]. Meticulous preparation of composting material is vital, as it provides nutrients for growth of both mushrooms and associated microflora. These nutrients include: sources of nitrogen, commonly supplied through chicken manure, brewer's grains, and/or cottonseed meal [7] complex carbohydrates, such as cellulose and lignin; water; vitamins; and minerals [5]. Developing the ideal substrate for *A. bisporus* is complicated by the obligate co-existence with microbes. Besides the fact that they also require nutrients for growth and metabolic processes, microbiota with that may either stimulate or inhibit fruiting may require additional factors for which is still unspecified [4]. While inherently difficult to achieve, the ideal compost provides a stimulatory environment for development of both the mushrooms and symbiotic microflora along with inhibiting growth of pathogens or competitors [7]. By investigating changes in casing bacterial population and understanding the roles of major groups in the growth of *A. bisporus*, the task of developing an ideal substrate will be more easily attainable.

## MATERIALS AND METHODS

### Cropping

Compost was prepared at the Mushroom Research Center (MRC) at The Pennsylvania State University following standard methods, with Phase I taking place in forced aerated bunkers. The standard composting formula consisted of straw-bedded horse manure, switch grass straw, poultry manure, dried distillers grain and gypsum. The aerated Phase I composting period lasted 6 days and was turned on day 3 and filled on day 6 into wooden trays and moved into a Phase II room at the MRC. Temperatures were maintained according to MRC standards for 8 days and pasteurization and conditioning were performed. After 8 days in Phase II, the substrate was spawned with a commercial off-white hybrid strain at a rate of 2.5% and supplemented with a commercial supplement at the manufacturer's recommended rate. 22.7 kg (50 lbs.) of wet substrate was layered into 0.26 $m^2$ (2.75 $ft^2$) plastic growing tubs and the substrate in each tub was pressed tightly with a hydraulic press. Fifty four tubs were placed on metal racks and moved to an environmentally controlled production room at the MRC where temperature and humidity were maintained for the duration of the 17 day spawn run period.

Standard MRC casing consisted of sphagnum peat moss mixed with agricultural limestone added @ 22.7 kg limestone/ 0.17 $m^3$ peat (50 lbs./ 6 $ft^3$) with a commercial casing inoculum (CI). The room was flushed with fresh air and temperature lowered 5 days after casing to initiate primordial development. First break harvest began 10 days after the fresh air flush and 16 days after casing. Mushrooms were harvested and yield data recorded for 3 growing cycles or breaks.

### Sample collection and preparation

Casing layer samples were collected from the *A. bisporus* crop (MRC 1303) at the MRC. Samples of the casing layer were obtained at four different periods over the cropping cycle: day 6, 13, 22, and 29. Day 6, or the sixth day after casing, is when primordial formation typically occurs. Day 13, 22, and 29 after spawning represent the onset of the first, second, and third flush, respectively. On each sampling date, a #10 plug core sampler was used to obtain 3 plugs of the casing layer from 3 separate tubs, totaling 9 samples. The plugs were then combined into a pooled sample and stored at -20°F. This process was repeated twice more to provide a total of 3 pooled casing samples for each date. Frozen samples were then ground in liquid nitrogen prior to extracting DNA. DNA was extracted using the MoBio PowerSoil® DNA Isolation Kit. DNA extractions were carried out three times from each pooled sample. Extracted DNA was subsequently amplified for pyrosequencing using the 16S rRNA universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG–3') and 907R (5'-CCCCGTCAATTCMTTTGAGTTT-3').

**Metagenomic and statistical analysis**

Metagenomic analyses of the extracted DNA were conducted at the Penn State Bioinformatics Consulting Center. The three pooled samples representative of each collection date were sequenced individually, and the results were combined into a single set. Using MEGAN, statistical comparisons between metagenomic data sets were completed to determine significant changes in bacterial populations during a cropping period. Default parameters were used to assess the lowest common ancestry (abbrev. LCA) among bacteria, with the exception of the minimum probability value. This was set to 0.95 to exclude matches with less than 95% certainty of being correctly identified. Statistical comparisons were carried out as described by Suparna Mitra *et al*. [8] using a critical *p*-value of 0.01. The corrected Holm-Bonferroni method was utilized to compare the following sets of data: Day 6 vs. 13; Day 13 vs. 22; and Day 22 vs. 29.

## RESULTS

Fig. 1 provides a comprehensive list of the bacterial phyla identified in the casing samples taken on the four sampling dates. Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were found to be the four predominant phyla of casing microbiota. As shown in Table 1, the populations of both Actinobacteria and Firmicutes decreased significantly after Day 6 but increased on Day 22 and 29. Levels of Bacteroidetes present in the casing layer increased over the cropping period until Day 22, with a subsequent decrease on Day 29. Proteobacteria, the largest bacterial population identified in the samples, showed an increased population on Day 13, which was followed by decrease on Day 22 and 29. Populations of the Phylum Gemmatimonadetes and Planctomycetes increased significantly with each sampling date. The population of Chloroflexi increased significantly from day 6 to day 13 with a significant drop in numbers on day 22 and 29. These populations are not included in Table 1 due to their relatively minor contributions to the overall population size (each making up less than one percent of the total reads); however, these three groups exhibited significant changes in their populations. Interpretation of the ecological importance that the major 4 phyla identified in the casing layer has is a difficult undertaking. To have better understading on what role these phyla have in the casing layer, the significant orders identified with each group are listed in Table 2, according to comparative population size.

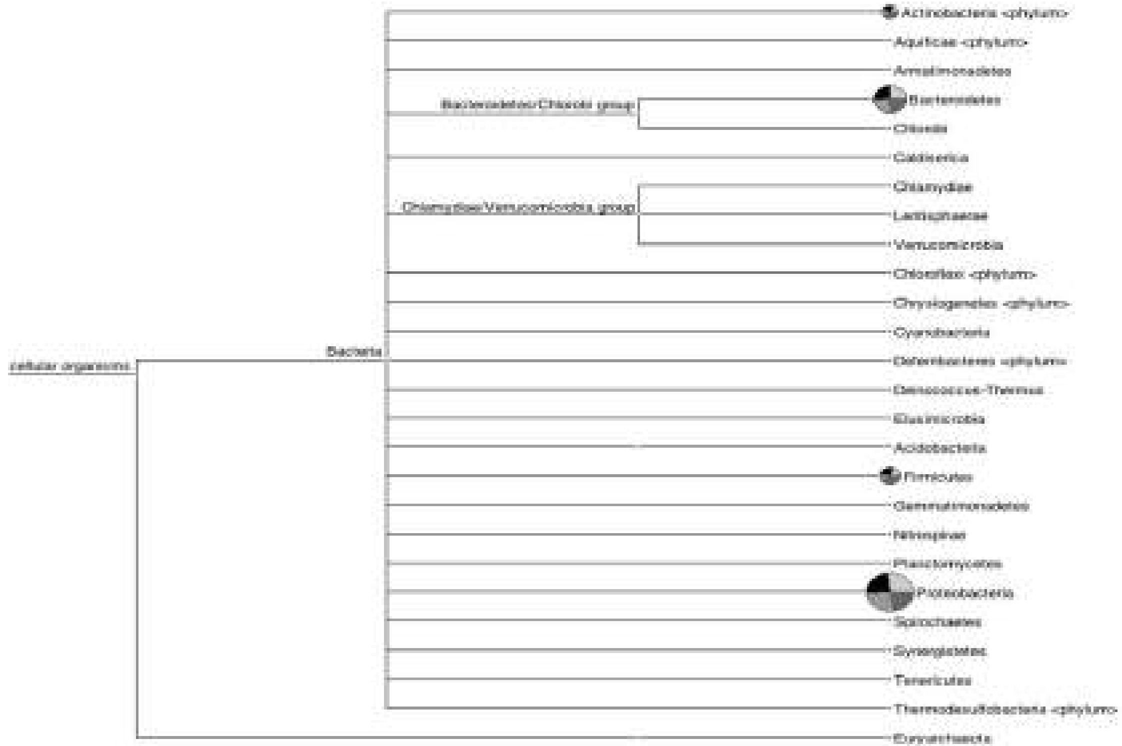


**Figure 1.** Taxonomy profile of casing microbiota. Comprehensive list of bacterial phyla found in casing samples from Day 6, 13, 22, and 29. Size of the circle preceding a group is representative of its relative population size. The four major groups identified were as follows: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria.

**Table 1.** Highlighted differences between different casing samples. "Difference" column represents the statistically significant increase (+) or decrease (-) in number of reads identified in each group

| Phylum | Day 6 to Day 13 | | Day 13 to 22 | | Day 22 to Day 29 | |
|---|---|---|---|---|---|---|
| | *Difference* | *P-value* | *Difference* | *p-value* | *Difference* | *p-value* |
| Actinobacteria | - | 0.01 | + | 0.01 | + | $9.3 \times 10^{-6}$ |
| Bacteroidetes/Chlorobi group | + | 0.01 | + | 0.005 | - | 0.001 |
| Firmucutes | - | 0.01 | + | 0.01 | + | 0.01 |
| Proteobacteria | + | 0.01 | - | 0.01 | - | $4.6 \times 10^{-20}$ |

**Table 2.** Significant orders within major phyla. Orders that form the majority of each phylum within the casing samples

| Phylum | Significant Orders |
|---|---|
| Actinobacteria | Actinomycetales; Coriobacteriales |
| Bacteroidetes/Chlorobi group | Flavobacteriales; Sphingobacteriales |
| Firmicites | Bacillales; Clostridiales |
| Proteobacteria | Rhizobiales, Caulobacteriales, Sphingomonadeles; Burkholderiales; Xanthomonadales, Pseudomonadales |

## DISCUSSION

In this initial survey of microbiota found in a sphagnum peat moss-based casing layer, the major groups were found to be Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Actinobacteria, a phylum that includes common soil microorganisms and nitrogen-fixing bacteria, represented up to 10 per cent of the casing bacterial community. Bacteria belonging to the phylum Bacteroidetes made up approximately 25% of the community profile in the casing layer. This group includes the orders Flavobacteriales and Sphingobacteriales. Members of Flavobacteriales are able to carry out *de novo* biosynthesis of nicotinamide adeosine diphosphate (NAD), which is an essential cofactor in many metabolic pathways. These bacteria also have roles in lipid degradation which may affect the nutrition of the substrate for *A. bisporus* (NCBI). Order Bacillales, belonging to phylum Firmicutes, contains species that produce vital coenzymes. Specifically, members of this order can synthesize folate, an important precursor required for amino acid, DNA or RNA biosynthesis (NCBI).

Bacteria belonging to the phylum Proteobacteria were predominant population present in the casing, making up between 45 and 55 per cent of the bacteria profile on the different sampling dates. This phylum contains multiple species, including one that has been studied for its role in mushroom production, specifically *Pseudomonas putida*, which has been proposed to play a role in primordia maturation [9]. Previous studies have indicated that *Pseudomonas* species play a role in metabolizing volatile organic compounds, produced by *A. bisporus* that are inhibitory to primordia maturation. Hayes and Nair [10], showed that *Pseudomonas* species increased from 34% of the population at casing up to 95% of the casing population at the end of the cropping cycle. The pseudomonas are in the Gamma proteobacteria class (phylum Proteobacteria) which, in this study, demonstrated a decrease in the population of the bacterial communities from day 6 through day 29, with the greatest decline occurring between day 22 and day 29. However, the Gamma proteobacteria still made up approx. 4-5 percent of the reads from each sampling period.

The majority of the previous studies investigating microbial populations within the substrate and in the casing have often been carried out using traditional microbiological techniques (i.e. plating). This allows us to look at nutritional requirements and changes of the bacterial communities that are able to grow in culture. However, it is estimated that as few as 2% of bacterial populations in environmental samples are able to be cultured on media [11]. Previous studies are very beneficial and give us a baseline on what role certain microbial communities play in mushroom nutrition and disease development, however, there is currently an enormous void in our true understanding as to what roles these microbes may actually play

in mushroom production. To expand on the meaningfulness of the results of this study, more detailed analyses of the casing microbiota should be done to elucidate bacterial compositions down to the genus level to determine what key role they may play in mushroom nutrition and development. This could eventually lead to targeted solutions for increasing yields. This study was intended to conduct a general survey of the casing microbiota found in a sphagnum peat-based casing material, and the results were presented with an assumption that the largest bacterial populations have the greatest influence on *A. bisporus*. It is quite possible that other bacterial communities identified in this study, especially those that also exhibited significant changes over the cropping period, play important roles in the growth and development of *A. bisporus* despite of their relatively small makeup of the total bacterial community. Furthermore, an investigation into the fungi present in the casing layer should also be made to determine what role these organisms play in *A. bisporus* production and disease development. Composition of the microflora is likely to vary with modification of compost and casing materials. Much more research is needed to better understand how these variables affect the potentially beneficial microbial communities present in the mushroom production system.

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# PERFORMANCE OF STRAW MUSHROOM (*VOLVARIELLA VOLVACEA*) RAISED AS AN INTERCROP IN COCONUT PLANTATIONS OF COASTAL ODISHA

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## ABSTRACT

Paddy straw mushroom (*Volvariella volvacea*) is an edible mushroom of the tropics and sub-tropics. In India, Odisha is the leading state in terms of straw mushroom production (8129 tonnes/annum). It is cultivated outdoor as an intercrop in the coconut plantations in the coastal belt from the month of February to November. However, the yields are unstable and low (10 %). In a study to ascertain the seasonal productivity of straw mushroom under natural conditions, beds were raised at monthly intervals round the year in the coconut plantation of the Central Research Station of the University using appropriate protocol. Analysis of data indicated that the crop raised in the month of July gave significantly high yield of 1771 g/bed with a biological efficiency of 25.30 %. Appreciable higher yield levels obtained during the period from June to September (rainy season) were in the range of 1263-1771 g/bed in comparison to the summer crop (724-1097 g/bed). However, the yields realized from the months of December and January were substantially low (165.33 and 137 g/bed, respectively) with the biological efficiency of 2.36 and 1.95 %, respectively. Hence, it was concluded that the crop grown during the months of December and January are less productive and non-remunerative.

**Keywords:** *Volvariella volvacea*, coconut plantation, intercrop, productivity

Paddy straw mushroom (*Volvariella volvacea*) ranks sixth among the cultivated mushrooms of the world contributing to 3 % of total production [1]. It is a fast growing mushroom and under favourable growing conditions, the total crop cycle is completed within three weeks time. This mushroom can use wide range of cellulosic materials with a C:N ratio of 40-60. Presently, this mushroom is more popular in the coastal states like Odisha, Andhra Pradesh, Tamil Nadu, Kerala and West Bengal. In Odisha, paddy straw mushroom is largely cultivated as an intercrop in the coconut plantations of the coastal agro-ecological region of the state from the month of February to November. The hot and humid coastal climate experienced during rainy and summer months is favourable for raising paddy straw mushroom outdoor. However, conventional method of farming in tree shade leads to unstable and low yields (10 %) in the predominantly mushroom growing coastal belt of Odisha [2]. In this context, the present study was undertaken to ascertain the seasonal productivity of straw mushroom under natural climatic conditions and to correlate the yields with the prevalent weather conditions.

## MATERIALS AND METHODS

The experiment was conducted in Randomized Block Design with 12 treatments each with six replications in the coconut plantation of the Central Research Station, Orissa University of Agriculture and Technology, Bhubaneswar, Odisha. Straw mushroom beds, each comprising of 7 kg dry paddy straw were raised after processing of good quality straw as per standard procedure. Bundles (1.5′ length) were soaked in clean and cold water supplemented with one per cent calcium carbonate for six hours. Excess water was drained-off and beds of 1.5′ x 1.5′ x 1.5′ (length x breadth x height) were prepared with 3 % each of spawn as well as wheat bran in three layers employing conventional method of cultivation. Beds were covered with transparent polythene sheets and appropriate aftercare was taken. After completion of the mycelial growth, beds were uncovered and adequate light and ventilation were ascertained in the growing areas to facilitate induction of fruit bodies. Appropriate substrate moisture was maintained to prevent desiccation of young buttons. The fruits of the first flush were harvested followed by providing the polythene cover once again. Then second flush was harvested. Observations were recorded on time taken (days) for emergence of pinheads, time taken for first harvest (days post-spawning), number of fruit bodies, average fruit body weight and total yield of mushrooms for three week cropping period

(kg/100 kg dry substrate).

The multiple regression analysis was performed to find out the impact of mean day temperature and relative humidity on mushroom productivity. The combined effect of mean day temperature and relative humidity on biological efficiency was represented by the prediction equation, $y = -50.587 + 0.748\ X_1 + 0.581\ X_2$ where

$y$ = Biological efficiency

$X_1$ = Mean day temperature

$X_2$ = Mean relative humidity

## RESULTS AND DISCUSSION

Data recorded on time taken (days) to pin head emergence, first harvest, number of sporophores, mean weight of fruit bodies (g) and total weight of fruit bodies (g) are presented in Table 1.

**Table 1.** Influence of time of spawning on yield of *V. volvacea*

| Sl.No. | Month and year | Days to pin head emergence | Days to first harvest | Number of sporophores/ bed | Mean wt. of sporophores (g) | Weight of sporophores/ bed (g) | Biological efficiency (%) |
|---|---|---|---|---|---|---|---|
| 1 | January, 2011 | 16.83 | 21.50 | 9.83 | 13.66 | 136.66 | 1.95 |
| 2 | February, 2011 | 14.00 | 19.50 | 41.33 | 17.16 | 724.00 | 10.34 |
| 3 | March, 2011 | 8.50 | 14.66 | 60.66 | 18.16 | 1097.50 | 15.67 |
| 4 | April, 2011 | 7.83 | 14.50 | 55.16 | 17.16 | 951.66 | 13.59 |
| 5 | May, 2011 | 8.50 | 13.66 | 53.16 | 17.66 | 942.83 | 13.46 |
| 6 | June, 2011 | 7.50 | 13.50 | 63.66 | 20.00 | 1263.00 | 18.04 |
| 7 | July, 2011 | 8.33 | 14.66 | 84.16 | 21.50 | 1771.16 | 25.30 |
| 8 | August, 2011 | 8.83 | 14.83 | 73.16 | 18.83 | 1372.33 | 19.60 |
| 9 | September, 2011 | 9.00 | 14.50 | 65.00 | 20.83 | 1356.83 | 19.38 |
| 10 | October, 2011 | 8.66 | 14.00 | 62.66 | 16.33 | 1019.16 | 14.55 |
| 11 | November, 2011 | 11.66 | 16.50 | 50.33 | 15.16 | 766.66 | 10.95 |
| 12 | December, 2011 | 18.00 | 22.16 | 14.50 | 13.50 | 165.33 | 2.36 |
| | C.D. (0.05) | 0.61 | 0.75 | 3.09 | 1.50 | 8.49 | - |
| | C.V. (%) | 5.00 | 4.04 | 5.06 | 7.44 | 0.76 | |

Analysis of data indicated that there was significant variation among the treatments in respect of all the parameters recorded. Significantly high yield of 1771 g/bed was obtained from the crop raised in the month of July. Superiority of the treatment was observed in terms of number of sporophores (84.16) and mean weight of sporophore (21.5 g) with a modest crop duration of 14.66 days. Further, it was observed that crop raised during the period from June to September (rainy season) recorded comparatively higher yields (1263–1771 g/bed) as compared to the yield levels obtained during the winter months of October to January (137-1019 g/bed). The yields realized from the summer crops raised during February to May (724-1097.5 g/bed) was better than the yields realized from winter crops. The lowest yield of 137 g/bed was recorded from the crop raised in the month of January. It appeared that the winter months are not favourable for raising paddy straw mushroom in outdoor conditions.

The multiple regression analysis performed to find out the impact of independent variables on dependant variables (Table 2) indicated a positive correlation between mean day temperature and biological efficiency wherein the correlation coefficient 'r' recorded at 0.633 was found to be significant at 5% level. Similarly the mean relative humidity influenced the biological efficiency positively and the correlation coefficient 'r' calculated at 0.785 was significant both at 5% and 1% level. The overall contribution of mean day temperature and relative humidity on biological efficiency was calculated to the tune of 72.50% and the correlation coefficient 'R' calculated at 0.88 was found to be significant at both 5% and 1% level.

**Table 2.** Correlation coefficient studies in *V. volvacea*

| Sl.No. | Month and year | Mean day temperature (°C) | Mean relative humidity (%) | Biological efficiency (%) |
|---|---|---|---|---|
| 1 | January, 2011 | 21.39 | 63.10 | 1.95 |
| 2 | February, 2011 | 26.83 | 63.25 | 10.34 |
| 3 | March, 2011 | 30.07 | 71.12 | 15.67 |
| 4 | April, 2011 | 32.88 | 67.90 | 13.59 |
| 5 | May, 2011 | 31.90 | 76.00 | 13.46 |
| 6 | June, 2011 | 31.03 | 80.62 | 18.04 |
| 7 | July, 2011 | 26.20 | 84.60 | 25.30 |
| 8 | August, 2011 | 29.88 | 82.12 | 19.60 |
| 9 | September, 2011 | 29.25 | 82.87 | 19.38 |
| 10 | October, 2011 | 27.69 | 80.80 | 14.55 |
| 11 | November, 2011 | 26.36 | 75.12 | 10.95 |
| 12 | December, 2011 | 20.67 | 70.87 | 2.36 |

Biological efficiency verses temperature correlation coefficient 'r' = 0.633*
Biological efficiency verses relative humidity correlation coefficient 'r' = 0.785**
Prediction equation $y = -50.587 + 0.748 X_1 + 0.581 X_2$
Coefficient of determination $R^2 = 0.775$
$R^2$ (adjusted) = 0.725
Multiple regression coefficient 'R' = 0.880**

Data indicated that the mean day temperature recorded during March to October was in the range of 26.2 to 32.88°C which was favourable for the fungus to grow and reproduce. The impact of climatic conditions on mushroom yield showed a positive correlation in between yield and mean day temperature and relative humidity. However, significantly high yield (1771.16 g/bed) was obtained in the month of July when the mean day temperature was 26.2°C with relative humidity of 84.6%. Earlier experiments recorded higher yields from *Volvariella volvacea* (15.9%) raised during the month of July in the temperature and relative humidity range of 25.1-33.1°C and 73–92 %, respectively [3]. Further, the development of deformities in *Volvariella volvacea* can be avoided by the maintenance of a constant growing temperature in the range of 22-28°C [4]. The optimum temperature and humidity for fruitification of *Volvariella volvacea* was found to be 28-32°C and 80%, respectively under conventional method of cultivation [5]. Findings of the present investigation was more or less in agreement with the findings of several workers[6, 7]. Moderate temperature (25-38°C) and high humidity (> 85 %) requirement of *Volvariella* spp. were once again proved in the above investigation.

## CONCLUSION

Rainy season (June to September) was found to be the appropriate one for raising *V. volvacea* crop having recorded comparatively higher yields (1263-1771 g/bed) than the summer and winter seasons. The summer crop raised during the

months of February-May recorded a modest yield of 724-1097.5 g/bed as compared to the yield levels (137-1019 g/bed) obtained during the winter months of October-January. The highest and lowest yields of 1771 and 137 g/bed, respectively were obtained from the crop raised during the month of July and January, respectively. It appeared that the winter months are not favourable for raising paddy straw mushroom in outdoor conditions.

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# THE RESEARCH, DEVELOPMENT AND APPLICATION OF THE FERMENTATION ACCELERATOR, A NEW MATERIAL FOR EDIBLE MUSHROOM CULTIVATION ON UNSTERILIZED SUBSTRATE

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## ABSTRACT

The fermentation accelerator is a product based upon the differences in biological characteristics of various fungi. The purpose of the research was to develop a substance that can accelerate fermentation of the substrate, thus realize sterilization at a lower temperature and in a shorter time period, along with saving of energy, cost and labor. We found that by using it in the traditional sterilization system, the fermentation accelerator could help in production of ammonia and beneficial micro-organisms to accelerate the killing of harmful micro-organisms like weed moulds at a temperature between 70-80 $^{o}$C in 5-7 hours, realizing the purpose of sterilization, The production of 10,000 bags of *Lentinula edodes* (shiitake) or *Auricularia auricula* -judae (black wood ear) could save 720 kgs of coal and 2 labor forces and increase the yield by over 10%. Our conclusion was that edible mushrooms can also be cultivated on unsterilized substrate with the help of the fermentation accelerator, which bring about considerable economic and ecological benefits.This achievement passed the expert appraisal of scientific and technological administration sector and over 2 billion bags were spread and used in 26 provinces (regions, municipalities) across China.

**Keywords**: fermentation accelerator, edible mushrooms, cultivation on unsterilized substrate, new material

## INTRODUCTION

### Concepts of unsterilized substrate and mushroom cultivation

Edible mushroom cultivation on unsterilized substrate has many advantages and it has the potential to reform the cultivation procedures and the development of its industry. Cultivation on unsterilized substrate is a concept comparative with cultivation on sterilized substrate. The former refers to the cultivation procedures in which mushroom mycelia is inoculated on the unsterilized substrate and it can grow well and contaminants are inhibited or killed. The latter refers to the cultivation procedures of substitute substrate in which mushroom mycelia is inoculated on the steam sterilized substrate [1,2].

The ancestors of Chinese nationality began to cultivate edible mushrooms on unsterilized substrate a long time ago. *Auricularia auricula*- judae (black wood ear) and *Lentinula edodes* (shiitake) cultivation on wood logs were the cultivation techniques on unsterilized substrate. The primitive cultivation on unsterilized substrate depends on the primitive materials, hardwood logs, and an optimal natural environment, so it is limited by climatic conditions, comprising humidity and temperature. Furthermore, cultivation on wood logs has shortcomings as low biological efficiency, unstable mushroom quality and the destruction of forest resources, so it is banned at many places and is gradually replaced by the cultivation technique on substitute substrate (sterilized substrate) emerged in the late 1970s.

Although cultivation on substitute substrate has the advantages of high yield, easy control by man and stable fruiting, however, it also has following disadvantages: (i) wastage of raw materials: equivalent amount of firewood or alternative energy source is needed to sterilize 1 kg of sawdust substrate; (ii) heavy nutrient loss of the raw materials at a high temperature or pressure; (iii) varied quality of mushrooms from different flushes: loose and soft tissue, poor mouth feel; and (iv) a long sterilization period and requirement of manpower input. Therefore, cultivation technique on unsterilized substrate even after being dreamed by mushroom growers long ago, has not been successfully popularized and applied due to the severe contamination problem after inoculation. The few so-called successful cultivation examples had very high costs and much sequel. All the above set forth a research requirement for cultivation technique on unsterilized substrate.

## BASIC OBJECTIVES OF MUSHROOM CULTIVATION ON UNSTERILIZED SUBSTRATE

Compared with cultivation on sterilized substrate, cultivation on unsterilized substrate has the following advantages: simple procedures, save labor and cost, safety from the injury by disinfectants in the inoculation room, reduced the use of energy and maintenance of eco-balance. Hangzhou Huadan Agricultural Products Company Limited developed a fermentation accelerator after over 20 years of constant research. It can basically meet the above-mentioned requirements in edible mushroom cultivation.

## FUNCTIONS AND PRINCIPLES OF THE FERMENTATION ACCELERATOR

### The sterilization principle and function

The fermentation accelerator is a product resulted from the research on the differences in biological characteristics various fungi. On the substrate not thoroughly sterilized, the major contaminants in the growing bags are *Aspergillus* and bacteria. But on those thoroughly sterilized, they are mainly *Trichoderma* spp. and green moulds. The cultivated edible mushrooms are macro-fungi, the fermentation accelerator will primarily kill *Aspergillus* and bacteria, and will also kill contaminants such as *Trichoderma* spp., green moulds and *Neurospora* spp. (Agent Orange).

### The fermentation acceleration principle and function

At a temperature between 70 and 80 $^{o}$C, the fermentation accelerator will volatilize onto the substrate to speed up fermentation and realize the effectiveness of composting for over 1 week. The ammonia and beneficial microorganisms produced in a short time period accelerate the killing of contaminants like weed moulds, thus realize effective sterilization. The nutrient loss of substrate is quite low in this process [3].

### The catalytic fermentation principle and function

At a substrate temperature between 70 and 80 $^{o}$C, the fermentation accelerator can decompose the substrate into carbon dioxide, water, urease and special–effect catalytic enzyme, which will volatilize and penetrate in the substrate consisting mainly of sawdust, straw and cottonseed husk, etc. After mixing with these ingredients, the fermentation accelerator can be decomposed into $NH_4^+$ and $OH^-$ with the help of enzyme. $OH^-$ can break the ester bond and the mosaic structure between the molecules of lignin and cellulose in the substrate, thus dissolves them into semi-cellulose and cellulose. The $NH_4^+$ generated from the fermentation accelerator can be adsorbed on the surface and fracture of the substrate, and form a compound of $NH4^+$— N with the free nitrogen. During the mycelia, growth, this kind of compound is synthesized into the microorganism protein, which will be freely adsorbed on the surface of the substrate and is more optimal for the growth of mushroom mycelium. The research showed that the fermentation accelerator can increase the biological efficiency of the substrate by about 10% ~ 20% and the mushroom yield by more than 10%.

## EFFECTS OF THE FERMENTATION ACCELERATOR APPLIED TO MUSHROOM PRODUCTION

The fermentation accelerator is suitable for all wood-rotting mushrooms cultivated with the technique of normal pressure sterilization (steam production boiler, earth stove). It can also be used in compost making for *Agaricus bisporus* to make the compost temperature rise and ferment in a short time period. After a long period of experimentation and extension, it showed the following effects:

(1) A low sterilization temperature at 70 ~ 80 $^{o}$C and a short sterilization period in 7 or 5 hours. Fuel, time and labor were saved by about one half, but effect was improved by one fold.

(2) A wide-range application. It can be applied to almost all edible mushrooms cultivated at present (with very few exceptions). The percentage of mushrooms fruited and harvested in large-scale cultivation exceeded 95%.

(3) The amount of bran used in the regular formula could be reduced to 10% ~15%, thus decreased the cost. Twenty per cent of wastes could be used to increase the resource utilization.

(4) The robust growth and fast colonization of the mycelium could make the spawn run advanced by 7 days.

(5) Sterilization at a low temperature would cause less nutrient loss of the substrate and less deformed mushrooms as well as high quality and yield.

(6) The improved resistance to high temperatures would reduce the number of rotten bags, slow down the shrinking and loosening of growing bags, strengthen the after– effect and prolong the fruiting period.

(7) It could resist the infestation of *Neurospora* spp. (Agent Orange) and Fastigiated bacteria,which are massive and severely harmful [4].

(8) The reduced occurrence of pests and diseases saves the use of pesticides.

(9) The application to straw rotting mushroom cultivation could make the temperature of fermented substrate rise in a short time period, thus promoted the substrate fermentation.

## MAIN POINTS OF OPERATING THE FERMENTATION ACCELERATOR IN EDIBLE MUSHROOM CULTIVATION

The fermentation accelerator is simple to use. What you need to do is to add it and other ingredients successively from a small amount to a large amount based on the percentages in the formula, then mix them evenly, and finally stir the mixture with water.

(1) Add 0.35% ~ 0.5% of the fermentation accelerator to the total dry weight of the substrate. Take the Black wood ear as an example: 1 bag of the fermentation accelerator weighing 1,000 grams can be formulated in about 600 growing bags of $17\times33$ cm.

(2) Mix the raw materials: Add the fermentation accelerator and other ingredients in the sequence from a small amount to a large amount based on the proportions in the formula: the fermentation accelerator $\rightarrow$ gypsum powder $\rightarrow$ bran (corn meal, soybean meal etc.) $\rightarrow$ sawdust, straw (cottonseed husk, corncob etc, which can be pre-wetted). Mix these ingredients evenly in sequences, and then stir the mixture with water.

(3) Sterilization (i) 2 ~ 3 cm of gap should be maintained between rows when stacking the bags in the container so as to facilitate efficient steam circulation and reduce the dead spaces. (ii) during sterilization, the temperature displayed on the thermometer should be the actual substrate temperature inside the bag, so the thermometer should be inserted inside the bag at the bottom of the container on the stove. (iii) when the substrate temperature inside the bag reaches 80 $^{o}$C, maintain it for 5 hours, and then let it cool down naturally to a proper temperature and take out the bags from the container. During the cooling period, the container is allowed to be tightly closed and the artificial logs should be prevented from dust contamination. (iv) the temperature of 80 $^{o}$C inside the sterilization container is not constant, so the fire can be stopped when the temperature exceeds 80 $^{o}$C and should be continued when it is reduced to 80 $^{o}$C. The effective temperatures of the fermentation accelerator range between 50 and 150 $^{o}$C, so it will not lose its efficacy at a temperature of 100 $^{o}$C.

(4) Inoculation: It should be carried out in accordance with the regular sterile operation methods. Good quality spawn at a proper fungus age should be used for inoculation.

(5) Sealing the inoculation bags: The cotton plugs and rubber bands (plastic rings) are not proper for sealing the inoculation bags, because in the same container as the substrate, they will be contaminated instead of sterilized at a temperature of 80 $^{o}$C. For edible mushrooms growing on bags, it is suggested that the bags be sealed by tying or folding them. The sticky tape or white wax (20% of leather belt wax can be mixed) is suitable for sticky sealing the inoculation holes in the bags for Shiitake and *Tremella fuciformis* (Silver ear) cultivation. The surface mycelium on the inoculated squares shall be kept in a good, wet and aseptic microclimate to speed up their germination and colonization. If the inoculation holes are sealed with the spawn itself, more spawn shall be used and no gap is allowed on the surface, which shall be kept dry. The incubation room shall be frequently disinfected to kill contaminants in the air and shall have a good ventilation and aeration to maintain hygienic conditions.

(6) Spawn running: During the first 7-day after inoculation, the spawn used with the fermentation accelerator colonizes the substrate slower than that of the regular substrate, but after that period, it becomes faster. The mycelium can colonize the entire bag 7 days earlier so that the regular substrate. The temperature in the artificial log pile should be monitored all the time to prevent it from overheating.

(7) Compost: It shall be done according to the requirements of the regular production for different edible mushrooms. The quantity of the fermentation accelerator to be added is @0.5%.

(8) Storage: The fermentation accelerator is very easy to be moist and stuck together, so it should be stored in a closed, shady, cool and dry place.

(9) Bans: The fermentation accelerator cannot be used directly by adding water to it, as it becomes moist and compact, making it unsuitable for use.

## BENEFIT ANALYSIS ON THE FERMENTATION ACCELERATOR APPLICATION

Take Shiitake, a typical wood rotting mushroom as an instance, to produce 1,000 bags adding the fermentation accelerator, the direct input of 111 Yuan would be reduced. A cost comparison of the sterilized substrate and that of adding the fermentation accelerator in Shiitake cultivation are listed in Table 1.

**Table 1.** A comparison of cost calculation forthe recommended formulas of per ton substrate

| Ingredients of the substrate | Sterilized substrate (CK) (100 °C for 12~16 hours) | | | The fermentation accelerator (70 °C for 7 hours or 80°C for 5 hours) | | |
|---|---|---|---|---|---|---|
| | Quantity (kg) | Unit price (Yuan/kg) | Amount (Yuan) | Quantity (kg) | Unit price (Yuan/kg) | Amount (Yuan) |
| | | | **Costs of raw materials** | | | |
| Hardwood sawdust | 780 | 0.44 | 343 | 850 | 0.44 | 374 |
| Wheat bran | 200 | 1.1 | 220 | 140 | 1.1 | 154 |
| Gypsum powder | 10 | 0.8 | 8 | 10 | 0.4 | 8 |
| Brown sugar | 10 | 3.6 | 36 | … | … | … |
| Fermentation accelerator | … | … | … | 3.5 ~ 5 | 20 | 70 ~ 100 |
| Firewood for sterilization | 300 | 0.4 | 120 | 100 | 0.4 | 40 |
| Laborer for sterilization | 2 | 30 Yuan/ laborer | 60 | 1 | 30 Yuan/ laborer | 30 |
| Total | … | … | 787 | … | … | 676 ~ 706 |
| Variable costs | | | | | | |
| Survival rate | 80% | … | 984 | Survival rate | 90% | 751 |
| | 85% | … | 926 | | 95% | 711 |
| | 90% | … | 874 | | 98% | 690 |

Notes: (1) Formula of sterilized substrate: sawdust 78%, wheat bran 20%, gypsum powder 1%, brown sugar 1%; (2) Formula using the fermentation accelerator: sawdust 85%, wheat bran 14%, gypsum powder 1%, the fermentation accelerator 0.4%; (3) The expenditures for plastic bags, production and miscellaneous laborers are the same; (4) 1 ton of the above substrate can be filled in 1,000 15×55×0.0045 ~ 0.005 cm polyethylene bags for Shiitake production; (5) The figures in this Table are for reference only, the specific figures are subject to the cultivation varieties and local prices.

## ADVANTAGES TO USE THE FERMENTATION ACCELERATOR

(1) Simple procedures: High pressure sterilization is unnecessary, so the operation is not limited by sites.

(2) Save labor and costs: With the help of the fermentation accelerator, sterilization can be realized at a lower temperature in a shorter time period, so it can reduce the consumption of energy and costs. A strictly disinfected inoculation room (cabinet) is also not required. The unsterilized substrate bag cultivation can save a cost of 12% ~ 35% than the sterilized substrate bag cultivation. In mushroom cultivation on substitute sterilized substrate, it consumes a large amount of firewood or other fuel to sterilize at the normal pressure. Calculated at 1,000 bags on 1 sterilization unit, 300 kgs of firewood are to be burnt. Cultivation on unsterilized substrate can make use of the firewood saved to produce more Shiitake so that the contradiction between mushroom cultivation and forests can be alleviated and the eco-balance is maintained, which is advantageous for the sustainable development of mushroom industry.

(3) Improved inoculation conditions: The sterile room (cabinet) required for the inoculation of sterilized substrate is replaced by a clean room, which can save the use of disinfectants in inoculation room.

(4) Save time: Unsterilized substrate bag cultivation saves time for sterilization and multiple handling procedures.

(5) The change from bag cultivation to tray cultivation facilitates and simplifies spawn running, coloring, fruiting and management.

(6) There is no limit to production scales. The unsterilized substrate cultivation can be done in bags or on ridged fields according to labor availability, busy or slack seasons and sizes of the incubation rooms. The production scale is unlimited, which can be small or large. Cultivation can be flexibly adjusted according to local altitudes and climatic conditions in any season so long as the temperature is in the optimal range for themycelium growth of the spawned mushroom strain.

(7) 95% of mushrooms can be fruited and harvested from the unsterilized substrate cultivation technique, which considerably increases the economic benefit.

Under the authorization of Zhejiang Provincial Department of Science and Technology, Hangzhou City Science and Technology Bureau hosted the appraisal meeting for the project of Research on the Application Technique of the Fermentation Accelerator on August 6, 2006. The project appraisal committee consisted of 8 experts from the Microbiology Research Institute of the Chinese Academy of Sciences, Jilin Agricultural University, Edible Mushroom Research Institute of Shanghai Academy of Agricultural Sciences, Zhejiang University and Zhejiang Provincial Academy of Agricultural Sciences etc. Wei Jiangchun, an academician, was appointed to host the appraisal as chairman of the committee.

The expert committee gave the following evaluations on the technique: compared with traditional sterilization method, the application technique of the fermentation accelerator had such characteristics as a low sterilization temperature, a short time period, low costs and high efficiency. Ideal results were obtained in applying it to sterilize the substrate for mushroom cultivation at a substrate temperate of 70 ~ 80 °C for 5 ~ 7 hours.

The formulation of Enterprise Standards of the Fermentation Accelerator provided a technical support for safe and standardized production of the new product. The research results were applied in Zhejiang, Anhui, Gansu and Fujian Provinces, etc, with a total of 290 million bags and an accumulative of 116 million Yuan were earned from saving labor and costs, and increased efficiency. All these showed that it had significant social and economic benefits. The experts approved of the product's passing the appraisal and suggested that the result be transformed and spread as soon as possible to create larger social and economic benefits.

Mushrooms can be cultivated on unsterilized substrate using the fermentation accelerator. Producing 10,000 bags of Shiitake or Black wood ear can save 720 kgs of coal and 2 laborers and increase the output by over 10%, realizing substantial economic and ecological benefits. During last more than 1 year period since the appraisal meeting, over 2 billion bags of the fermentation accelerator were supplied to 26 provinces (municipalities, regions) across China, getting obvious benefits.

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# EFFECT OF *PLEUROTUS OSTREATUS* COLONIZED SUBSTRATE ON BROILER CHICKEN GROWTH

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## ABSTRACT

Alternative compounds have been used in ethnoveterinary to enhance growth performance and/or prevent common bacterial infections to poultry. However few of them have used mycelial-colonized substrate to partially replace standard diet in broiler chickens. The objective of this study was to evaluate broiler chicken production with partial replacement of standard diet by *Pleurotus ostreatus*-colonized substrate. The replacement of the standard diet by 100 and 200 g $kg^{-1}$ (P100 and P200) of colonized substrate for 21-day-old chickens presented up to 35% and 40% higher feed intake ($p \leq 0.05$), respectively, than the control. For body mass of P100 and P200, it was 50% and 58% higher ($p \leq 0.05$), respectively, than control for 21-day-old. The use of *P. ostreatus*-colonized substrate in chicken feeding is an alternative to improve broiler chicken production.

**Keywords:** *Pleurotus ostreatus*, broiler performance, chicken diet, basidiomycota.

## INTRODUCTION

Some basidiomycetes are known for their immunomodulating, antimicrobial and antitumor activity [1] and could be an alternative to improve poultry production. Alternative compounds have been used in ethnoveterinary to prevent common bacterial infections to poultry and/or enhance growth performance [2]. Poultry industry has improved the investments on animal health by using sub-therapeutic levels of antibiotic for subclinical disease control despite of people have been infected with bacteria that are resistant to those antibiotics [3].

*Pleurotus ostreatus* (Jacq.) P. Kumm. (Basidiomycota, Agaricales, Pleurotaceae) produce edible mushrooms recognized as a functional food due to its biological activity in the immunological system [4]. Different sources of fungi have been used on chicken production such as spent substrate from the cultivation of *Pleurotus sajor-caju* [5] or *Agaricus blazei* [6]; mushrooms from *Agaricus bisporus* [7] and mushroom extracts from *Lentinula edodes* and *Tremella fuciformes* [8]. However, the use of spent substrate from mushroom production has disadvantages for chicken feeding because of the reduced nutritional value and contaminating microorganisms that produce mycotoxins, common at the end of the mushroom cultivation and not always visible. The use of mushrooms or their extracts have a high production cost [5, 6], limiting their use for chicken feeding.

The use of vegetative mycelium grown in the feed itself has been little reported for chicken feeding [9], and presents the advantage of having the same biocompounds produced by mushrooms [4]. Moreover, mycelium production can be done in a shorter time, with high control of the cultivation [4], and is an alternative for chicken feeding. In addition *P. ostreatus* is a laccase producer, an enzyme widely used for hydrolysis of lignin, facilitating exposure and access to nutrients [10]. Thus, the objective of this study was to evaluate broiler chicken production with partial replacement of the standard diet by *P. ostreatus*-colonized substrate.

## MATERIALS AND METHODS

*P. ostreatus* from the culture collection of *Paranaense* University, cryopreserved at – 20 °C was used as inoculum for the experimental phases [11]. *P. ostreatus*-colonized substrate was used for partial replacement of standard diet for feeding from zero-to-21-day-old chickens.

This study was approved by the Ethics Committee of Research with Animal Experimentation of *Paranaense* University. 150 one-day-old male Cobb chicks were randomly distributed into five treatments. Each treatment had three replicates, with 10 birds per replicate per box, totalizing 30 birds. Each box had 1.5 m x 1.5 m with wood shavings on a concrete pad. The experimental groups were fed standard diet partially replaced by *P. ostreatus*-colonized substrate. Standard diet replacement was of 5, 10, 100 or 200 g $kg^{-1}$ of *P. ostreatus*-colonized substrate,

Feed intake and body mass of poultry production were recorded on the 21st day. The feed conversion ratio was calculated by the feed intake divided by the body mass gain. Chickens were slaughtered by cervical dislocation with monitoring of the local Sanitary Surveillance Agency to allow consumption of approved carcasses at the university restaurant. The results were submitted to analysis of variance and differences among averages were determined by Scott-Knott's test with significance level at 5%.

## RESULTS AND DISCUSSION

### Broiler chicken production

Treatments P100 and P200 for 21-day-old chickens presented up to 35% and 40% higher feed intake ($P < 0.05$), respectively, than the control. For body mass of P100 and P200, it was 50% and 58% higher ($P < 0.05$), respectively, than control for 21-day-olds. In conclusion, the replacement of *P. ostreatus*-colonized substrate in standard diet is effective for chicken body mass gain and production. Body mass gain increases when replacement in standard diet is higher than 10 g $kg^{-1}$ of colonized substrate. Feed conversion ratio is more effective for 21-day-old chicks when the replacement of the standard diet is 100-200 g $kg^{-1}$ of colonized substrate.

## ACKNOWLEDGEMENTS

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# DRIP IRRIGATION, A NEW WAY FOR WATERING, DURING *AGARICUS BISPORUS* CULTIVATION: INCREASED PRODUCTION AND LOWER CARBON FOOTPRINT

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## ABSTRACT

Drip irrigation was invented in Israel, more than 50 years ago. Today, it is the most efficient way for irrigation of field crops and orchards worldwide. Nevertheless, there was no attempt to adjust this way for water supply, to mushroom cultivation, probably due to technical constrains. The development of new low flow, pressure adjusted, leak less, drippers enabled the needed accurate water spread and opened the way to their use in mushroom cultivation. Drip irrigation overcomes the watering obstacles for mushroom cultivation. Drip irrigation enabled the keeping of optimal water contents in compost and casing during entire crop cycle. The value of mushroom yields (quantity and quality), was found to be higher with drip irrigation, mainly at the third picking flush. Decreasing relative humidity (RH), in the cultivation room, after spraying, is avoided with drip irrigation, decreasing the energy needs for room's drying. Higher mushroom quality, using drip instead of spray irrigation, mainly at the second and third flushes, increased the entire crop value. The reduction in energy needs and in thickness of casing reduces the carbon "footprint" of mushroom cultivation. The mentioned data collected until now, reveals the commercial advantage of drip irrigation, over spray watering for mushrooms cultivation.

**Keywords:** drip irrigation, watering system, mushroom yield, Mushroom quality, carbon footprint

## INTRODUCTION

In recent years we have seen continual attempts to improve quality and reduce picking costs, mainly by growing bigger and heavier mushrooms, without wetting the cups. Most of mushrooms are being sold as "medium cup" at a size of 35-60 mm. In order to achieve this, we need to supply: suitable compost, growing space for the cups, evaporation conditions and available water. Sonnenberg and Blok [1] demonstrated that the drops in compost, humidity during the first and second flushes were the limiting factor in mushroom production during the end of 2nd and the 3rd flushes. Dry substrate can lead to stalk conditions such as 'hollow stem' (excessive water up take followed by rapid evaporation), 'early-openers' (restricted water at a critical stage in mushroom enlargement), 'hard gill' (excessive evaporation with insufficient substrate moisture) and clefts and craters in mushroom fruits, thought to be related to low moisture, between mushroom's flushes [2].

We have recently reported, that the use of drip irrigation can overcome, the above mentioned obstacles in mushroom cultivation [3,4], since watering is done inside the casing, below the developing mushrooms and water can be added, to fit the needs at each growing stage. Drip irrigation allows careful management of moisture balance in the compost and casing layers. Excessive moisture in the substrate, can lead to anaerobic conditions, that can prevent lignin degradation and cause significant abiotic symptoms such as 'false mummy' (dried mummified mushrooms similar to those of Mummy disease caused by *Pseudomonas* spp.), or waterlogging (clear glassy patches on the fruit surface),'weepers' (moisture exudation from the marginal edge of the mushroom cap) or 'brown stems', bruised waterlogged stem tissue [5].

In the present work we report on further studies that were carried out in order to develop the optimal use of the drip irrigation system, together with picking management, to increase the size and quality of the picked mushrooms, mainly at the second and third flushes grown for "medium cup" size.

Optimal mushroom growth is dependent on continuous water supply, since the ability to evaporate water, is essential for mushroom growth. It is therefore crucial to insure that high water availability remains, during the entire cultivation cycle.

## MATERIALS AND METHODS

Irrigation experiments took place in commercial mushroom farm "The Champignon Farm" in Zarit at northern Israel. The rooms, where the experiments took place, were of 385m$^2$ shelf growing area, in two rows of six shelves high. The compost was phase 1 compost, produced on farm. Casing soil was composed of (mainly) black peat and limestone (Hartpeat - Ireland). The white *Agaricus bisporus* strain "Lambert 901", was used (8 liter/ton compost). At the end of spawn run nutrition supplement at 15 kg/ton compost (Champfood, The Netherlands) was added to the compost on the shelves and they were cased. Casing was done mechanically, with mixing some compost into the casing, a method called: compost added at casing (Cacing). The thickness of the casing layer was 5cm in the spray irrigated parts. In drip irrigated parts this thickness was reduced by 35%.

**Experimental Design:** In each experiment two mushroom cultivation rooms were used for the two treatments: the control, spray watering room, and the drip irrigation room. In each room there were six plots of 64.166 m$^2$ shelf area. Every plot was separately picked and yield's quantity and quality were recorded. The mushrooms were picked at the "stretch stage" as can be seen in Fig.1, for "medium cup" at a size of 35-60mm. Each mushroom was measured for its diameter and weight.

As demonstrated in Fig. 1, at the logarithmic phase of the growth curve, the mushroom weight is increasing faster than its cap size. Mushrooms are picked at the stretch stage to ensure quality and shelf live. The shape of the mushrooms at this stage is demonstrated in Picture 1.

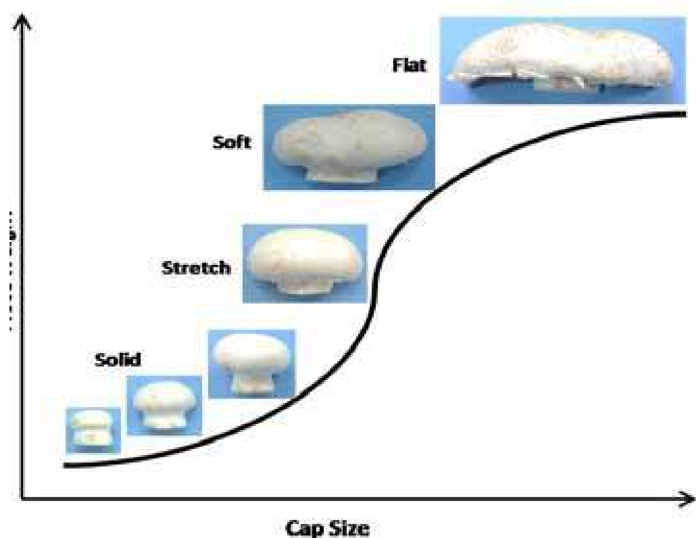



**Figure 1.** Mushroom growth stages

**Picture 1.** Medium cup at a size of 35-60 mm Diameter and Weight changes

**Irrigation Methods.** The control rooms were irrigated by Dofra automatic watering systems (The Netherlands). In the drip irrigated rooms a system, that was recently developed [4] in cooperation with Netafim Irrigation Systems (Israel) was used. The drip pipelines with Uniram 0.7 liter/hr drippers were incorporated into the casing, during Cacing, into the middle of the casing depth. Water amounts of 30-50 liter/m$^2$ were added to the treatments, during the cropping cycle. During the periods when spray irrigation is halted (pin-head development, flush beginning), drip irrigation continued, in order to avoid water content decrease in the casing and to minimize compost drying.

**Energy consumption.** The use of cooling and heating was measured separately from the Dalsem climate control system, in each room, in order to compare, energy consumption, by the two different irrigation systems. Cooling coil output was 75 kW heating coil output 37 kW.

## RESULTS

Mushroom's growth is measured as the increase in diameter and weight of each mushroom. The ratio between mushroom's diameter and weight growth, under the two irrigation methods, is presented in Figures 2.a, b, c.

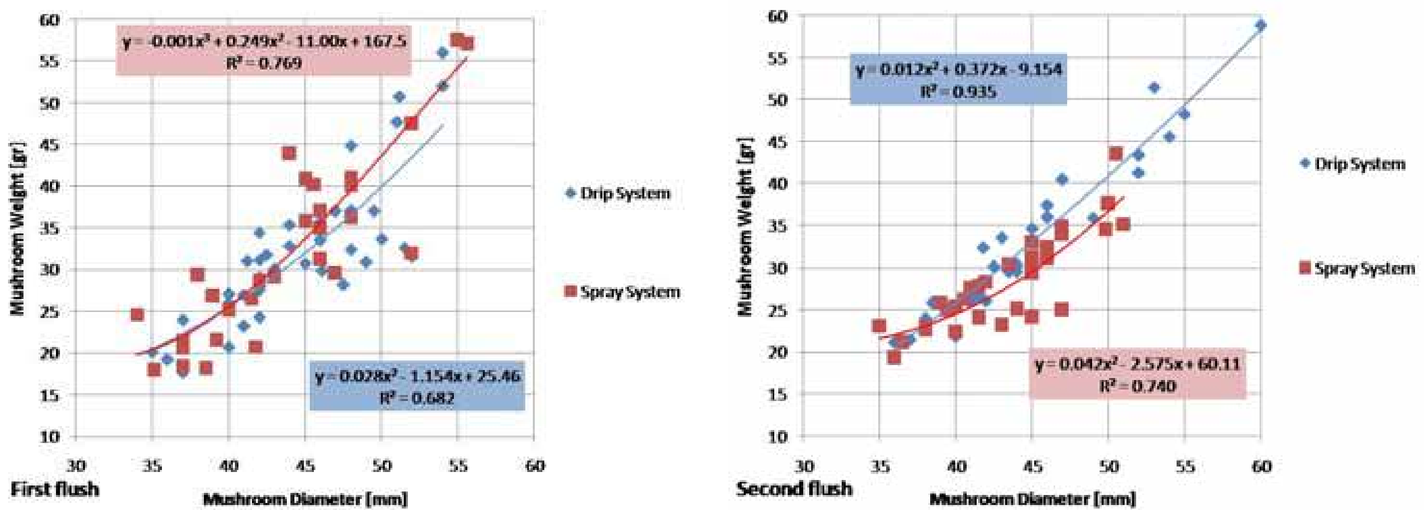



**Figure 2a.** The ratio between mushroom's cups diameter and weight during the first flush

**Figure 2b.** The ratio between mushroom's weight and cups diameter at the second flush

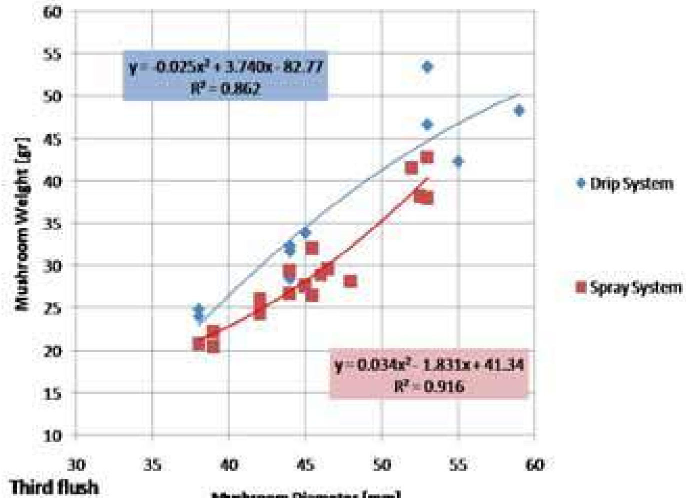
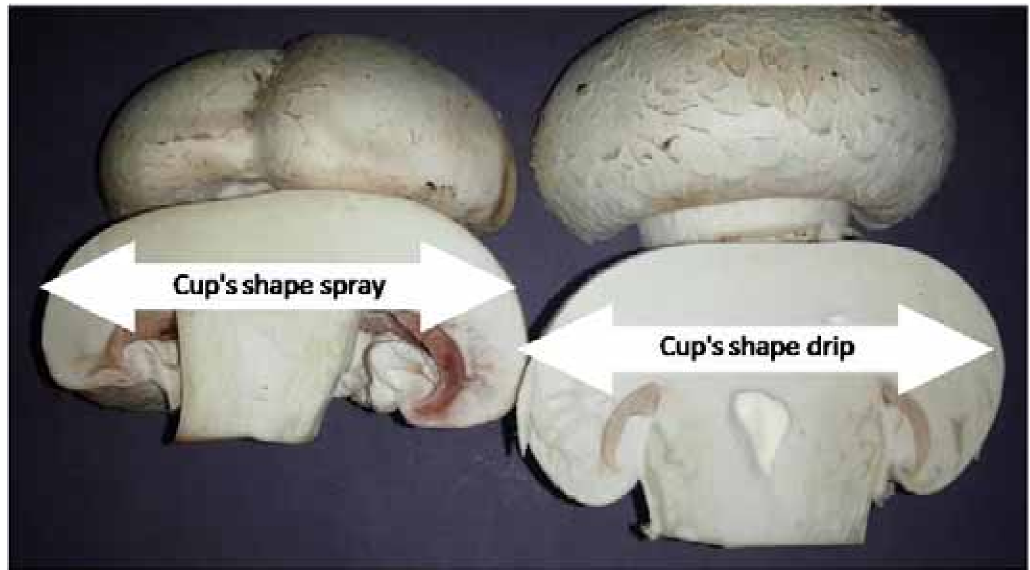



**Figure 2c.** The ratio between mushroom's weight and cups diameter at the third flush

In Fig. 2a it can be seen that in the first flush, increasing of the cups diameter from 35 mm to 50 mm increased the cups average weight from 20 gram/mushroom to 40-45 gram/mushroom in both treatments. Cups were picked at a diameter of 55 mm, with an average weight of 55 gram/mushroom.

In Fig. 2b it can be seen that in the second flush, increasing of the cups diameter from 35 mm to 50 mm, increased the cups weight from 20 gram/mushroom to 37 gram/mushroom in the spray system treatment and to 41 gram/mushroom with the drip irrigation treatment. In the drip irrigation treatment only, 55 mm cups were picked with an average weight of 50 gram/ mushroom.

In Fig. 2c it can be seen that in the third flush, increasing of the cups diameter from 38 mm to 53 mm, increased the cups weight from 20 gram/mushroom to 40 gram/mushroom in the spray system treatment and to 41 gram/mushroom with the




**Picture 2.** The effect of the watering system on cups shape in the third flush

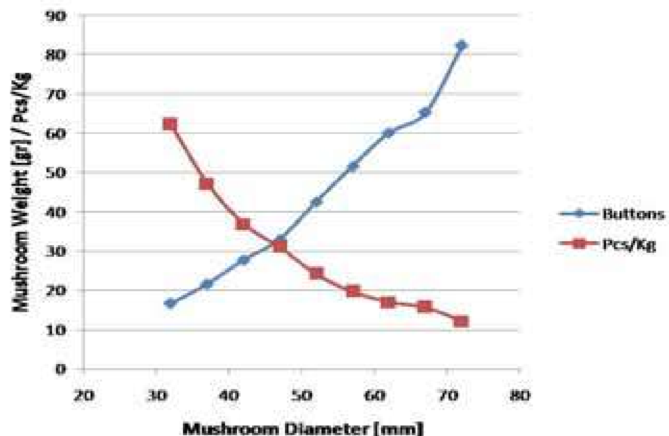



**Figure 3.** The relationship between mushroom's cup size and number of mushrooms in 1.0 kg

drip irrigation treatment, but only in the drip irrigation treatment 55 mm cups were picked with an average weight of 50gr/mushroom, as demonstrated in Picture 2.

It is demonstrated, that the use of drip irrigation during mushroom cultivation allows managing carefully the moisture balance in the compost and casing layers during the 2nd and 3rd flushes. This allows the cups to grow to bigger size as "medium cup" and to gain more weight compared to the same size of caps that were spray irrigated.

The use of drip irrigation allows the cups to grow to bigger size as "medium cup" and to gain more weight compared to the same diameter of caps that were spray irrigated. The shape of the cups of drip irrigated mushrooms is full and therefore they heavier than the opened shape of the spray irrigated mushrooms.

Increasing mushroom's diameter from 35mm to 50mm is accompanied by reducing the number of mushroom in a kg from 60 to 30 cups. Accordingly this phenomenon is followed by increased picking rate and reduced picking costs. The continuous water supply by drip irrigation leads to this situation.

**Table 1.** The average energy use, in spray and drip irrigation rooms, during the summer

| Treatment | % Cooling | % Heating | Cooling [kW] | Heating [kW] | % Difference in average summer energy consumption |
|---|---|---|---|---|---|
| Spray Irrigation | 76 | 21 | 57 | 10 | |
| Drip Irrigation | 44 | 26 | 33 | 8 | -26 kW Cooling and Heating |

In table 1 it can be seen, that during the summer, in spray irrigation rooms the need for cooling was higher than in the drip Irrigation rooms, since drying the cultivation room, after spraying, is avoided with drip irrigation. In order to transfer this data to energy saving costs, energy meters will be installed in the future.

## DISCUSSION

Water is taken up by the mushroom mycelium and fruiting bodies and evaporated during the entire cultivation cycle. Water had to be replaced by applying water to the casing. That is, however, halted during certain periods (aeration and flushing). To overcome this situation the concept of drip irrigation was developed. The use of under-surface drip irrigation, allows continuous water supply to the casing and compost throughout the cultivation cycle. It was proved in our previuos studies that the use of this system minimized the decrease in casing and compost water content. Furthermore, the use of drip irrigation allowed a decrease of up to 35% in thickness of the casing layer, and reduction of bacterial blotch disease.

In the present study we examind the impact of drip irrigation on the development of the individual fruit body. The use of drip irrigation with proper growth conditions (growth space and evaporation conditions) avoided the decrease of mushroom's quality at the second and third flushes. This quality decrease, is mainly due to low water availabilty, that cause faster growth in the fruit body dimention (diameter ) than in its weight. This is demonstrated, when drip irrigation is used by decrease in ratio between "weight/"diameter. These results enable increase in the size and quality of the picked mushrooms, mainly at the second and third flushes grown for "medium cup" size. The increased mushroom weight enhanced their picking rate and reduced picking costs. Drip irrigation allows this option, insuring better mushroom quality and higher yields.

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# AN INNOVATIVE METHOD OF PREPARATION OF HEALTHY GRAIN SPAWN

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## ABSTRACT

Mushroom spawn is prepared on cereal grain media by inoculating them with pure cultures of selected mushroom species under sterile conditions, yet the problem of contamination of spawn is a major bottle-neck in the growth and spread of mushroom farming in developing countries. Species of *Trichoderma*, *Aspergillus, Penicillium, Rhizopus,* etc. besides wet spot causing bacteria are known to be major contaminants of mushroom spawn in eastern India, including Jharkhand affecting commonly grown mushrooms viz., *Pleurotus* spp, *Hypsizygus ulmarius* and *Volvariella volvacea*, which need urgent action for their management. In view of reports of appearance of fungicide-resistant strains of *Trichoderma*, therefore, a Neem- based herbal formulation, viz., Mahaneem containing 0.15% Azadirachtin was tried as a prophylactic pre-treatment of the wheat grains used for the spawn medium together with some empirical changes in the current method of preparation of the grain spawn medium. The modified method tried successfully for raising healthy, contamination-free and productive master and planting spawns of oyster and paddy straw mushrooms has been discussed.

**Keywords:** spawn, oyster-mushroom, azadirachtin

## INTRODUCTION

Grain spawn is a medium that is impregnated with mycelium made from a pure culture of the chosen mushroom strain, and spawn production is a fermentation process in which the mushroom mycelium increases by growing through a solid organic matrix. Although, grain spawn is currently prepared by inoculating sterilized grains with complete sterile precaution, yet small and medium size spawn- laboratories still face the problems of contamination i.e. unwanted intrusion of harmful and competing organisms in the spawn, making them useless and dangerous for mushroom production due to their potential for infecting the beds and the crop as a whole. Mushrooms have since long been there in the diet of various tribes of Jharkhand, mostly drawn from wild collections during monsoon season, and hence several mushroom farming units have come up during last few years both in the urban and rural areas of the state. Gradually spawn laboratories both in public and private sectors are also coming up in major towns to serve the growers. However, due to humid and wet weather conditions particularly during rainy season, 20-40% losses are reported to be caused by bacterial and fungal contaminants of commercial spawn, restricting the availability of healthy and quality spawn in time and in required quantities, which is seriously hampering the growth and spread of mushroom farming in the area.

It has been roughly estimated that about 1 million microbes are killed while preparing only 1 kg of pure spawn. Moreover, during the process of spawn making, contamination is likely to come from various sources like aerospora of the lab, impure inoculum, incomplete sterilization of grain medium and contaminated spawn bags/tools/hands of the workers, etc, which obviously make pure spawn making a highly risky activity. Moreover, cereal grains carry lacs of microbes in them, particularly those escaping full sterilization, either due to their thermo-tolerance, insect damage, mechanical injury or those ruptured by over-boiling. Yet, in the prevalent methodology of spawn-preparation, the grains are not given any sort of pre-treatment to reduce their inoculum-load, although while preparing the beds for oyster and milky mushrooms, their substrates (wheat or paddy straw etc) are widely being treated with chemicals/fungicide [1] or with Neem-oil based bio-formulations [2] for the same purpose. In the year 1985, the button mushroom Farms of Ireland and again in 1990s of Europe, America, Canada, Mexico, etc faced devastating on slaughts of Green Mould disease which led to the use of systemic fungicides to control the malady. Grogan *et al.* [3] reported that grain-spawn treated with benomyl or carbendazim or thiabendazole could effectively control the causal fungus *Trichoderma harzianum* biotype Th2. However, large scale use of Benzimidazole fungicides soon led to the emergence of a fungicide-resistant biotype Th4 of *T. harzianum* (syn. *T. aggressivum, f. aggressivum),* against which the same treatments proved ineffective [4], which led to the banning of

those fungicides in several affected countries. Obviously, such an action has left the commercial growers of those areas under the possible threat of another epiphytotic.

The small growers of eastern states of India are mostly raising seasonal crops of oyster, milky and paddy straw mushrooms with low cost and low input technology and are more prone to diseases and pests. Yet, only some preventive measures to manage them are likely to be adopted both by the growers and spawn producers of the area. Hence, in the present investigation experiments have been carried out to get rid of as much of the contaminating microbial load on the grains as possible by their pre-treatment with potent bio-formulations together with suitable modification in the spawn making methodology so as to ensure effective sterilization of the spawn medium.

## MATERIALS AND METHODS

### Modified method of spawn preparation

Spawn is a potent source of contamination to cultivated mushrooms [5,6] . Hence, with a view to remove the contaminating microbes from the spawn, an attempt to modify the standard method of spawn preparation now in practice was made in this investigation. Required quantities of good quality wheat grains were procured from the local market and their initial moisture content was determined. Subsequently, the grains were washed 2-3 times to remove any dirt and artifacts and soaked in water for 30 minutes to get rid of any pesticide-residues. Neem oil (Mahaneem) dilutions containing 1.5 and 0.15 ppm Azadirachtin were prepared in quantities just sufficient to submerge the grains and to absorb most of the solution so as to make their final moisture-content to not less than 55% as calculated on the basis of their initial moisture-content. The initial pH of the 2 sets of dilutions were kept at 8.5 and 9.0, in which the grains were soaked for more than 18 hours or overnight and all the treatments were replicated 4 times with two controls, viz., grains soaked i) in plain water, and ii) in 0.05% carbendazim solution. During soaking, the containers were covered with plastic sheets to prevent any loss of the liquid. The soaked grains were later spread thinly on a clean surface in shade to dry the free water sticking on the grains, to which calcium carbonate was added @ 2% and mixed thoroughly to make the grain spawn medium ready. The medium was filled in 1 litre glass bottles up to 2/3rd capacity and plugged with non-absorbent cotton, which were autoclaved at a pressure of 20 lb psi for not less than 45 minutes. The bottles were then shaken to avoid clumps and kept over-night in the inoculation-room before inoculating them with 1x1mm inocula taken from the growing edges of freshly raised pure cultures of *Pleurotus florida* and *Hypsizygus ulmarius*. For *Volvariella volvacea* also the grain medium was similarly prepared except that wheat grains were replaced by Paddy grains. The inoculated media bottles were incubated at 28±2 °C/30±2 °C for oyster and paddy straw mushrooms respectively.

### Growth performance of modified grain spawn

The inoculated bottles during incubation were shaken at regular intervals to enhance and ensure uniform growth of the mushroom mycelium. After 2 weeks of incubation their final growth-performance, vigor, abnormality /contamination were noted by visual observation. Further, the spawn bottles were monitored during storage in refrigerator for 3 weeks at regular intervals for any late contaminants.

### Yield-performance of spawn prepared by modified method

Spawn prepared by modified method were tested for their productivity by raising crops of *P. florida,* and *H. ulmarius* on paddy-straw in polythene bags as per Vijay and Sohi [1]. Experiment on *P. florida* was laid with modified spawn treated with dilutions of Neem-oil @ 1.5 and 0.15 ppm Azadirachtin adjusted to pH 8.5, and followed by straw treatment with 500 ppm formalin- solution. Two controls viz., i) Modified spawn treated with 1.5 and 0.15 ppm of Azadirachtin at their natural pH 5.6 -5.9 followed by straw treatment with 500 ppm formalin; and ii) Modified spawn without treatment but straw treated with solution of 75 ppm Bavistin and 500 ppm formalin. Experiment on *H. ulmarius* was however designed to assess the performance of the Modified spawn with grains treated with 0.1% Bavistin instead of Neem oil, and straw treated with 37.5 ppm carbendazim and 500 ppm of formalin and the 2 controls kept were: 1) untreated spawn + treated straw with carbendazim and formalin; and 2) both spawn and straw untreated. The crops were grown in an

improvised seasonal growing room in the RKMV university campus, with temperature ranging between 26-29 °C during the day.

## RESULTS AND DICUSSION

### Modified grain spawn medium

Soaking of wheat grains in measured quantity and known strength of Azadirachtin dilutions for a long duration of 18 hrs or so, ensured maximum possible absorption and retention of the bio-pesticide in the internal tissues of the seeds making them least prone to attacks by the contaminants. Moreover, prolonged soaking of the grains could avoid the process of 30-35 minutes boiling, now in practice, saving thereby leaching of nutrients of the grains and their cracking, as well as the costly fuel and valuable time spent over boiling. Besides, the possibility of killing of the heat- resistant bacterial endospores during autoclaving was also very much enhanced due to their germination to vegetative phase during prolonged soaking. This was amply revealed by the absence of bacterial soft-rot infection in any of the spawn-bottles prepared by the modified method.

### Growth performance of modified grain spawn

Grain spawns of 3 mushroom species, viz. *P. florida*, *H. ulmarius* and *V. volvacea* were prepared by the modified method using two dilutions of Azadirachtin adjusted at 2 different pH in case of former 2 species. However, for all the 3 species 2 controls were kept, one soaked in 0.05% carbendazim and the other in sterile water only. In Tables 1, 2 & 3, observations on their mycelial growth attained 2 weeks after incubation have been given. It is noted that *H. ulmarius* attained faster growth at pH 8.5 as compared to pH 9.0, irrespective of dilutions of the Neem-oil. carbendazim soaking at 0.05% concentration also supported faster growth-rate of the spawn. Data in Table 2 exhibit the same trend of growth response by *P. florida* as noted for *H. ulmarius* both in the case of Azadirachtin as well as carbendazim. The spawn of *P. florida* also grew faster at pH 8.5 than at pH 9. However, the growth-rate of *V. volvacea* was slightly retarded due to soaking in 1.5 ppm Azadirachtin as compared to its lower conc. (0.15 ppm) and also 0.05% carbendazim (Table 3). It is notable that none of the Modified spawn bottles under different treatments/replications exhibited any contamination what so ever (Figs.1 & 2).

**Table 1.** Performance of *Hypsizygus ulmarius* spawn prepared by modified method and treated with Neem oil at different pH

| Treatments | Azadirachtin (ppm) | | | | Carbendazim | Water |
|---|---|---|---|---|---|---|
| | pH 8.5 | | pH 9 | | | |
| | 1.5 | 0.15 | 1.5 | 0.15 | Control | Control |
| Growth on 13th day | ++++ | ++++ | +++ | +++ | ++++ | +++ |
| Remarks | No Contamination | | | | | |

++++ = Complete, +++ = Incomplete

**Table 2.** Performance of *Pleurotus florida* spawn prepared by modified method and treated with Neem oil at different pH

| Treatments | Azadirachtin (ppm) | | | | Carbendazim | Water |
|---|---|---|---|---|---|---|
| | pH 8.5 | | pH 9 | | | |
| | 1.5 | 0.15 | 1.5 | 0.15 | Control | Control |
| Growth on 13th day | ++++ | ++++ | +++ | +++ | ++++ | +++ |
| Remarks | No Contamination | | | | | |

++++= Complete, +++= Incomplete

**Table 3.** Performance of *Volvariella volvacea* spawn prepared by modified method and treated with Neem oil at different pH

| Treatments | Azadirachtin (ppm) | | Carbendazim* | Water |
|---|---|---|---|---|
| | 1.5 | 0.15* | | |
| Growth on 14th day | +++ | ++++ | ++++ | +++ |
| Contamination | No Contamination | | | |
| Remarks | *Profuse formation of chlamydospores by 18th day | | | |

++++= Complete, +++= Incomplete

**Figure 1.** *Hypsizygus ulmarius* spawn with Neem Oil $10^{-3}$ dilution at pH 9

**Figure 2.** *Volvariella volvacea* spawn treated with Neem Oil dilution $10^{-4}$ and carbendazim 0.05%

Further it was also noted that 0.15 ppm Azadirachtin and 0.05% carbendazim treated spawn profusely developed brick-red colored chlamydospores, which are known to be indicators of healthy and vigorous spawn [7].

## Yield-testing of spawn prepared by modified method

Spawn sprepared by Modified method were used to raise crops of *Hypsizygus ulmarius, Pleurotus florida* and *Volvariella volvacea* to test their productivity under *in vivo* conditions on paddy straw as substrate (Figs.3, 4 & 5). Data obtained from replicated Experiments on the former two species in Tables 4 & 5 indicated that the modified spawn treated either with Azadirachtin (1.5 & 0.15 ppm) or carbendazim (0.05%) could give good yield of *P. florida* (71-78.33% BE) and *H. ulmarius* (56.73-79.20% BE), respectively. However, in case of both the mushroom species, even untreated modified spawn gave equally good yield either on formalin+carbendazim treated straw or untreated straw, which might possibly be due to the vigorous and healthy nature of the spawn grown on a more nutritious medium. The health and vigor of the spawn was also indicated by the high yield obtained from *P. florida* bags even though most of the bags with treated spawn(but only formalin-treated straw), got locally infected with green mould below the points where holes were made for fruiting. Another important observation noted in the performance of *H. ulmarius* was the negative effect of carbendazim on the productivity of that mushroom giving the lowest yield, when spawn and straw both were treated with the fungicide (Table 5).

A crop of *Volvariella volvacea* was also raised with Azadirachtin treated modified spawn by the Cage-culture method , but it could not be replicated and its yield performance could not be assessed. However, good fruiting was recorded with attractive and healthy sporocarps (Fig. 5). Use of cereal grains for mushroom spawn was introduced by Sinden [8] as early as in1931 the superiority of which was established by Szudyga and Mallinowske [9] even in the year 1974, although, various modifications continued to be proposed therein. Lemke [10] suggested that the grains should contain 50% moisture and the pH of the spawn medium be adjusted between 6.5-6.7, while Kumar *et al.* [11] recommended the use of

**Figure 3.** *H. ulmarius* in fruiting

**Figure 4.** *P. florida* in fruiting

**Figure 5.** *V. volvacea* in fruiting

**Table 4.** Yield Performance of *P. florida* spawn prepared by modified method and treated with Neem oil on chemically pasteurized straw

| Treatments | | | Room Temp. | Yield | B.E | Remarks |
|---|---|---|---|---|---|---|
| Spawn | Straw | | (°C) | (g) | % | |
| | Carbendazim (ppm) | Formalin (ppm) | | | | |
| Azadirachtin (1.5ppm) @ 8.5 pH | - | 500 | 27-29 | 1175 | 78.33 | Contamination of *Trichoderma* sp. |
| Azadirachtin(0.15ppm) @ 8.5 pH | - | 500 | 27-29 | 1140 | 76.00 | |
| Azadirachtin(1.5ppm) at natural pH (5.6-5.9) | - | 500 | 27-29 | 1085 | 72.33 | |
| Azadirachtin (0.15ppm) | - | 500 | 27-29 | 1065 | 71.00 | |
| Untreated control natural pH | 37.5 | 500 | 27-29 | 1115 | 74.33 | No contamination |
| CD 5% | | 46.76 | | | | |

calcium carbonate and gypsum in the ratio of 1:3 for better spawn growth. Mantel [12] earlier in 1973 had perfected the method of grain spawn preparation in India, which is still being practiced all over the country and its vicinity with minor changes only, with little attention to quality and health.

Besides high productivity and consumer-friendly traits of the mushroom strain, maintenance of health and vigor of spawn are very important aspects, which are inter-related. A healthy spawn must be free from contaminants to be vigorous.

**Table 5.** Yield Performance of *H. ulmarius* spawn prepared by modified method and treated with carbendazim on chemically pasteurized straw

| Treatments | | Room Temp. | Yield | B.E | Remarks |
|---|---|---|---|---|---|
| Spawn | Straw | (°C) | (g) | % | |
| Carbendazim 0.1% | Carbendazim + Formalin | 26- 29 | 851 | 56.73 | No contamination observed |
| – Do - | Untreated | 26- 29 | 967 | 64.47 | |
| Untreated | Carbendazim + Formalin | 26- 29 | 1075 | 71.67 | |
| Untreated | Untreated | 26- 29 | 1188 | 79.20 | |
| CD 5% | | 101.15 | | | |

Similarly, only a vigorous spawn can remain contamination-free [13]. Obviously, both these aspects depend heavily on nutrients available in the spawn-medium. Avoidance of boiling of the grains and absorption of almost all the water used for soaking in the modified method being discussed here very well prevent the loss of valuable nutrients by leaching and hence the grain medium so prepared is more nutritious and well set to support a healthy and vigorous spawn. Further, prolonged soaking in aqueous solution is most likely to induce the heat-resistant endospores of *Bacillus* sp, causing wet-spot disease, to germinate into heat-sensitive vegetative phase leading to their elimination, as also of some thermo-tolerant species of *Aspergillus*, during the standard process of autoclaving. Thus, the modified grain spawn medium is more likely to be contamination-free.

The current scenario of spawn health and quality in India and abroad is, however, worrying. Soriano [6] surveying the Central Luzon recorded the problem of spawn contamination among 91% mushroom growers, 67% of whom referred it as occasional, and 16% each as frequent and seldom. In India, 5-years data from a public sector Mushroom Research and Development Laboratory in Haryana, reported 5-25% spawn contamination by various moulds as well as wet-spot causing *Bacillus* sp. [14]. Earlier, Ahlawat *et al*. [15] also reported appreciable damage to *A. bisporus* spawn due to *Bacillus subtilis* infection at NRCM, Solan. From the North Eastern State of Assam also, 8 fungal and 1 bacterial species were reported as common contaminants of oyster mushroom spawn, being more frequent during the monsoon months [16].

Thus, in the present challenging scenario [17] of disease management, every effort needs to be made right at the starting point, i.e. preparation of spawn by keeping them fully free from the contaminants as well as maintaining their quality and health. Also, mushrooms themselves being fungi as also most of the contaminants, they cannot be managed with fungicides and other hazardous chemicals alone for obvious reasons [18]. Therefore, preventive measures like preparation of contamination- free grain spawn medium like the one described here, together with observance of full sterile precautions, cleanliness and hygiene are essential to be adopted in all spawn laboratories, which might ensure the availability of healthy and vigorous spawn to the growers at large.

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# COMPARATIVE CULTIVATION EXPERIMENTS OF *PLEUROTUS ERYNGII* ISOLATES

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## ABSTRACT

Sixteen *Pleurotus eryngii* isolates, mostly of Hungarian origin, were cultivated on lignocellulose substrate containing soy bean based enrichment. Composition of the substrate: 65% beech sawdust; 17% bran; 9% beech woodchips; 3.5% gypsum; 5.5% soybean supplement (Promycel 480). Water content was adjusted to 60%, and then the mixture was filled into plastic bags, sterilized and spawned. The blocks were cased by peat-based commercial *Agaricus* casing soil. We determined the amount of yield, number of fruiting bodies, period of flushes, average weight of fruiting bodies, biological efficiency (BE, %) and productivity (P, %) for each strains. The amount of yield was given for 100 kg of substrate. The highest yield was produced by the Ple-4V (41.5 kg/100 kg) and Ple-5V (39.5 kg/100 kg) strains, whereas the lowest yield was found in case of the PEL (9 kg/100 kg) and PEG (11 kg/100 kg) strains. The average yield of the strains was 27,53 kg calculated for 100 kg substrate. The number of fruiting bodies was 1488 pcs, the average weight of fruiting bodies (concerning the species) was 19.95 g. Very high biological efficiency was produced by the Ple-4V (156.2%) and Ple5V (140%) strains. The lowest efficiency was found at the PEL (28.5%) and PEG (37.8%) strains. The average yield was over 25 kg/100 kg spawned substrate.

**Keywords:** king oyster mushroom, *Pleurotus eryngii,* cultivation experiment, strain, isolate, biological efficiency

## INTRODUCTION

*Pleurotus* species can be found on the northern hemisphere between latitude 30 and 50, mainly in steppes, in arid grasslands or sometimes in mountainous areas, and always in association with umbelliferous plants [1, 2]. In their natural habitat, taxa of the species complex are facultative biotrophs, white rot fungi of certain plant species belonging to the families *Apiaceae (Umbelliferae)* and *Asteraceae* (*Compositae*) [1, 3-8]. In spite of the fact that fruit bodies are primarily formed in association with umbelliferous plants, in cultivation they do not need the presence of these plant species. A number of lignocellulose based substrates can be used for intensive cultivation [9-13]. Numerous varieties are listed in the *P. eryngii* species complex: var. *eryngii* (DC.: Fr) Quel.; *ferulae* Lanzi /syn.: *P. fuscus* var. *ferulae*/; *elaeoselini* Venturella *et al.*; *nebrodensis* (Inzenga) Sacc.; *tingitanus* Lewinsohn *et al.*; *tuoliensis* C. J. Mou; *P. hadamardii* Constantin; *P. fossulatus* (Cooke Sacc.) [1, 6-8, 14-16].

Nowadays *P. eryngii* is cultivated in several Asian countries and in many European mushroom farms, and it is present in the European markets as well. In the USA *P. eryngii var. eryngii* is grown since 2000 [2]. Pasteurization and sterilization are the common procedures in substrate preparation. The substrate is than filled into bags, blocks, PP bottles or boxes. For achieving higher yields it is possible to apply peat-based casing, but the mushroom is able to grow on the surface of the lignocellulose substrate itself as well [2, 17, 18].

Different international sources report a wide range of biological efficiency depending on the substrate composition, application of casing and quality and quantity of supplementation. According to Sonnenberg *et al.* [19], the biological efficiency in Germany and in the Netherlands is rather low (10-15 %), but in their experiments they could achieve 20-25 %. Kirbag and Akyuz [20] used different agricultural byproduct based substrates and reported 45 % biological efficiency. Rodriguez *et al*. [21] succeeded179 % with applying different combinations of supplementation and casing.

Nowadays spawn producing companies use wild strains selected by growers, but in the future breeding will hold the key for *P. eryngii* production. From the species complex, *P. eryngii* is an exceptional choice for cultivation, as it is considered to have the best taste amongst all oyster species [21]. It is relatively easy to cultivate, has a fairly long shelf life, it also has a reduced spore load and a quite high price on the market [22]. Our aim was to conduct a comparative growing experiment of different, mainly Hungarian isolates of *P. eryngii* to identify the growing properties and the biological efficiency of the 16 isolates under the same ambient conditions, on the same substrate.

## MATERIALS AND METHODS

### Strains

The majority of the strains were collected from the grasslands and hills of the eastern part of Hungary (Table 1). Following collection of tissue samples of the fruit bodies were transferred onto malt agar, and by repeated inoculations clean vegetative mycelia cultures were achieved.

**Table 1.** *Pleurotus eryngii* isolates and their origin used in trial

| Code | Origin | Cultivated/collected |
|---|---|---|
| PEA | Tószeg; Hungary | collected |
| PEP | Eger, Pásztorvölgy; Hungary | collected |
| PEG | Tószeg; Hungary | collected |
| PEF-i | Demjén, Vas-tanya; Hungary | collected |
| PEF | Kecskemét; Hungary | collected |
| PEC | Eger, Pásztorvölgy; Hungary | collected |
| Ple1V | Novaj; Hungary | collected |
| Ple2V | Novaj; Hungary | collected |
| Ple3V | Bogács; Hungary | collected |
| Ple4V | Heves; Hungary | collected |
| Ple5V | Novaj; Hungary | collected |
| Ple6V | Novaj; Hungary | collected |
| PES | Netherlands | cultivated |
| PE-SZM | Malaysia | cultivated |
| PEL | Italy | cultivated |
| PEK | China | cultivated |

### Propagation material (spawn)

Rye-based spawn was made in case of all 16 strains by the prevalent standard spawn production process.

### Substrate

2000 ml capacity polypropylene bags were filled with 900 g wet substrate. The substrate consisted of 65 % beech sawdust, 17% wheat bran, 9% beech chips, 3.5% gypsum and 5.5% soy supplement (Promycel 480) (values referring to dry weight). Water content of the substrate was adjusted to 60%. The bags were closed with paper plugs and covered by tinfoil before sterilization in autoclave on 121°C for 2.5 hours. Following cooling the substrate was inoculated with spawn (10 m/m% spawn rate) in a laminar flow box.

Samples of fresh and spent substrate of the 16 strains were taken and dried to constant weight on 105 °C for measuring dry matter content. The dry matter content of the fresh and spent substrate and the difference in water content of the material before and after cropping were determined. Nitrogen content was measured by Kjeldahl method using a Büchi B-324 distillation unit.

**Growing conditions**

The bags were placed in a room with 25 °C temperature for spawn run. Since in case of each strain spawn run was evenly completed by the 10th day following spawning, the temperature was lowered to 10 °C. On the 12th day the bags were opened and folded and 3 cm casing was applied using sterilized peat based button mushroom casing soil. Following irrigation the bags were covered by *vlies*. From this day 12-12 hours light and dark periods were set and relative humidity was adjusted to 95%. On the 15th day the temperature was raised to 17.5 °C while keeping the surface covered and the humidity set on 95%. By the 17th day the mycelium appeared on the edge of the surface of the first bags. On the 19th day the vlies was removed, which lowered the $CO_2$ level below 800 ppm inducing fruit body formation.

After the primordia have appeared temperature was adjusted to 19 °C for the cropping period. During cropping, the surface of the bags, the floor and the walls of the room were irrigated daily to maintain proper humidity level. Ambient conditions in the cropping cycle: 19 °C (±2 °C) growing room temperature, 95% relative humidity and $CO_2$ level below 800 ppm.

**Determination of growing properties**

The yield and number of fruit bodies were registered during the entire cropping period; the values are given in kg and pcs per 100 kg substrate. The flushes, days of picking and average fruit body weights were determined in case of each strain. BE% and P% were also measured on fresh and spent substrates [21, 22].

**Correlation and cluster analysis**

Based on the results of substrate analysis and yield values the following questions were formed: a) Does the dry weight loss of the substrate correlate with the yield? b) Does the weight loss of wet materials correlate with the yield? c) Is there a correspondence between the total nitrogen content and the yield? To answer these questions Pearson's correlation analysis was conducted by using SPSS 15 program. Classification of the strains based on their yields was done by Tukey's correlation analysis and SPSS 15 program. The aim of the analysis was to determine which strains belong to the same group based on yields.

## RESULTS

**Yields**

The highest yields were registered in case of strains Ple-4V (41.5 kg/100 kg) and Ple-5V (39.5 kg/100 kg), while the lowest in case of strains PEL (9 kg/100 kg) and PEG (11 kg/100 kg). Based on the results of this experiment *P. eryngii* can be characterized with an average 27.53 kg/100 kg yield, a total number of 1488 fruit bodies and an average fruit body weight of 18.5-19.95 g (Fig. 1). Fig. 1 also shows that based on the Tukey cluster analysis only two strains (PE-SZM and PES) could be placed in the same group, the others had various yields from lower to higher values.

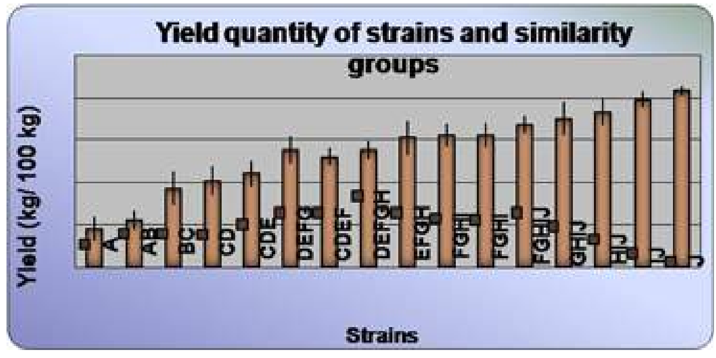


**Figure 1.** Yields of strains calculated for 100 kg substrate (kg), average weight of fruiting body (g), value of similarity and dissimilarity between strains. (The more identical characters in the name of the strains mean higher degree of similarity, while less identical character refers to less similarity).

In our opinion high yields (besides being genetically determined) were influenced by the following factors: casing

prevented the drying of the lignocellulose substrate, provided equalized water supply and constant humidity for the mushrooms on the surface of the bags. The 12-16 days long spawn run described [21] was shorter (only 8-10 days) in our experiment due to the higher (10%) spawn rate. According to Stamets [22] the first flush is to be expected on the 20-29th day, but in spite of the shorter spawn run, we could not pick fruit bodies of the first flush sooner than 27-28 days. It has to be noted that others only picked on the 38th [20], or even on the 37-54th days [19] from different substrate compounds.

## Biological efficiency and productivity

When taking the spent compost weight into account, relatively high biological efficiency can be reported in case of most strains. Especially high BE% was observed for instance by strains Ple4V (156%) and Ple5V (140%), while the lowest by PEL (29%) and PEG (38%). The average biological efficiency was 98% (based on spent substrate weight) and average productivity was 44%. If BE% and P% are calculated based on fresh materials, both values are lower (26% and 17%) because of the forthcoming weight changes during cultivation. In our opinion it is important to calculate these parameters in case of both the fresh and the spent substrates, since in international research papers it is not stated whether the presented BE% and P% values are calculated based on measurements before or after cropping. This fact might be the reason of the significant differences experienced in various researches. Table 2 shows the number and average weight of fruit bodies, biological efficiency and productivity (calculated for 100 kg substrate).

**Table 2.** Important data of comparative cultivation experiments, calculated for 100 kg substrate

| Strains | Yield (kg/100 kg) | Total no. of fruit bodies (pcs/100 kg) | Av. weight of fruit bodies (g) | BE (%) (spent) | P (%) (spent) | [a]BE (%) (diff.) | [b]P (%) (diff.) |
|---|---|---|---|---|---|---|---|
| PEL | 9 | 1050 | 8.6 | 28.52 | 11.11 | 4.8 | 2.11 |
| PEG | 11 | 1500 | 7.3 | 37.82 | 14.29 | 8.9 | 3.29 |
| PEP | 18.5 | 550 | 33.6 | 56.31 | 20.56 | 7.6 | 2.06 |
| Ple6V | 20.35 | 1050 | 19.4 | 74.35 | 32.82 | 20.8 | 12.47 |
| PEFi | 22 | 1100 | 20 | 72.29 | 31.65 | 14.4 | 9.65 |
| Ple1V | 26 | 1400 | 18.6 | 97.33 | 44.83 | 28.9 | 18.83 |
| PEC | 27.5 | 1600 | 17.2 | 100.28 | 45.45 | 27.9 | 17.95 |
| Ple2V | 27.6 | 1400 | 19.7 | 104.38 | 45.62 | 31.7 | 18.02 |
| Ple3V | 30.5 | 2450 | 12.4 | 109.43 | 58.65 | 29.1 | 28.15 |
| PES | 31 | 1500 | 20.7 | 111.2 | 43.06 | 29.6 | 12.06 |
| PE-SZM | 31 | 2150 | 14.4 | 120 | 55.36 | 38.4 | 24.36 |
| PEA | 33.5 | 1700 | 19.7 | 118.44 | 45.58 | 30.2 | 12.08 |
| PEF | 35 | 1400 | 25 | 119.7 | 53.44 | 27.5 | 18.44 |
| PEK | 36.5 | 950 | 38.4 | 128.26 | 65.18 | 32.2 | 28.68 |
| Ple5V | 39.5 | 1400 | 28.2 | 140.03 | 65.29 | 36 | 25.79 |
| Ple4V | 41.5 | 2600 | 16 | 156.18 | 76.85 | 46.9 | 35.35 |
| Mean (species) | 27.53 | 1488 | 19.95 | 98.41 | 44.36 | 25.9 | 16.83 |
| Deviation | 9.401 | 541.140 | 8.258 | 35.773 | 18.640 | 11.713 | 9.973 |

[a]BE% (diff.): BE% calculated to spent substrate – BE% calculated to fresh substrate.
[b]P% (diff.): P% calculated to spent substrate – P% calculated to fresh substrate.

## Correlation analysis

Fig. 2 shows a more notable weight loss of the substrates in case of strains with higher yields. It can be concluded that productive strains have more efficient enzyme systems that are capable of degrading and utilizing the substrate more efficiently up to a certain point. The most likely explanations of the weight loss of the fresh substrate are: a) the mushroom utilizes the components of the substrate to build its own "body" and we remove this mass from the system by picking the mushrooms; b) the mushroom oxidizes the substrate and the carbon compounds (originating primarily from the substrate) leave the growing material in $CO_2$ form which leads to the weight loss of the substrate. The average weight loss is 9.6 kg/100 kg (lowest: PEP – 5.126 kg/100 kg; highest: PE-SZM – 12.15 kg/100 kg). After a certain point the strains with higher yields are not able to a more intense degradation, regardless to the fact that the yield continued to increase. This is probably explained by other genetic differences of the strains.

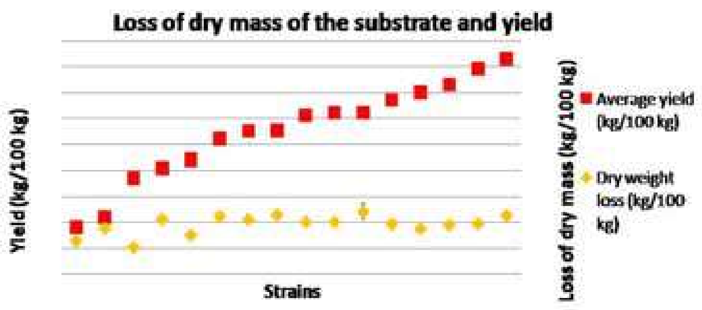


**Figure 2.** Differences between dry mass of substrate before and after cultivation (dry mass loss of substrate) and changes in the yield.

The decrease in dry substrate mass and the yield was analyzed with Pearson's correlation. The Pearson's correlation coefficient of the 16 samples showed that there is a positive correlation (r = 0.542) between the weight loss and the yields by 5% significance level, thus the correlation is significant ($\alpha = 3\%$). There is a linear connection between weight loss and yields.

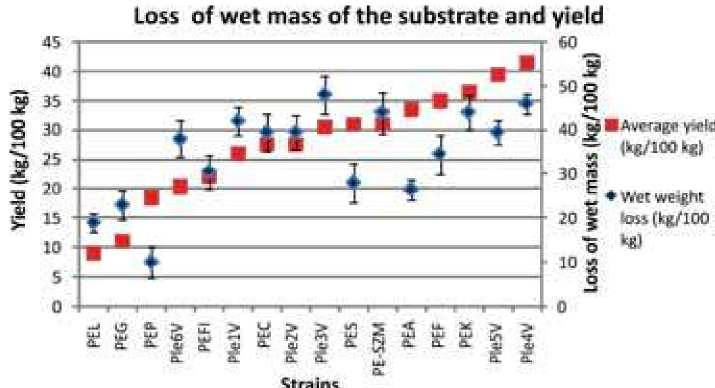


**Figure 3.** Differences between wet weight of substrate before and after cultivation (weight loss of wet substrate) and changes in the yield

Similar results can be seen on Fig. 3 where the connection between yields and the wet weight change of the substrate before and after cultivation is presented. Besides the picking of the mushrooms, other factors could serve as reasons for the weight loss of the wet substrate: a) intensity of evaporation, influenced by ambient conditions (primarily relative humidity and airflow); b) thickness of casing applied; c) water content of the casing soil; d) water transport from the casing soil towards the fruit bodies and evaporation intensity of the fruit bodies. For *P. eryngii* it is not yet known whether there is a water transport from the casing soil to the fruit bodies like in case of button mushrooms and if there is such, in what extent. Another question is how much does the evaporation capacity of the different strains vary?

Since *P. eryngii* has sufficient quantity and quality crop on supplemented substrate, we were looking for a connection between the total nitrogen content and yields. The results are shown on Fig. 4.

The Pearson's correlation coefficient of the strains showed that there is also a significant correlation between the nitrogen content and the yield. The correlation is negative (r = -0.593), the level of significance is at least 5 % ($\alpha = 1.5\%$). According to the results strains with lower yields had higher nitrogen levels in their spent substrates, while in case of the strains with higher yields the nitrogen level was lower.

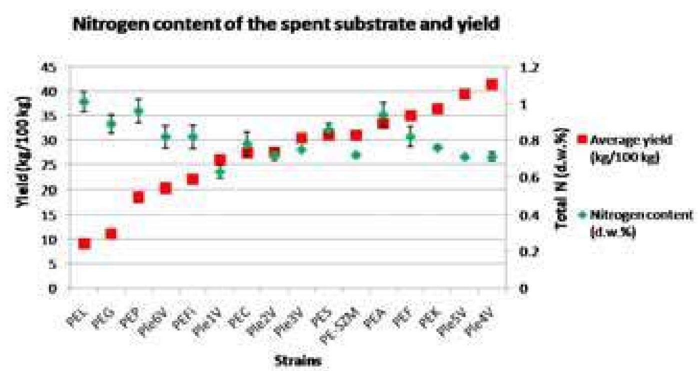


**Figure 4.** Correlation between total nitrogen content of substrate and amount of yield

## Qualitative and quantitative elements

The results presented above mainly refer to physiological features and yields of the strains and the species. For the morphological and qualitative evaluation a photo documentation and a detailed cultivation guide was prepared, from which the following elements should be highlighted. Based on the days necessary to form the first flush and fruit bodies the quickest strains are: Ple-3V, Ple-4V, Ple-5V, PES and PEA. The first mature fruit bodies of these strains could be picked on the 27-28$^{th}$ day. The mushrooms reached mature state on the 30-31$^{st}$ day in case of strains PEP, PEC, PE-SZM, Ple-1V, Ple-2V and Ple-6V; 32-34$^{th}$ in case of PEK, PEF and PEF-i and 37$^{th}$ for strains PEL and PEG.

Strain PEC had the most intense development in the vegetative state, but its mycelium was apt to become stoma-like. Despite this phenomenon, numerous fruit bodies were formed in the stoma-like areas as well. Some distorted mushrooms appeared in case of strains Ple-1V and Ple-2V. PEL and PEG were the two strains with the lowest quality and quantity features. Strain PEL formed only a few regular but small fruit bodies, while PEG had pencil-shaped, curved fruit bodies without caps. These strains should be excluded from future breeding materials. By using sterilized substrate and providing maximal hygienic conditions no mycoparasites, competitor moulds or pests appeared in the course of the experiment. Only in one case did high relative humidity cause bacterial (*Pseudomonas* sp.) spots to form on the cap of a single fruit body of Ple-5V.

## Description of strain PES

On the 23$^{rd}$ day, next to the edge of the bags a number of nice pea-sized, sometime walnut-sized fruit bodies developed. On the 26$^{th}$ and 27$^{th}$ day spherical, brown-greyish, marble patterned fruit bodies with curled up edges appeared. The first flush was ready to be picked on the 28$^{th}$ day. The *cap* of the immature fruit body is hemispherical, the edge is curled up at first, then it becomes flat and in the end funnel like, with a small lump in the middle. The color of the cap is brown-grayish with a hint of purple in it; the surface is radially filamentous, moderately felty and drily matt. By time the edge of the cap is less prone to discoloration (yellowing). The *stem* is usually centered. The *gills* are quite dense, whitish when young, then turn greyish. The gills run down on the top third of the stem and then continue as veins, fibers towards the base. The spore is white. The strain (Fig. 5) is recommended for commercial use. Picking dates: day 28, 30, 32, 37, 47, 56 and 60$^{th}$. Other features: BE% = 111.2; P% = 43.06; average yield: 31 kg/100 kg; average number of fruit bodies: 1500 db/100 kg; average fruit body weight: 20.7 g.

**Figure 5.** Fruiting bodies of PES strain that was found to be appropriate for cultivation

**Description of strain PEF**

The first flush was ready to be picked on the 34th day. At first the *cap* is hemispherical, then is flattens and become funnel like. On the older fruit bodies the irregular, wavy edge is clearly apparent. The color is brownish with a slight grey shading, lighter on the edge. The surface of the cap is radially filamentous and matt.The stem is white, centered or eccentric, sometimes flattened on the sides. The gills are white when young, then become greyish. The gills are quite dense and run down deep on the stem. The spores are white. The fruit bodies grow in groups. The groups are sometimes big because of both the number and the size of the fruit bodies (Fig. 6). In spite of the few days longer growing period, this strain is highly recommended for cultivation. Picking dates: day 3, 47 and 56th. Other features: BE% = 119.7; P% = 53.44; average yield: 35 kg/100 kg; average number of fruit bodies: 1400 pcs/100 kg; average fruit body weight: 25 g.

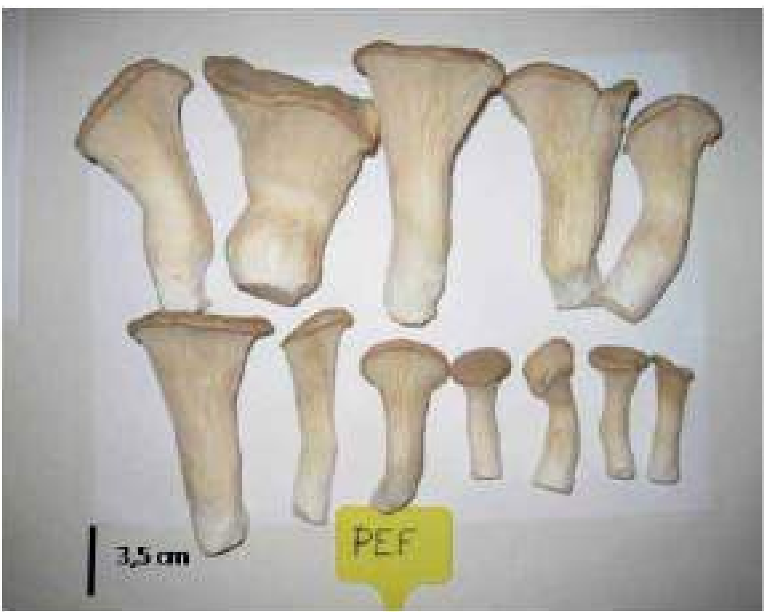


**Figure 6.** Fruiting bodies of PEF strain that was found to be appropriate for cultivation

## CONCLUSION

Future improvements of cultivation technologies should focus on the following problems. Besides using selected wild strains, breeders should produce hybrids for the industry. It would be advised to find the proper supplementation process and to adapt heat treatment procedures to the *P. eryngii* substrate. Another possible way of improving cultivation technology is to define the exact specific ambient conditions of the species or even the strains.

Nowadays the mushrooms in the fruit body stage ready to be picked are not evenly mature and so the picking date has to be adjusted the smaller fruit bodies would better be left on the bags to grow and most mature mushrooms can be plucked. If we just pick the big mushrooms and leave all the other on the bag, in most cases the remaining pieces of the group die. It would be necessary to find a way to synchronize fruit body development to have mushroom groups that are evenly mature.

During fruit body induction a high number of primordia appear, but only a few become full-grown mushrooms. The remaining fruit body initials die and serve as ground for bacterial and fungal diseases. An improved way of fruit body induction should be found to reduce primordia size and to promote equalized development. To prevent mushroom groups to form and to achieve uniform fruit body development, growers should try ruffling (a technique already used in button mushroom cultivation) in case of *P. eryngii* as well. Another solution is to apply a form of CACing, where kind of a 'Phase III. substrate' or casing spawn is mixed into the casing soil.

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# COMPARATIVE EVALUATION OF CASING MIXTURES ON THE YIELD POTENTIAL OF BUTTON MUSHROOM *(AGARICUS BISPORUS)*

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## ABSTRACT

In order to study the effect of different casing mixtures on the growth and yield of common button mushroom (*Agaricus bisporus)* an experiment was carried out with seven casing mixture formulations. Maximum sporophore (12.33) were obtained by casing mixture of CCP + VC + FYM + SD + Sand and minimum sporophore ( 5.67) were obtained on casing mixture CCP + FYM. The highest yield of first flush was obtained from the casing mixture of CCP + VC + FYM + SD + Sand (270.33 g) followed by CCP + FYM + SD, VC + FYM, VC + SD + FYM + Sand and CCP + VC + Sand ( 266.67, 216.67, 213.33 and 196.67), respectively. Casing mixture CCP + VC + FYM + SD + Sand recorded the highest yield (320 g) whereas CCP + FYM ( 250 g) showed lowest yield in the harvesting of second flush. The total highest yield (1112.26 g) was obtained from casing mixture, CCP + VC + FYM + SD + Sand and lowest yield (736.67 g) from CCP + FYM. Casing mixture of CCP + FYM + SD recorded second highest yield (1033.67 g).Finally all the casing materials were evaluated for their effect on growth parameters and yield of *Agaricus bisporus*. Among the different casing mixtures tested CCP + VC + FYM + SD + Sand and CCP + FYM + SD were found to be better in yield when compared to other casing mixtures. These studies will help to mushroom growers for selecting the most suitable casing materials for better growth behaviour and optimum yield potential of common button mushroom (*A. bisporus*) grown in our country.

**Keywords:** *Agaricus bisporus*, casing mixtures, growth parameters, yield

## INTRODUCTION

India is blessed with varied agro-climatic regions from temperate, tropical to subtropical. This makes India as a suitable place for the production of different types of mushrooms. *Agaricus bisporus* (Lange) Sing. is popularly known as the button mushroom, is extensively cultivated throughout the world and contributes about 40% of the total world production of mushroom. Mushrooms are considered to be healthy food because of their relatively high and qualitatively good protein content and also because of the presence of good vitamins, minerals and low fat content. Verma *et al*. [1] reported that mushrooms are very useful for vegetarian diet because they contain some essential amino acids which are mostly found in animal proteins. In this context mushroom cultivation represent one of the economically viable processes for the bioconversion of agricultural and agro-industrial wastes into a protein rich food, making it a potent weapon against malnutrition in developing countries like India which has lowest per capita consumption of protein in the world [2-5].

Button mushroom is one of the largely grown mushrooms and has the good demand in the market and world trade too. Keeping this view in mind the choice of the farmers for growing of button mushroom depends on easily and locally available casing mixtures. Bhatt *et al*. [6] evaluated seven different casing mixtures prepared from 5 materials viz., FYM, spent compost, Vermicompost, coir pith and press mud, for their yield potential on *A. bisporus*. In a similar studies Dhar *et al*. [7] also reported effect of casing materials on the yield and quality of the fruiting body of button mushroom (*A. bisporus*). Huge quantities of farm yard manure, vermicompost, saw dust and other organic wastes are generated annually through the activities of agricultural, forest and food processing industries. Mushroom yield canbe increased if these locally available casing mixtures are used to produce button mushrooms. Therefore, present investigation was carriedout to see the effect of different casing mixtures on the yield potential and growth parameters of button mushroom (*A. bisporus*).

## MATERIALS AND METHODS

### Spawn Preparation

Well cleaned wheat grains were boiled for 30 minutes until the grain became soft and were mixed with 2% Chalk (calcium carbonate) and 2% Gypsum (calcium sulphate) [8]. Then grain filled bags were sterilized in an autoclave for 1 h at 121 °C. After sterilization, the bags were allowed to cool down at room temperature and then inoculated with bits of mushroom mycelium (10 days old cultures) of *A. bisporus*. Inoculated bags were incubated at 25±2 °C in a B.O.D. incubator for 16 days. These bags were shaken at 4 days intervals to spread the mycelia and completely impregnated the grains.

### Compost preparation

For the basic materials for compost, wheat straw were collected from Agro Farm, Banaras Hindu University. Other ingredients like wheat bran, urea, potassium (Murate of Potash), phosphorus (Single Super Phosphate), gypsum, molasses and lindane were procured from market.

**Table 1.** Formulation of the compost for button mushroom

| Ingredients | Quantity (kg) |
|---|---|
| Wheat straw | 600 |
| Wheat bran | 60 |
| Urea | 7.5 |
| Murate of potash | 6.0 |
| Single Super phosphate | 6.0 |
| Molasses | 9.0 |
| Gypsum | 60 |
| Lindane Dust | 0.5 |

### Procedure

The compost was prepared by long method of composting (LMC), using the method developed by Mantel *et al*. [9]. Wetted wheat straw was spread thinly over entire floor of the composting yard and then gradually wetted by sprinkling water, till the straw was taking no more water. The straw was then turned for even wetting at this stage and water content was maintained at 75 per cent. The moist straw was mixed with wheat bran and fertilizers uniformly scattered over the straw. A heap was made after each turning but not compressed tightly so as to maintain the aerobic condition in the compost heap. Gypsum was mixed at the third turning and at each turning. Lindane was mixed at 7th turning for prevention of insects pests. Total eight turnings were done and each turning at four days interval. The compost was then ready for spawning i.e. it was dark brown in colour and without any smell of ammonia and had sufficient moisture content (68-70%) when pressed between the palms.

### Spawning

The compost was mixed with mushroom spawn @ 2.5 kg/quintal compost and spawned compost was filled (4 kg/bag) in a cylindrical polythene bag (size 40 x 40 cm) and compost filled bags were covered with newspaper sheets to prevent loss of moisture content from mushroom beds, placed in growing chamber, where temperature ranged between 22-28 ºC (Ram and Holkar [10].

### Preparation of casing mixtures

The selected basic material for preparation of casing soil such as farm yard manure was obtained from Agro-farm, B.H.U. and other casing materials viz., coir pith, saw dust, vermi-compost and sand were obtained from local market.

### Procedure

Seven casing mixture formulations were used as casing for investigations.

i. Coconut coir pith + FYM + Saw dust (1:1:1).

ii. Vermi compost + Saw dust + FYM + Sand (1:1:1:1)

iii. Coconut coir pith + FYM (1:1)

iv. Coconut coir pith + Vermi compost + FYM + Saw Dust + Sand (1:1:1:1:1)

v. Vermi compost + FYM (1:1)

vi. Coconut coir pith + Vermi compost + Sand (1:1:1)

vii. Coconut coir pith + Vermi compost + FYM (1:1:1)

First Coconut coir pith was soaked in water for 24 hours before mixing. Initially individual casing materials viz., FYM, sawdust, coconut coir pith, sand and vermicompost were sterilized in a horizontal autoclave at a temperature of 121 ºC for 20 minutes. Sterilized casing materials were taken out to cool down at room temperature by spreading on well cleaned cemented floor.

**Observations and Measurement**

The newspaper sheets were removed and the surface of compost was uniformly layered about 3.5 cm by casing formulations. Mushroom beds were sprayed regularly with water to keep the casing soil adequately moist. The recorded temperature of cropping room was ranged 18-24 ºC during harvesting period. Observations were recorded on fruiting bodies of *Agaricus bisporus* in each bag on the following parameters.

i. Initiation of pin heads (Days after spawning)

ii. Average diameter of fruit bodies (cm)

iii. Average weight of fruit body (g).

iv. Yield from each flush and total yield (g).

Each treatment was replicated three times and the yield data were statistically analyzed by Complete Randomized Design (CRD).

**RESULTS AND DISCUSSION**

Mushroom bags were completely colonized by mushroom mycelium within 20 days and then covered by different casing soil for following observation of growth stages.

**Initiation of pin head**

Data pertaining to the time taken for initiation of pin heads are presented in Table 2. The casing mixture of VC+ FYM took maximum time (i.e. 38.67 days) CCP + VC + FYM + SD + sand took minimum time period (35 days). These casing mixtures differed non-significantly among themselves.

**Time taken for harvesting of different flushes**

The harvesting of first flush ranged between (40.67 to 44.47 days). It is evident from the Table 2 that application of two casing mixture CCP + FYM + SD and CCP + VC + SD + FYM + Sand showed significant difference in time taken for harvesting of first flush. The casing mixture CCP + VC + FYM + SD + Sand was taken minimum time (40.67 days) for harvesting of first flush and maximum time (44.67 days) by application of CCP + FYM+ SD.

The harvesting of second flush in two casing mixture i.e. VC + SD + FYM + Sand and VC + FYM took the same time period (62.67 days) and these casing mixture differed non-significantly among themselves. The range of time taken for harvesting second flush was found between (60.33-66.67 days). Third flush was harvested in the range of 77.33 to 85.00 days on various casing mixtures. The performance of casing CCP + FYM + SD was better than other casing mixture. Application of casing mixture CCP + VC + FYM + SD + Sand differed significantly in time taken for harvesting of third flush with that of CCP + FYM + SD + Sand.

The data evaluated the time taken for harvesting of fourth flush showed the non-significant difference between casing mixtures. It revealed information regarding total crop period. The crop period was observed maximum (i.e. 95.66 days) with application of CCP +VC + FYM + SD and VC + SD + FYM + Sand, whereas, the crop period was minimum (90.33 days) with application of CCP + VC + Sand casing mixture.

**Table 2.** Effect of casing mixtures on growth behaviour of button mushroom (*Agaricus bisporus*)

| Casing mixtures | Initiation of pin heads (days) | Time taken for harvesting of flushes (days) | | | | Total crop period (days) |
|---|---|---|---|---|---|---|
| | | Ist flush | IInd flush | IIIrd flush | IVth flush | |
| CCP + FYM + SD (1:1:1) | 38.33 | 44.67 | 60.00 | 77.33 | 94.66 | 94.66 |
| CCP + FYM (1:1) | 36.67 | 43.33 | 61.33 | 77.67 | 93.33 | 93.33 |
| VC + SD + FYM + sand (1:1:1:1) | 37.00 | 41.67 | 62.66 | 80.67 | 95.66 | 95.66 |
| CCP + VC + sand (1:1:1) | 35.67 | 41.33 | 61.66 | 81.00 | 90.33 | 90.33 |
| VC + FYM (1:1) | 38.67 | 43.33 | 62.67 | 76.67 | 94.00 | 94.00 |
| CCP + VC +FYM + SD + sand (1:1:1:1:1) | 35.00 | 40.67 | 60.33 | 85.00 | 95.66 | 95.66 |
| CCP + VC + FYM (1:1:1) | 36.33 | 42.67 | 60.33 | 76.67 | 92.33 | 92.33 |
| $SEm_{\pm}$ | 2.24 | 0.78 | 1.07 | 1.32 | 2.96 | 2.96 |
| C.D. (P= 0.05) | 6.90 | 2.41 | 3.30 | 4.07 | 9.12 | 9.12 |

CCP = Coconut coir pith, FYM = Farm yard manure, SD= Saw dust, VC = Vermi compost.
Initiation of pin head: Days recorded after spawning
Harvesting of flushes: Days recorded after spawning

## Number and Weight of sporophore

Data pertaining to the number of sporophores per bag from different flushes has been given in Table 3. Maximum sporophore (12.33) were obtained by casing mixture of CCP + VC + FYM + SD + Sand and minimum sporophore (5.67) were obtained with application of casing mixture CCP + FYM. The results indicate that there was significant difference between the number of sporophore with casing of CCP + VC + FYM + SD + Sand and VC + FYM, CCP + VC + Sand with CCP + FYM and CCP + FYM + SD with CCP + FYM.

It was intended that number of sporophore was always found to be reduced in the fourth flush as compared to Ist, IInd and IIIrd flushes. Similarly, the casing mixture CCP + VC + Sand showed minimum sporophores (2.67) and CCP + VC + FYM + SD + Sand showed maximum sporophores (6.33). Casing mixture CCP + VC + Sand and CCP + VC + FYM + SD + Sand differed significantly with respect to number of sporophores.

The weight of sporophores was calculated as average weight for each treatment Table 3. In observation, heavy weight of sporophores were obtained with the casing of CCP + VC + FYM (1:1:1) (i.e. 36.00 g) and less weight of sporophore were obtained with casing mixture CCP + FYM (1:1) (27 g).

## Length of stalk

The length of stalk was measured in average for each treatment as given in Table 3. The maximum stalk length (3.05 cm) was recorded in the casing of CCP + VC + FYM (1:1:1) where as minimum stalk length (2.03 cm) was observed in the casing of VC + SD + FYM + sand (1:1:1:1) The length of stalk in casing CCP + VC + FYM (1:1:1) was found highly significant to CCP + FYM + SD (1:1:1) and other casing soils.

## Yield of flushes and total yield

It is evident from the Table 3 that the highest yield of first flush was btained from the casing mixture of CCP + VC + FYM + SD + Sand (270.33 g) followed by CCP + FYM + SD, VC + FYM, VC + SD + FYM + Sand and CCP + VC + Sand (266.67, 216.67, 213.33 and 196.67), respectively. Casing mixture CCP + VC + FYM + SD + Sand showed the highest (320 g) yield whereas CCP + FYM (250 g) showed lowest yield in the harvesting of second flush. In the 3rd flush, maximum yield was obtained from casing CCP + VC + FYM + SD + Sand (305.33 g) where as lowest yield was obtained

**Table 3.** Effect of casing mixtures on growth parameters and yield of button mushroom (*Agaricus bisporus*)

| Casing mixtures | No. of sporophores per bag at diff. flushes | | | | Wt. of sporo-phores (g) | Length of stalk (cm) | Yield of different flushes (g) | | | | Total yield (g) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ist | IInd | IIIrd | IVth | Av. | Av. | Ist | IInd | IIIrd | IVth | |
| CCP + FYM + SD (1:1:1) | 8.00 | 8.33 | 9.67 | 4.33 | 28.33 | 2.17 | 266.67 | 302.00 | 298.3 | 216.6 | 1083.67 |
| CCP + FYM (1:1) | 5.67 | 5.33 | 4.67 | 3.67 | 27.00 | 2.07 | 183.33 | 200.00 | 256.6 | 196.6 | 836.67 |
| VC + SD + FYM + sand (1:1:1:1) | 8.00 | 11.67 | 10.67 | 5.00 | 29.67 | 2.03 | 213.33 | 246.67 | 226.6 | 200 | 886.00 |
| CCP + VC + sand (1:1:1) | 7.33 | 6.33 | 6.33 | 2.67 | 32.00 | 2.07 | 196.67 | 233.3 | 216.6 | 186.6 | 833.33 |
| VC + FYM (1:1) | 8.00 | 8.33 | 8.33 | 3.33 | 31.67 | 2.07 | 216.67 | 250.0 | 233.3 | 183.3 | 883.33 |
| CCP + VC + FYM + SD + sand (1:1:1:1:1) | 12.33 | 13.67 | 11.00 | 6.33 | 30.33 | 2.05 | 270.33 | 320.00 | 305.33 | 216.6 | 1112.26 |
| CCP + VC + FYM (1:1:1) | 6.67 | 6.33 | 5.33 | 4.33 | 36.00 | 3.05 | 190.33 | 205.00 | 250.0 | 190.67 | 836.00 |
| SEm± | 0.91 | 1.00 | 0.69 | 0.77 | 0.72 | 0.15 | 56.42 | 19.03 | 17.30 | 16.29 | |
| C.D. (P= 0.05) | 2.81 | 3.08. | 2.14 | 2.37 | 2.26 | 0.45 | 24.23 | 41.47 | 27.33 | 35.50 | |

from CCP + FYM (196.6 g). There was significant difference in yield obtained between casing mixture VC + FYM and CCP + FYM and between CCP + FYM + SD and VC +SD + FYM + Sand.

The highest total yield (1112.26 g) was obtained from casing mixture, CCP + VC + FYM + SD + Sand and lowest yield (736.67 g) was obtained from CCP + FYM. The second highest yield (1033.67 g) was obtained from CCP + FYM + SD casing mixture.

This finding was in accordance with the result of Dhar *et al*. [11] who used eight commonly available casing materials in India viz, FYM, SMC, CCP, MG, VC, Terracare-A, Terracare-B and FYM + SMC to identify the suitable casing materials for use in button mushroom cultivation. Coir pith results in early pinning and significantly higher number of fruit bodies and total yield. Our result also confirmed the findings of Pardo *et al*. [12] who evaluated different casing materials for the cultivation of button mushroom. All the casing materials were evaluated for their effect on growth parameters and yield of *Agaricus bisporus*. Among the casing mixtures, CCP + VC + FYM + SD + Sand and CCP + FYM + SD were found to be better in yield compared to other casing mixtures. The finding of present study will help the farmers for selection and better utilization of locally available casing materials for obtaining higher yield of button mushroom (*A. bisporus*) to increase their income.

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# EVALUATION OF DIFFERENT STRAINS OF OYSTER MUSHROOM FOR THEIR CULTURAL, MORPHOLOGICAL AND YIELD ATTRIBUTES

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## ABSTRACT

Oyster mushroom is one of the important edible mushrooms grown in India as well as in world having high nutritive value and manifold uses. Different strains of oyster mushroom (PL-1, PL-3, PL-4 and PL-5) were evaluated for their cultural, morphological and yield attributes. Strain PL-3 grew best (maximum mycelium growth) at temperature 30 °C and at pH 6 however, higher temperature (30 °C) and higher pH level (pH 8) suited PL-1. The low temperature (20 °C) and higher pH 8 suited for PL-5 strain. Among different media tested, the maximum radial growth of mycelium was observed on oat meal agar (OMA) medium in PL-3 and PL-1 strain. The strain PL-3 had maximum stipe length, whereas shortest stipe was recorded in PL-5 strain. The maximum pileus diameter was recorded in strain PL-1 whereas minimum in PL-5 strain. On the basis of yield performance, strain PL-4 was found the best producing 395.42 g/2 kg wet substrate with biological efficiency 65.90% on wheat straw + waste paper (1:1). It was observed that the supplementation of waste paper (50%) to the wheat straw is suitable for PL-1, PL-3 and PL-4. However, supplementation of palm leaves (50%) to wheat straw was suitable only for PL-5 with 53.94% biological efficiency.

**Keywords:** oyster mushroom, physiological requirements, substrate, yield performance

## INTRODUCTION

Among the various cultivated mushrooms, oyster mushroom is easy to cultivate due to its strong enzymatic action towards the utilization of various kinds of organic substrates. It has gained importance only in the last decade and is now cultivated in many countries in the subtropical and temperate zones. Generally *Pleurotus* is referred to as 'oyster mushroom' all over the world, while in India as 'Dhingri'.

Oyster mushrooms are a diverse group of saprotrophic fungi belonging to the genus *Pleurotus* [1]. This mushroom is a good source of non-starchy carbohydrates, with high content of dietary fiber and moderate quantity of proteins, including most amino acids, minerals, and vitamins [2]. The protein content varies from 1.6 to 2.5%, and the niacin content is about ten times higher than that of any other vegetable. Oyster mushroom significantly reduced serum triglyceride and serum cholesterol in diabetic subjects [3]. Oyster mushroom diet effectively prevented the progress of hypercholesterolemia (decreased by 38%) and cholesterol accumulation in liver (decrease by 25%) that were induced by the cholesterol diet in rats [4]. This mushroom is gaining popularity day by day considering the nutritional and medicinal importance of this mushroom, an attempt was made to evaluate different strains for their physiological requirements and the substrate suited for their production

## MATERIALS AND METHODS

### Culture and culture maintenance

The culture of different strains of oyster mushroom i.e. PL-1, PL-3, PL-4 and PL-5 obtained from Mushroom Research and Training Center (MRTC), GBPUA&T, Pantnagar which obtained cultures from Directorate of Mushroom Research, Solan were used. The cultures were maintained on potato dextrose agar (PDA) medium at 28±1 °C temp for further studies. To determine a suitable temperature for the mycelial growth of different strains of oyster mushroom, the Petri plates containing 20 ml PDA medium were inoculated and incubated at four different temperature viz.,15 °C, 20 °C, 25 °C and 30 °C. Three replications were maintained for each temperature and radial growth of each replication was taken till the colony covered the full plate.

Pre-sterilized PDA medium was adjusted at 5, 6, 7, and 8 pH levels. Poured Petri plates inoculated with 5 mm culture disc of test mushroom cultures were incubated at 28 ± 1 °C temperature. For each treatment, there were three replications. The radial mycelial growth of the fungus was measured as described earlier. Six different media *viz.* oat meal agar (OMA), malt extract agar (MEA), potato dextrose agar (PDA), Czapek's dox agar (CDA), wheat extract agar (WEA) and water agar (WA) were used to investigate the mycelial growth of the strains. Petri plates containing 20 ml of the sterilized medium were inoculated at the centre with 5 mm diameter disc of actively growing mycelium under aseptic conditions and incubated at 28±1 °C temp. For each strain and each medium, three replications were maintained and radial growth was recorded.

## Evaluation of substrate

The substrates used for the experimentation were wheat straw (WS), WS + palm leaves (1:1), and WS + waste paper (1:1). All the substrates were chemically treated with carbendazim 37.5 ppm + formalin 500 ppm for 18 hrs. The tank was covered with polythene sheet to prevent the evaporation of formalin. Thereafter, substrate was taken out from the tank and spread on cemented floor already treated with 2 per cent formalin and left for 2-3 hours to drain out excess water [7].

Spawning was done under clean conditions with already prepared commercial wheat grain spawn @ 2 per cent on wet weight basis of the substrate. The spawned substrate was filled in 2 kg capacity polypropylene bags and the mouth of the bags was folded and stapled. After spawning, bags were kept in the cropping room at relative humidity of 70-80 per cent and a temperature of 25-28°C for spawn run. After complete spawn run, it becomes a compact mass and bags were cut open to expose the substrate. The relative humidity in the crop room was maintained by sprinkling of water twice/thrice a day. The fruit bodies were harvested at the time of maturity. Biological efficiency was calculated using the following formula: Biological efficiency (BE) % = (Fresh weight of fruit body/Dry weight of substrate 4 x 100

## RESULTS AND DISCUSSION

Diametric growth of the four strains of *Pleurotus* was studied at temperature range from 15 °C - 30 °C on potato dextrose agar (PDA) medium. The results revealed that out of four strain, strain PL-3 showed maximum diametric growth of mycelium (9.0 cm) with average growth rate of 1.13 cm per day followed by PL-1 and PL-4 which were at par to each other but significantly higher than the mycelial growth recorded in PL-5 strain at 30 °C temperature. However at 25 °C the fastest mycelial growth was recorded as 8.33 cm from the strain PL-1 in 8 days followed by PL-3 and PL-4, whereas minimum of 3.23cm in PL-5 which is significantly lower than other strains. The maximum mycelial growth of strain PL-5 (3.73 cm) recorded at 20 °C followed by 25°C temperature (Table 1).

**Table 1.** Effect of different temperatures on mycelial growth of different strains of oyster mushroom

| SI. | Strain | Days | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2th | | | | 4th | | | | 6th | | | | 8th | | | |
| | | 15°C | 20°C | 25°C | 30°C | 15°C | 20°C | 25°C | 30°C | 15°C | 20°C | 25°C | 30°C | 15°C | 20°C | 25°C | 30°C |
| 1 | PL-1 | 0.57 | 0.80 | 1.23 | 1.53 | 1.80 | 1.87 | 3.37 | 3.23 | 1.97 | 3.93 | 5.97 | 6.93 | 4.13 (0.52) | 4.97 (0.62) | 8.33 (1.04) | 8.97 (1.12) |
| 2 | PL-3 | 1.63 | 1.90 | 1.23 | 1.73 | 2.17 | 1.90 | 2.77 | 3.17 | 2.50 | 3.50 | 5.17 | 7.30 | 3.40 (0.43) | 4.57 (0.57) | 7.50 (0.94) | 9.00 (1.13) |
| 3 | PL-4 | 1.77 | 2.03 | 1.57 | 1.83 | 2.33 | 2.03 | 3.50 | 4.03 | 2.70 | 4.27 | 5.53 | 6.63 | 4.33 (0.54) | 5.17 (0.65) | 7.17 (0.90) | 8.67 (1.08) |
| 4 | PL-5 | 0.60 | 1.00 | 0.63 | 0.63 | 0.87 | 1.00 | 1.00 | 0.93 | 0.93 | 2.63 | 2.20 | 1.83 | 2.53 (0.32) | 3.73 (0.47) | 3.23 (0.40) | 2.80 (0.35) |
| CD at 5% | | a (Temp,) 0.13<br>b (Strain) 0.13<br>a* b 0.25 | | | | a(Temp.) 0.23<br>b (Strain) 0.23<br>a* b 0.45 | | | | a (Temp.) 0.49<br>b (Strain) 0.49<br>a* b 0.99 | | | | a (Temp.) 0.53<br>b (Strain) 0.53<br>a* b 1.054 | | | |

**Note**: figures in parenthesis are growth rate per day (cm)

These findings are in accordance with the results reported by earlier worker that the temperature ranges of 25-30°C is the best for the mycelial growth of *P. sajor caju* [4]. Of the six temperature conditions tested, i.e. 15, 20, 25, 30, 35 and 40°C, all hybrids and parental species exhibited maximum growth at 25°C, slowest at 15°C and almost no growth at 35°C and above. From the data (Table 2) it is clear that the strain PL-3 showed maximum diametric mycelial growth (8.57 cm) at pH 6.0 with average growth rate of 1.07 cm followed by pH 7.0. The strains PL-1, PL-3 and PL-4 showed approximately similar diametric growth ranges from 8.03-8.40 cm with average growth rate of 1.0 -1.05 cm per day at pH 7.0 i.e. most suitable pH level for all the strains. It was observed that the strain PL-1 grows faster at all pH level tested and showed diametric growth ranges from 8.27-8.40 cm with average growth rate of 1.03-1.05 cm per day. The strain PL-5 showed slow diametric growth at all the pH level. The strain PL-1 and PL-5 favored higher pH level 8.0 however, low pH level of 5.0 favor by strain PL-1. These result are in agreement with the findings of earlier workers who reported the best growth of *Pleurotus djamor, P. ostreatus, P. sajor-caju, P. cystidiosus* and *V. volvacea* on MEA medium [6, 8,9].

**Table 2.** Effect of different pH on the diametric growth of different strains of oyster mushroom

| SI. | Strain | Days | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2th | | | | 4th | | | | 6th | | | | 8th | | | |
| | | pH5 | pH6 | pH7 | pH8 | pH5 | pH6 | pH7 | pH8 | pH5 | pH6 | pH7 | pH8 | pH5 | pH6 | pH7 | pH8 |
| 1 | PL-1 | 1.97 | 1.87 | 1.60 | 1.57 | 3.13 | 3.20 | 3.20 | 2.73 | 6.53 | 6.13 | 5.90 | 5.37 | 8.33 (1.04) | 8.27 (1.03) | 8.27 (1.03) | 8.40 (1.05) |
| 2 | PL-3 | 1.83 | 1.90 | 1.70 | 1.40 | 3.47 | 3.13 | 2.93 | 2.30 | 5.80 | 6.30 | 6.30 | 5.13 | 7.73 (0.97) | 8.57 (1.07) | 8.40 (1.05) | 7.00 (0.88) |
| 3 | PL-4 | 1.63 | 1.83 | 1.87 | 1.47 | 2.63 | 2.97 | 3.10 | 2.60 | 5.40 | 5.57 | 6.03 | 4.10 | 7.57 (0.95) | 7.37 (0.92) | 8.03 (1.00) | 5.93 (0.74) |
| 4 | PL-5 | 0.53 | 0.57 | 0.57 | 0.37 | 0.83 | 0.90 | 0.90 | 0.67 | 1.60 | 1.40 | 1.97 | 1.67 | 2.23 (0.28) | 2.03 (0.25) | 2.80 (0.35) | 2.83 (0.35) |
| CD at 5% | | a (pH)<br>b (Strain)<br>a * b | 0.14<br>0.14<br>0.28 | | | a (pH)<br>b (Strain)<br>a * b | 0.15<br>0.15<br>0.29 | | | a (pH)<br>b (Strain)<br>a * b | 0.37<br>0.37<br>0.73 | | | a (pH)<br>b (Strain)<br>a * b | 0.59<br>0.59<br>1.17 | | |

**Note:** figures in parenthesis are growth rate per day (cm).

## Morphological characterization

Stipe length is the most important character of oyster mushroom, as shorter the stipe length, better the quality. The stipe length varied significantly from strain to strain. The average stipe length recorded as short as 0.97 cm in strain PL-5 and as long as 5.97 cm in PL-3 strain. The maximum width was recorded in the strain PL-4 (1.77 cm) and minimum 0.47 cm in PL-5 strain. However, the pileus diameter was maximum (10.97 cm) in strain PL-1 and minimum (5.25 cm) in strain PL-3. The margin of the pileus of strains PL-1, PL-4, and PL-5 were wavy except PL-3 which produced the irregular margin.

The hybrids of *P. djamor* were different with each other in pileus morphology. The pileus length and width ranges from 5.2-8.4 cm and 6.0-12.1 cm, respectively. It was reported that the stipe length and width varied among the recombinant strains of *P. djamor* [7]. In terms of number of fruiting bodies produced, PL-5 produced significantly higher number of fruiting bodies than other strain on all the substrate tested. The minimum number of fruiting bodies was recorded in PL-3 on Ws and Ws+Pls. In terms of yield (fresh weight), all the strains produced higher yield on Ws+Wp except PL-5 that produces maximum yield on WS+Pls. On wheat straw, the strain PL-5 gave higher yield (193.12 g/2kg wet substrate) with biological efficiency of 32.19 per cent followed by the strain PL-1 (141.33 g/ 2 kg wet substrate with biological efficiency 23.56 per cent) and PL-3 (120.49 g/2 kg wet substrate with biological efficiency 20.08 per cent) and minimum yield was recorded from PL-4 (119.9 g/2kg wet substrate) with biological efficiency 19.98 per cent. However, the significantly higher yield (323.64 g/2 kg wet substrate) with biological efficiency 53.94 per cent was recorded from Ws+Pls in strain PL-5. Strain PL-3 gave poor yield on Ws+Pls substrate (Table 5). In terms of average weight per fruiting body the strain PL-1 average

**Table 3.** Effect of different media on diametric mycelial growth (in cm) of different strains of oyster mushroom

| Sl. | Strain | Days | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2th | | | | | | 4th | | | | | | 6th | | | | | | 8th | | | | | |
| | | OMA | PDA | MEA | WA | WEA | CDA | OMA | PDA | MEA | WA | WEA | CDA | OMA | PDA | MEA | WA | WEA | CDA | OMA | PDA | MEA | WA | WEA | CDA |
| 1 | PL-1 | 1.87 | 1.27 | 1.10 | 0.57 | 1.13 | 0.77 | 4.07 | 3.97 | 3.20 | 1.47 | 1.90 | 1.30 | 6.83 | 6.23 | 5.13 | 2.10 | 2.77 | 1.53 | 8.47 (1.06) | 8.47 (1.06) | 7.20 (0.90) | 2.40 (0.30) | 3.57 (0.45) | 1.87 (0.23) |
| 2 | PL-3 | 1.77 | 1.33 | 1.20 | 0.50 | 1.20 | 1.50 | 4.17 | 3.77 | 3.33 | 1.53 | 2.70 | 3.30 | 7.27 | 6.00 | 5.73 | 2.77 | 3.70 | 4.50 | 9.00 (1.13) | 8.30 (1.04) | 7.80 (0.98) | 3.30 (0.41) | 5.03 (0.63) | 5.13 (0.64) |
| 3 | PL-4 | 1.60 | 1.27 | 1.20 | 0.93 | 1.70 | 1.40 | 3.97 | 3.60 | 3.20 | 1.63 | 3.20 | 2.73 | 6.13 | 5.67 | 5.27 | 2.47 | 4.13 | 3.67 | 7.90 (0.99) | 7.93 (0.99) | 7.37 (0.92) | 3.13 (0.39) | 5.37 (0.67) | 4.03 (0.50) |
| 4 | PL-5 | 1.80 | 0.57 | 0.37 | 0.50 | 0.77 | 1.27 | 3.53 | 2.00 | 1.63 | 1.50 | 2.53 | 2.23 | 4.17 | 2.57 | 2.60 | 2.37 | 4.00 | 3.20 | 4.53 (0.57) | 3.47 (0.43) | 3.70 (0.46) | 2.80 (0.35) | 4.83 (0.60) | 3.80 (0.48) |
| **CD at 5%** | | | | | | | | | | | | | | | | | | | | | | | | | |
| a (Media) 0 .14 | | | | | | | | a (Media) 0.24 | | | | | | a (Media) 0.32 | | | | | | a (Media) 0.37 | | | | | |
| b (Strain) 0.12 | | | | | | | | b (Strain) 0.19 | | | | | | b (Strain) 0.26 | | | | | | b (Strain) 0.30 | | | | | |
| a*b 0.28 | | | | | | | | a*b 0.48 | | | | | | a*b 0.63 | | | | | | a*b 0.74 | | | | | |

**Note:** figures in parenthesis are growth rate per day (cm).

**Table 4.** Morphological characters of different strains of oyster mushroom on wheat straw + waste paper substrate

| SI.No. | Strains | Stipe length (cm) | Stipe width(cm) | Pileus diameter(cm) | Margin of fruit body | Colour of fruit body |
|---|---|---|---|---|---|---|
| 1. | PL-1 | 5.53 | 1.00 | 10.97 | wavy | Dark creamy white |
| 2. | PL-3 | 5.97 | 0.98 | 5.25 | irregular | creamy white |
| 3. | PL-4 | 4.23 | 1.77 | 7.37 | wavy | creamy white |
| 4. | PL-5 | 0.97 | 0.47 | 5.93 | wavy | Dark creamy white |
| CD at 5% | 0.11 | 0.13 | 0.14 | | | |

**Table 5.** Yield performance of different strains of oyster mushroom on available agrowaste.

| Strain | Wheat straw | | | Wheat straw + Waste Paper | | | Wheat straw + Palm leaves | | |
|---|---|---|---|---|---|---|---|---|---|
| | Av. No. of fruit body | Av. Yield (g/2kg wet subs.) | BE (%) | Av. No. of fruit body | Av. Yield (g/2kg | BE (%) | Av. No. of fruit body | Av. Yield (g/2kg wet subs.) | BE (%) |
| PL-1 | 32.74 | 141.33 | 23.56 | 29.56 | 311.82 | 51.97 | - | - | - |
| PL-3 | 14.3 | 120.49 | 20.08 | 49.23 | 249.93 | 41.66 | 14.97 | 81.59 | 13.60 |
| PL-4 | 15.5 | 119.9 | 19.98 | 52.26 | 395.42 | 65.90 | - | - | - |
| PL-5 | 137.16 | 193.12 | 32.19 | 98.02 | 193.18 | 32.20 | 271.05 | 323.64 | 53.94 |
| CD at 5% | 26.87 | 45.59 | — | 35.35 | 141.32 | — | 58.93 | 122.29 | — |

weight of 10.55 g per fruiting body on Ws +Wp and minimum on Ws 4.32g per fruiting body. In case of strain PL-3, the highest average weight 8.43g per fruiting body was recorded from Ws followed by on Ws + Pls 5.45 g per fruiting body and minimum on Ws 5.08 g per fruiting body. In case of PL-4 maximum average weight 7.74 g per fruiting body was recorded from wheat straw followed by on Ws+Wp 7.57 g per fruiting body. The PL-5 strain, proved poor as it produced fruiting body with minimum average weight ranged between 1.19-1.97 g per fruiting body on the tested substrate.

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# OPTIMIZING MUSHROOM SPAWN PRODUCTION IN UGANDA

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## ABSTRACT

Most farmers in Uganda own less than an acre of land, which is typically used to grow crops such as bananas, pineapples and cassava. Mushroom cultivation requires less space, takes place near the home and has the potential to improve the lives of thousands of small land holders, especially women. However, for various reasons, farmer groups in Uganda are presently unable to meet the demand for high quality, fresh and dried mushrooms. This project, initiated in March 2014 and funded through the AgriTT (Agricultural Technology Transfer) programme, is directed at addressing current deficiencies in, and impediments to, the development of the Ugandan mushroom industry. The project introduced several key innovations that have made China the world's pre-eminent mushroom producer. These include: the provision of uninterrupted supplies of robust, high quality, genetically-stable spawn (through the Mushroom Training and Resource Centre [MTRC] and registered spawn producers) capable of generating high yields of quality mushrooms and using a wide range of cheap, readily-available cultivation substrates under the diverse climatic conditions prevailing in different regions of Uganda. It also includes establishing a germplasm bank to protect and conserve indigenous mushroom resources and a comprehensive breeding programme for enhanced strain performance. In addition, it incorporates research, educational and training elements involving MTRC, the Uganda Industrial Research Institute, Makerere University and Guizhou Academy of Agricultural Sciences, and a Field Technical Service to serve as a conduit to facilitate feedback from the growers based on their practical experiences as well as technology transfer between farmers and researchers. The programme will ensure a sustained supply of 'home-grown' mushrooms and well-trained mushroom biologists, and expedite engagement with the Ugandan government aimed at establishing a National Mushroom Policy and Strategy.

## INTRODUCTION

Present activities of mushroom farmers active in the region of Kabale, Southwest Uganda, are directed at *Pleurotus ostreatus* cultivation, but yields are often poor due to problems associated with mushroom spawn production and quality including:

- Contamination of spawn during the production process.
- Poor quality spawn produced by local spawn producers.
- Limited spawn production levels that cannot meet demand.
- High cost of spawn.
- Losses due to inadequate spawn storage and distribution capacity.
- Unreliable power supplies.

## MATERIALS AND METHODS

In order to address the key problems facing Ugandan mushroom farmers, the following activities will be undertaken:

**Selection of newer, robust, genetically stable strains:** These strains possess organoleptic qualities acceptable to the targeted consumer, are suitable for cultivation on substrates that are locally available in plentiful supply, and for which the environmental conditions for growth and fruiting are readily achievable at low cost. In the short term, it is proposed that imported species of known provenance and cultivation characteristics (e.g. optimal environmental requirements, yields,

biological efficiency values on different substrates) should be adopted although the availability of indigenous species should be explored in the medium term. Attention will also be given to providing facilities for storing 'mother' cultures of selected strains under conditions that maintain viability and genetic stability.

**Development and optimization of systems for producing good quality, stable spawn:** Following selection of suitable fruiting cultures, a range of spawn substrates will be evaluated since various associated features (e.g. composition, particle size, texture) influences the rate of mycelial growth within the spawn medium as well as during spawn running following inoculation. It may also influence the storage qualities of the spawn and help it to withstand degeneration associated with handling and environmental fluctuations. Provision of facilities for producing, storing and transporting a continuous supply of good quality and reliable spawn will also be addressed.

**Development of differently formulated combinations and pre-treatments of readily available lignocellulosic materials to provide suitable cultivation substrates:** These will be designed to promote growth of the mushroom mycelium to the practical exclusion of other microorganisms: i.e. be rich in essential nutrients in forms that are readily available to the mushroom, devoid of spawn growth inhibitors, of appropriate moisture content and pH value, and provide for good gaseous exchange between the substrate and the surrounding environment.

**Optimisation and management of mushroom development conditions:** Details of current practices relating to cultivation methods (e.g. substrate composition and preparation, spawn inoculum size, size of cultivation bags, temperature control during spawn running, management of mushroom development) adopted by individual farmers will be analyzed and appropriate improvement strategies introduced.

**Introduction of a system of registered spawn producers:** Poor spawn quality is often traceable to untrained and unqualified spawn producers using sub-standard materials and unsophisticated methodology. In order to eliminate inferior sources of supply, it is proposed to introduce a system of registered spawn producers. Higher costs that may ensue will be minimised by the introduction of credit/partial payment schemes which, in the latter case, will also act as an incentive for the spawn producers to maintain standards.

**Infrastructure improvements at MTRC:** These will involve upgrading laboratories and inoculation rooms, and the installation of a solar-power system to ensure more stable energy supplies.

**Preparation of a catalogue of mushroom strains indigenous to Uganda:** This will be comprehensive and include, for example, data relating to the location, altitude and nature (forest, grassland, etc) of the collection site, prevailing climatic

Mushroom growers and researchers attending the 1st dissemination event at MTRC, Kabale, 8th May, 2014

*Pleurotus ostreatus* cultivation at MTRC

conditions, and mushroom distribution patterns. Canvassing of growers and the local population in general about sightings of mushroom fruit bodies growing in the wild will be employed in this context.

**Collection and collation by MTRC of data relating to the impact of programme activities on grower profitability:** MTRC will develop an accurate and representative system for the continuous collection and collation of data from farmers and in-house trials relating to improvements in, for example, spawn quality and stability, fruit body quality and yields, overall profitability emanating from the action strategies outlined above.

**Introduction of a Field Technical Service (FTS):** Coupled to research, educational and training elements involving MTRC, the Uganda Industrial Research Institute, Makerere University and Guizhou Academy of Agricultural Sciences, the FTS will serve as a conduit to facilitate feedback from the growers based on their practical experiences as well as technology transfer between farmers and researchers.

## RESULTS AND DISCUSSION

Expected outcomes of the proposed action strategies will ensure the future sustainability of Uganda's mushroom industry by:

i) Increasing the capacity of MTRC and other certified suppliers to produce high quality spawn at reasonable cost, thereby eliminating current shortages.

ii) Reducing losses incurred during the mushroom cultivation process, thereby raising the profitability of growers and MTRC.

iii) Aiding diversification into the production of other mushroom species which command higher market prices, and ensuring a sustained supply of 'home-grown' mushrooms.

iv) Improving the training capacity of MTRC.

v) Raising the standard of MTRC and its products within the region, thereby enabling the Centre to lead the drive for new innovations in research and development.

vi) Facilitating engagement with the Ugandan Government aimed at establishing a National Mushroom Policy and Strategy.

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# QUALITY IMPROVEMENT OF CASING MATERIAL AND YIELD IN MILKY MUSHROOM (*CALOCYBE INDICA*) BY USING BIOFERTILIZERS AND DIFFERENT SUBSTRATES

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## ABSTRACT

The study was undertaken to assess the effect of nitrogen fixing and phosphate solubilizing biofertilizers and different substrates for improvement of casing quality and fruit body yield in milky mushroom (*Calocybe indica*). The experiment was conducted in completely randomized design with eight treatments of biofertilizers viz., *Azotobacter* and Phosphate solubilizing bacteria (PSB) i.e. (*Bacillus megaterium* + *Pseudomonas striata*) and their combinations. The quality parameters of casing material for milky mushroom viz., mycelial growth, microbial count, C:N ratio and bulk density were analyzed in the laboratory and are interpreted.

The results revealed that inoculation of bacterial inoculants either in alone or in different combinations resulted in an increase in mycelial growth of *C. indica* compared to uninoculated control under *in vitro* conditions. Highest fresh (20.52 g) as well as dry weight (0.65g) of mycelia was obtained in consortium of *Azotobacter* + PSB i.e. (*B. megaterium* + *P. striata*), followed by treatment of PSB resulting in 20.08 g and 0.62 g fresh weight and dry weight of mycelia, respectively. The total nitrogen content of casing material increased significantly from 0.11 to 0.55% due to biofertilizers treatment of soil + FYM + coir pith based casing. However, organic carbon decreased from 1.46 to 0.55 per cent in same treatment. Further, the C:N ratio also decreased from 13.0 to 7.9 due to *Azotobacter* inoculation in casing soil upto crop harvesting. The microbial count of *Azotobacter* and PSB was also higher in the same treatment and it was recorded 21 x $10^5$ and 23 x $10^5$, respectively at casing, thereafter it decreased upto harvest of mushrooms with greater magnitude. The fruit body yield of milky mushroom increased from 12.89% to 79.81% due to inoculation of biofertilizers.

In another experiment, the wheat and soybean straws and their mixture were evaluated for optimization of fruit body yield of milky mushroom. The data revealed that the stipe length, pileus diameter and average fruit weight highest maximum in mixture of wheat and soybean straw (1:1). The highest fruit body yield/kg dry substrate was mixture of two straws followed by soybean and wheat straw alone and it ranged from 382 to 714 g/kg substrate and 38.2 to 71.4% respectively. The results revealed that $N_2$ fixing and phosphate solubilizing bacteria could increase the quality of casing material. Further, the mixture of wheat and soybean straw (1:1) as a substrate performed better than individual, reflected in higher fruit body yields of milky mushroom.

**Keywords:** milky mushroom, biofertilizers, substrate straw, yield

## INTRODUCTION

A lot of bio-waste is being generated in rural areas and this can be utilized directly through mushroom cultivation. The cultivation of mushroom is unique in the sense that it is the most efficient and economically viable biotechnological process for the conversion of lignocellulosic waste material into high quality protein rich palatable food. Nowadays, there is a need to promote the cultivation of mushroom to meet the challenges of increasing world population as well as energy crisis. Production of food in a cheaper and simpler way carries more significance in developing countries like India. Considering the growth requirements, milky mushroom fits very well to this situation.

*Calocybe indica*, a popular mushroom was first time reported from India in 1976 [1]. It can be easily gown in the temperature range of 25 to 35 $^oC$ with 75 to 85% relative humidity. It has a good biological efficiency (60 to 70%) under optimal growth conditions. Its sporophores have long shelf life. The major advantage with this mushroom is that, it can be

best fitted in the relay cropping when no other mushroom can be grown mainly due to its higher temperature optima. It has quite good scope and can substitute other tropical mushrooms like *Pleurotus* and *Volvariella* [2]. *P. sajor-caju* and *C. indica* contains higher protein, dry matter, fat, carbohydrate and ash than *P. sajor-caju* [3]. This results in higher energy value for *Calocybe indica.*

Therefore, the present investigation "Quality improvement of casing material and yield in milky mushroom (*calocybe indica*) by using biofertilizers and different substrates" was planned with the objectives to assess the effect of biofertilizers on casing material quality and different substrate on fruit body yield of milky mushroom.

## MATERIALS AND METHODS

The study was carried out at All India Coordinated Research Project on Mushroom, College of Agriculture, Pune-5. Wheat straw was used as substrate for mushroom cultivation. It was chopped into 4-5 cm pieces. The chopped straw was filled in gunny bags and soaked in fresh water for overnight. Then the excess water was allowed to drain off. This substrate was then pasteurized by steam at 80 °C for 1 h and allowed to cool. After cooling, the substrate was used for filling the polythene bags. Before filling, the polythene bags were disinfected with 5% formalin. The 20 days old spawn was used for spawning and it was done with 4% grain spawn in uniform layers [2, 4]. After inoculation the polythene bags were tied, labeled and perforated at 20 to 25 points with the help of sterile pin for aeration. The above procedure was carried out aseptically in sterile room. First of all, two years old sieved FYM, sandy loam soil about neutral pH (7.3-7.8) and coir pith were drenched with 5% formalin separately and kept in airtight condition for 24 hrs. Before application of casing material on spawn run bed, complete removal of residual formalin was ensured by smelling. Various casing mixtures were prepared by uniform mixing of sterilized sandy loam soil, FYM, coir pith and carrier based biofertilizers as per the treatments.

$T_1$ Soil + FYM (1:1), $T_2$ Soil + FYM + Coir pith (1:1:1), $T_3$ Soil + FYM + *Azotobacter* (1:1 + 1% *Azotobacter*), $T_4$ Soil + FYM + PSB (1:1 + 1% PSB), $T_5$ Soil + FYM + Coir pith + *Azotobacter* (1:1:1 + 1% *Azotobacter*), $T_6$ Soil + FYM + Coir pith + PSB (1:1:1 + 1% PSB), $T_7$ Soil + FYM + *Azotobacter* + PSB (1:1 + 1% *Azotobacter* + 1% PSB) and $T_8$ Soil + FYM + Coir pith + *Azotobacter* + PSB (1:1:1 + 1% *Azotobacter* + 1% PSB).

A uniform layer of casing material was applied over the surface of spawn run bed. The thickness of casing was kept 3 cm [2]. After casing, the beds were left for case run at ambient temperature. The casing layer was kept moist by spraying water regularly. Application of 0.01% Bavistin and 0.02% Nuvan was undertaken immediately after casing to avoid any incidence of pests and diseases during case run. The samples of casing materials were drawn from all treatments at the time of casing and at last harvest of milky mushroom. These samples were analyzed in the laboratory by adopting standard analytical procedures for organic carbon, total nitrogen, bulk density and microbial population in casing material.

Another experiment was laid out to assess the effect of different substrates on growth and fruit body yield of milky mushroom. The wheat, soybean and their mixture were used as substrate for cultivation of milky mushroom. The experiment comprised three treatments, each with seven replications in completely randomized design. Data was recorded for days required for spawn run, case run and fruiting, stipe length, pileus diameter, fruit body weight and total yield.

## RESULTS AND DISCUSSION

The effect of different bacterial cultures alone and in combinations, on growth of *C. indica* was assessed in liquid culture under *in-vitro* conditions by measuring mycelial growth. Highest fresh weight (20.52 g) as well as dry weight (0.65 g) of mycelia was obtained in treatment of *Azotobacter* + PSB (*B. megaterium* + *P. striata*) followed by PSB alone resulting in 20.08 g and 0.62 g fresh and dry weight of mycelia respectively. Among the individual bacterial inoculant treatments, PSB resulted in production of highest fresh weight (17.71 g) as well as dry weight (0.56 g) of mycelia growth. The least fresh weight (8.63 g) as well as dry weight (0.19 g) of mycelium was recorded in uninoculated treatment (Table 1).

The data clearly indicated that inoculation of bacterial inoculants either alone or in different combinations resulted in an increase in mycelia growth of *C. indica* as compared to uninoculated control.

While studying the effect of microbial inoculants on mycelial growth of white button mushroom under *in-vitro* conditions, similar trend of results have been reported [5]. Significantly higher fresh weight (23.83g) and dry weight (0.71g) of mycelia of *P. sajor-caju* due to the treatment of mixed bacterial culture viz., *Azotobacter chroococcum, B. megaterium* and *P. striata* in malt extract medium were obtained in *in vitro* condition [6].

**Table 1.** Effect of enrichment of casing material with *Azotobacter* and PSB on mycelial weight and bulk density of casing material.

| Treatments | Mycelial weight on PDA broth | | Bulk density (g/cm$^3$) | | Percent reduction in bulk density |
|---|---|---|---|---|---|
| | **Fresh (g)** | **Dry (g)** | **At casing** | **At harvest** | |
| Soil + FYM | 8.63 | 0.19 | 1.42 | 1.41 | 1.16 |
| Soil + FYM + Coir pith | 13.15 | 0.26 | 1.42 | 1.40 | 1.66 |
| Soil + FYM + *Azotobacter* | 14.54 | 0.31 | 1.40 | 1.36 | 4.12 |
| Soil + FYM + PSB | 17.71 | 0.56 | 1.41 | 1.37 | 3.96 |
| Soil + FYM + Coir pith + *Azotobacter* | 15.18 | 0.48 | 1.36 | 1.30 | 5.85 |
| Soil + FYM + Coir pith + PSB | 16.55 | 0.51 | 1.37 | 1.32 | 5.00 |
| Soil + FYM + *Azotobacter* + PSB | 20.08 | 0.62 | 1.39 | 1.33 | 6.24 |
| Soil + FYM + Coir pith + *Azotobacter* + PSB | 20.52 | 0.65 | 1.34 | 1.24 | 9.98 |
| **S.E. ±** | - | - | 0.003 | 0.001 | - |
| **C.D. at 5%** | - | - | 0.010 | 0.003 | - |

The bulk density of casing materials was determined at the time of casing and at the harvest of mushroom crop. The data revealed that the bulk density of casing materials decreased significantly in the treatments of biofertilizers compared to uninoculated treatments. The treatments of Soil + FYM and Soil + FYM + Coir pith showed significantly lower magnitude of decrease i.e. by 0.01g/cm$^3$ and 0.02 g/cm$^3$. The treatment of Soil + FYM + Coir pith + *Azotobacter* + PSB showed significantly highest magnitude of decline (by 0.10g/cm$^3$) among all the treatments (Table 1). Per cent reduction in bulk density (Table 1) was higher in casing mixtures inoculated with biofertilizers as compared to uninoculated treatments. The treatment of Soil + FYM + Coir pith + *Azotobacter* + PSB showed highest reduction (9.98%) in bulk density among all the treatments followed by treatment of Soil + FYM + *Azotobacter* + PSB (6.24%).

The effect of enrichment of casing material with *Azotobacter* and PSB on C:N ratio, organic carbon and total nitrogen contents of casing materials were determined at the time of casing and after harvest of mushroom crop. The data revealed that the total nitrogen content of casing materials increased significantly with greater magnitude in treatments inoculated with biofertilizers as compared to uninoculated treatments (Table 2). However, the treatment of Soil + FYM + Coir pith + *Azotobacter* + PSB showed significantly higher total nitrogen (0.55%) contents as compared to rest of treatments. The organic carbon content decreased from casing stage to harvesting stage and highest decrease was in soil + coir pith + FYM with all biofertilizers (1.46 to 0.55%). The C:N ratio of casing materials narrowed at harvesting stage contributed by an increase in total nitrogen content in increased and decreased organic carbon of casing materials at crop harvest stage (Table 2).

C:N ratio of casing mixture declined with greater magnitude in treatments inoculated with biofertilizers as compared to uninoculated treatments. The decline in C:N ratio was highest (13.0 :1 to 7.9 :1) in the treatment of Soil + FYM + Coir pith + *Azotobacter*.

Initial *Azotobacter* and PSB count in the treatments of Soil + FYM and Soil + FYM + coir pith was zero since we used sterilized soil, FYM and coir pith during casing. The *Azotobacter* and PSB count for same treatments at casing and harvest were 1 x $10^5$/g and 0.5 x $10^5$/g and 1.5 x $10^5$/g and 1 x $10^5$/g of casing material respectively (Table 3). Highest count of *Azotobacter* and PSB was estimated in the treatment of Soil + FYM + Coir pith + *Azotobacter* + PSB followed by treatment Soil + FYM + *Azotobacter* + PSB i.e. 21 and 14 and 23 and 14.5 x $10^5$/g of casing material, respectively. Population of *Azotobacter* and PSB of casing materials declined at harvest of mushroom crop (Table 3). A decline in microbial population of *Azotobacter* and PSB at crop harvest of white button mushroom has also been reported by earlier workers [5].

**Table 2.** Effect of enrichment of casing material with *Azotobacter* and PSB on organic carbon, total N content and C:N ratio of casing material.

| **Treatments** | **Organic carbon content (%)** | | **Total nitrogen content (%)** | | **C : N ratio** | |
|---|---|---|---|---|---|---|
| | **At casing** | **At harvest** | **At casing** | **At harvest** | **At casing** | **At harvest** |
| Soil +FYM | 0.71 | 0.229 | 0.05 | 0.20 | 13.6:1 | 11.6:1 |
| Soil + FYM + Coir pith | 0.80 | 0.301 | 0.06 | 0.27 | 14.1:1 | 11.3:1 |
| Soil + FYM + *Azotobacter* | 1.18 | 0.389 | 0.09 | 0.48 | 13.2:1 | 8.0:1 |
| Soil + FYM + PSB | 0.98 | 0.419 | 0.07 | 0.39 | 13.5:1 | 10.9:1 |
| Soil + FYM + Coir pith + *Azotobacter* | 1.22 | 0.386 | 0.09 | 0.49 | 13.0:1 | 7.9:1 |
| Soil + FYM + Coir pith + PSB | 1.02 | 0.424 | 0.08 | 0.39 | 13.3:1 | 10.8:1 |
| Soil + FYM + *Azotobacter* + PSB | 1.26 | 0.484 | 0.10 | 0.47 | 12.9:1 | 10.4:1 |
| Soil + FYM + Coir pith + *Azotobacter* + PSB | 1.46 | 0.555 | 0.11 | 0.55 | 12.8:1 | 10.2:1 |
| **S.E. ±** | 0.002 | 0.001 | 0.004 | 0.001 | 0.35 | 0.025 |
| **C.D. at 5%** | 0.006 | 0.004 | 0.011 | 0.004 | 1.02 | 0.072 |

The yield data (Table 3) as affected by different biofertilizers revealed that significantly higher average fresh fruit weight (61.46 g) was recorded in the treatment of Soil + FYM + Coir pith in conjunction with biofertilizers followed by the treatment of Soil + FYM + biofertilizers (60.87g). More or less similar trend of the results were also observed in respect of total fresh yield and it ranged from 216.38 to 389.06 g/kg substrate. The results indicated that the yield of milky mushroom increased from 12.89 to 79.81% due to inoculation of biofertilizers and highest increase was observed in the treatment of soil + FYM + coir pith with all biofertilizers. Similar trend of results have also been reported by earlier workers in *Pleurotus* sp. [6, 7] and in button mushroom [8].

The effect of different substrates on growth and yield (Table 4) of milky mushroom revealed that lowest time (days) i.e. 16.42, 8.14 and 18.00 for spawn run, case run and fruiting respectively were recorded in the treatment of wheat and soybean straw (1:1) as compared to individual substrates. The total no. of fruits/bed/kg substrate, stipe length, pileus diameter of fruit and average fruit weight were highest in the mixture of wheat and soybean straws i.e. 17.28, 13.64 cm 11.32 cm and 41.85 g, respectively followed by soybean and wheat straw alone.

The yield and biological efficiency (Table 4) of milky mushroom was highest in the substrate of wheat and soybean straw mixture (1:1), followed by soybean straw and wheat straw alone and ranged from 382 to 714 g/kg substrate and 38.2 to 71.4% respectively. The enhanced growth and yield of milky mushroom on mixture of soybean and wheat straw has also been reported by earlier workers [9-10].

**Table 3.** Effect of enrichment of casing material with *Azotobacter* and PSB on microbial population (*cfu* x $10^5$/g) and yield of milky mushroom.

| Treatments | Microbial population (*cfu* x $10^5$/g) of casing material | | | | Average fresh fruit body weight (g) | Total fresh yield (g)/kg substrate | Percent increase of yield |
|---|---|---|---|---|---|---|---|
| | *Azotobacter* | | PSB | | | | |
| | At casing | At harvest | At casing | At harvest | | | |
| Soil +FYM | - | 1.0 | - | 0.5 | 46.33 | 216.38 | - |
| Soil + FYM + Coir pith | - | 1.5 | - | 1.0 | 48.85 | 244.27 | 12.89 |
| Soil + FYM + *Azotobacter* | 13.5 | 6.0 | - | - | 56.63 | 301.86 | 39.50 |
| Soil + FYM + PSB | - | - | 17.0 | 8.5 | 57.42 | 344.52 | 59.22 |
| Soil + FYM + Coir pith + *Azotobacter* | 17.0 | 10.0 | - | - | 56.76 | 321.81 | 48.73 |
| Soil + FYM + Coir pith + PSB | - | - | 19.5 | 12.0 | 57.75 | 365.54 | 68.93 |
| Soil + FYM + *Azotobacter* + PSB | 18.5 | 10.5 | 21.0 | 13.0 | 60.87 | 365.22 | 68.79 |
| Soil + FYM + Coir pith + *Azotobacter* + PSB | 21.0 | 14.0 | 23.0 | 14.5 | 61.46 | 389.06 | 79.81 |
| **S.E. ±** | - | - | - | - | 0.93 | 5.25 | - |
| **C.D. at 5%** | - | - | - | - | 2.73 | 15.38 | - |

**Table 4.** Effect of different substtrates on days required for spawn run, case run, fruiting, in *Calocybe indica*.

| Treatments | Days for | | | Mean number of Fruits/ bed/kg substrate | Stipe lingth (cm) | Pileus diameter (cm) | Av. Fruit weight (g) | Yieldg/ kg substrate | Biological Efficiency (B.E.) % |
|---|---|---|---|---|---|---|---|---|---|
| | Spawn run | Case run | Fruiting | | | | | | |
| Wheat straw | 20.00 | 10.00 | 21.0 | 11.85 | 8.35 | 6.3 | 32.42 | 382 | 38.2 |
| Soybean straw | 18.42 | 9.00 | 19.57 | 14.42 | 11.11 | 8.9 | 36.0 | 510 | 51.0 |
| Wheat straw + Soybean straw | 16.42 | 8.14 | 18.00 | 17.28 | 13.64 | 11.32 | 41.85 | 714 | 71.4 |
| **S.E. ±** | 0.125 | 0.14 | 0.07 | 0.20 | 0.096 | 0.078 | 0.28 | 5.47 | 0.57 |
| **C.D. at 5%** | 0.38 | 0.45 | 0.21 | 0.61 | 0.29 | 0.24 | 0.86 | 17.70 | 1.77 |

## CONCLUSION

The conclusions drawn out from present investigation are elaborated as follows:

- *In vitro* studies revealed that the highest fresh weight (20.52 g) and dry weight (0.65 g) of mycelial growth of *C. indica* was in liquid culture inoculated with *Azotobacter* + PSB.
- Percent of reduction in bulk density was higher in casing mixtures inoculated with biofertilizers as compared to uninoculated treatments.
- The C:N ratio of casing materials narrowed towards harvesting stage as total nitrogen content of casing materials increased and decreased organic carbon decreased with greater magnitude.
- Decline in the population of *Azotobacter* and PSB was noticed in casing materials at harvest of mushroom crop. However, the higher count of *Azotobacter* and PSB at casing and harvest of the crop was observed in the treatment of Soil + FYM + Coir pith + *Azotobacter* + PSB.
- The results in general indicated that the quality of casing material improved due to inoculation of $N_2$ fixing and phosphate solubilizing bacteria in casing materials of milky mushroom as reflected in higher yields.
- The mixture of wheat and soybean straw (1:1) as a substrate was found suitable for maximization of fruit body yield in milky mushroom followed by soybean straw and wheat straw alone.

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# YIELD PERFORMANCE AND ELEMENT PROFILING OF DIFFERENT STRAINS OF *LENTINULA EDODES* (BERK.) PEGLER

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## ABSTRACT

Among five strains OE-388 took minimum period of 62 and 58 days for spawn run and produce maximum number of sporophore i.e. 80.16 and 69.50 and yield i.e. 2468.33 and 2070.50 g/800 g wheat straw which is significantly higher than the yield obtain from other strains in two seasons, respectively. Whereas strain OE-28 showed poor result in terms of taking maximum days for spawn run i.e. 68 days and produce minimum number of sporophore i.e. 65.67 and 47.33 and poor yield 830 and 715.67 g/0.8 kg wet wheat straw as compair to other strains. Amongst the five strains OE-388 contains high value of magnesium (109.51 mg/100 g dry wt.), iron (3.83 mg/100 g dry wt.), zinc (7.71 mg/100 g dry wt.), manganese (4.59 mg/100 g dry wt.) and copper (1.5 mg/100 g dry wt.) followed by OE-28. However toxic elements were not detected in any strains.

**Keywords:** mushrooms, shiitake, *Lentinula edodes*, lentinan, element profiling

## INTRODUCTION

*Lentinula edodes* (Shiitake mushroom) is the third most important edible mushroom in the world from the stand point of production after *Agaricus bisporus* and *Pleurotus* spp. It accounts for 17% of world production in terms of weight [1, 2]. It is one of the most popular medicinal mushrooms. It has high content of proteins, fibers, vitamins, minerals and low content of lipid specifically cholesterol [3]. It has been reported to boost the immune system, lower cholesterol, function as an anticoagulant and may have use in treatment of some cancers as it contains lentinan, a polysaccharide with strong anti-cancer properties [4, 5]. Mushrooms depend on substrates for their nutrition and the substrate is normally a source of lignocellulose material which supports growth, development and fruiting body of mushroom [1]. Shiitake mushroom is traditionally cultivated on the shii tree [*Castanopsis cuspidate* (Thunb.) Schott] or wood logs in Japan. Scarcity of theshii tree has necessitated a search for alternative substrates for shiitake cultivation. Large amounts of freely available wheat straw and sawdust offer a potential alternative substrate for mushroom cultivation.

Minerals (element) represent the ash left behind after complete incineration of the dry mushroom.The nutritive composition of the mushroom varies with species, strain, type of substrate on which it is grown, the maturity of the fruiting body, the method of analysis and the environmental conditions in which it is grown [6]. Minerals are very important because it is a co-factor of several enzymatic systems, being the most abundant macro-element in mushrooms [2, 7, 8]. Minerals such as iron, zinc, copper and manganese are essential metals since they play an important role in biological systems, whereas lead, nickel, arsenic and cadmium are nonessential metals as they are toxic, even in traces [9]. However, sodium is relatively less in mushroom species, thus mushrooms are said to be good for patients at hypertension [10].

In this context it is worthwhile to evaluate the available strains for their yield and metal content of the mushroom and assess the contribution of shiitake mushroom to the daily. The present study is focused on the analysis of five strains of shiitake mushroom for their yield performance on wheat straw and mineral composition.

## MATERIALS AND METHODS

### Yield performance of strains

The cultures of five strains of *Lentinula edodes* (OE 16, 22, 38 & 388) were obtained from Mushroom Research and Training Centre, Pantnagar (original source: DMR, Solan) and maintained using PDA medium at 25 °C. The spawn was

prepared using wheat grains as base material. The substrate wheat straw supplemented with 10% wheat bran was used to evaluate in two consecutive year.

The substrate was soaked in water for 16 h then taken out and kept for 2-3 h to drain out the excess and allowed to dry in shade for 6 h wet substrate (2 kg) filled in polypropylene bag, sterilized at 22lbs psi for 90 minute and allowed to cool down at room temperature. The sterilized substrate (bags) was inoculated aseptically by wheat grain spawn @5% on wet weight basis of the substrate. The bags were incubated in cropping room at 23$\pm$1 $^{o}$C temperature and 80-85% RH for spawn run. After complete spawnrun bag were cut open to expose the upper surface for fruiting and relative humidity of the cropping room maintained at 85-90% by sprinkling water twice or thrice a day. The observation recorded for spawn run period, number and weight of sporophores and average weight per fruitbody. Fruit bodies were harvested after maturity and biological efficiency (BE%) was calculated. BE (%) = (Fresh weight of fruit bodies/dry weight of substrate) x 100.

**Element profiling**

The fruting bodies of these mushrooms have been analysed for their element contents. Mushrooms were harvested at early maturing fruiting stage. The fruiting bodies of mushrooms were oven dried at 60 $^{o}$C for 1 hr and grounded in the blender. Fresh dried mushroom was used for nutritional analysis.

Total ash (1g of dried mushroom was burned in muffle furnace at 600 $^{o}$C for 8 hrs) was taken for the analysis of mineral content 2 ml of conc. nitric acid was added to the ash and heated for 2 min. one drop of hydrogen peroxide was added into the solution to remove turbidity. The solution then transferred into a volumetric flask and total volume was made 50 ml by adding deionised water. This was then used to analyse the contents of Cu, Fe, Zn, Mg, Cr, Mn, Ni and As by flame and graphite method with atomic absorption spectroscopy.

**Statistical analysis:** All the analyses were performed in triplicates, and these results were reported as means ± standard deviation (SD).

## RESULTS AND DISCUSSION

The data revelaed that out of five strains tested OE-388 took minimum period of 60 days for spawn run and produce maximum number of sporophore i.e. 74.83 and yield ie. 2269.54 g/2.5 kg wheat straw which is significantly hgiher than the yield obtain from other strainin with mean 90.78% biological efficiency. Whereas strain OE-28 showed poor result in terms of taking maximum period for spawn run i.e. 68 days and produce minimum number of sporophore i.e. 56.5 and poor yield 772.84 g/2.5 kg wheat straw as compared to other strains with 30.92% mean biological efficiency (Table 1). Very low yield with 6% biological efficiencywas obtained in saw dust [11]. It was reported that the biological efficiency of shiitake mushroom on wheat straw ranged from 25-56 per cent [12]. It was found that the shiitake mushroom strain $L_1$ and $L_2$ yielded maximum on wheat straw with 30% and 24% biological efficiency, respectively [13].

The fruting bodies of these strain mushrooms have been analysed for their element contents i.e. One major element (Mg), four trace elements (Fe, Mn, Zn and Cu) and two toxic elements (As and Cr) by using Atomic absorption spectroscopy.It was found that the strains OE-388 contains high value of magnesium (109.51 mg/100g dry wt), iron (3.83 mg/100g dry wt), zinc (7.71 mg/100g dry wt), manganese (4.59 mg/100g dry wt.) and copper (1.5 mg/100g dry wt.) followed by strain OE-28. Minimum value of Mn (1.09 mg/100g dry wt), Cu (0.87 mg/100g dry wt) and Fe (1.27 mg/100g dry wt) was found in OE-16 whereas least value of Mg (91.49 mg/100g dry wt) and Zn (5.08 mg/100g dry wt) was obtained from OE-38. However toxic elements (As & Cr) were not detected in any strains.

The Mg and Zn content recorded in the strains of shiitake mushroom were similar with the earlier findings [14-16]. Mg contents in these strains are high which is beneficial for human health. Shiitake mushroomis also good source of micro- and macro elements, such as K, Mg, P, Zn, Fe, or Cu [17].

**Table 1.** Yield performance of different strain of Shiitake mushroom (*Lentinula edodes*) on wheat straw

| Strain | Days taken for spawn run | | | Avg. yield (g/2.5kg dry substrate) from 40 days harvesting | | | | | | Wet per fruit body (g) | | | Biological efficiency (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2011 | 2012 | Mean | Number | | | Weight | | | 2011 | 2012 | Mean | 2011 | 2012 | Mean |
| | | | | 2011 | 2012 | Mean | 2011 | 2012 | Mean | | | | | | |
| OE-16 | 67 | 63 | 65 | 69.17 | 60.66 | 64.915 | 1010 | 922.67 | 966.34 | 14.60 | 15.21 | 14.91 | 40.4 | 36.91 | 38.66 |
| OE-22 | 66 | 59 | 62.5 | 77.33 | 67.5 | 72.415 | 1380 | 1282.5 | 1331.25 | 17.85 | 19.00 | 18.43 | 55.2 | 51.3 | 53.25 |
| OE-28 | 68 | 68 | 68 | 65.67 | 47.33 | 56.5 | 830 | 715.67 | 772.84 | 12.64 | 15.12 | 13.88 | 33.2 | 28.63 | 30.92 |
| OE-38 | 66 | 61 | 63.5 | 70.17 | 63.33 | 66.75 | 1090 | 1016 | 1053 | 15.53 | 16.04 | 15.79 | 43.6 | 40.64 | 42.12 |
| OE-388 | 62 | 58 | 60 | 80.16 | 69.5 | 74.83 | 2468.33 | 2070.5 | 2269.45 | 30.79 | 29.79 | 30.29 | 98.73 | 82.82 | 90.78 |
| CD at 5% | - | - | - | 11.40 | 719.18 | - | 14.19 | 284.5 | - | - | - | - | - | - | - |

**Table 2.** Element concentration (mg/100g on dry weight basis) in strains of *Lentinulaedodes* (shitake mushroom)

| Elements | Strain | | | | |
|---|---|---|---|---|---|
| | **OE-16** | **OE-22** | **OE-28** | **OE-38** | **OE-388** |
| **Major Elements** | | | | | |
| Mg | 104.92±0.78 | 99.74±0.73 | 100.32±0.58 | 91.49±0.75 | 109.51±0.53 |
| **Trace Elements** | | | | | |
| Mn | 1.09±0.01 | 3.88±0.12 | 4.49±0.46 | 1.86±0.28 | 4.59±0.56 |
| Zn | 7.38±0.19 | 7.50±0.12 | 7.42±0.05 | 5.08±0.13 | 7.71±0.07 |
| Cu | 0.87±0.18 | 1.33±0.13 | 1.23±0.11 | 0.95±0.08 | 1.50±0.07 |
| Fe | 1.27±.09 | 3.12±0.15 | 3.62±0.24 | 2.98±0.28 | 3.83±0.25 |
| **Toxic metal** | | | | | |
| As | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Cr | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

Each value is the mean of three replicate determinations ± standard deviation

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# USE OF *TRICHODERMA* ENRICHED BUTTON MUSHROOM SPENT SUBSTRATE (TEBMSS) FOR ENHANCING YIELD AND QUALITY OF KINNOW MANDARIN

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## ABSTRACT

The left over compost after harvesting full or remunerative crop of button mushroom is generally called as spent mushroom compost (SMC). An investigation was carried out at Integrated Farming Systems unit of Project Directorate for Farming Systems Research, Modipuram, Meerut, India, during 2013-14 to evaluate the alternative use of SMC for mass multiplication of *Trichoderma harzianum* and its further evaluation in the form of *Trichoderma* enriched button mushroom spent substrate (TEBMSS) for enhancing growth and yield parameters of potential fruit crop Kinnow mandarin. One month old *Trichoderma* enriched button mushroom spent substrate (TEBMSS) was applied in root zone of Kinnow @ 25 kg/plant in the month of April (flowering/fruiting stage). Uninoculated plants were treated as control. The cfu of *T. harzianum,* which was in the range of $10^4$/g substrate at starting (0 day), reached 8.72 x $10^6$/g substrate at the end of 4th week. It indicates better growth and sporulation of *T. harzianum* in the SMC. In Kinnow, the average leaf area in treated plants was 24.40 $cm^2$ against 14.23 $cm^2$ in control. The SPAD and NDVI values were also significantly higher in treated plants. The number of fruits/plant (395), average fruit circumference (22.87 cm) and fruit weight (148.57 g) were significantly higher in treated plants as compared to control. There was over 3 fold higher fruit dropping in control plants during May-June and September-October when Kinnow fruits are generally vulnerable for dropping. The increased leaf area, general greenness and canopy cover; increased fruit numbers, circumference and weight and decreased fruit dropping in TEMBSS treated Kinnow plants clearly exhibited the alternative use of SMC for multiplication of the fungal bioagent and its potential use in increasing the growth and yield of fruit crops like Kinnow mandarin.

**Keywords:** SMC, *Trichoderma harzianum*, TEBMSS, Kinnow mandarin

## INTRODUCTION

The left over compost after harvesting full or remunerative crop of button mushroom is generally called as spent mushroom compost (SMC). It is also known as spent mushroom substrate (SMS). On an average, 5 kg of SMC is produced after harvesting 1kg of fresh button mushroom. SMC is good quality organic manure rich in major and minor nutrients required for plant growth. Several modes have been documented to recycle the SMC for various purposes i.e. use as organic manure in field and horticultural crops, reclamation of soil and bioremediation of contaminated soil and water [1,2,3]. *Trichoderma* is a mycoparasitic fungus endowed with several beneficial effects in crop production i.e. plant disease management and yield enhancement. It is also a major contaminant and competitor mould in mushroom cultivation. The present investigation was aimed to recycle the SMC of button mushroom for mass production of *Trichoderma* and its utilization in the cultivation of important fruit crop, Kinnow mandarin.

## MATERIALS AND METHODS

The experiment was conducted at Integrated Farming Systems unit of Project Directorate for Farming Systems Research, Modipuram, Meerut, India during 2013-14. The spent compost of button mushroom was obtained from mushroom unit and inoculated with a local strain of *Trichoderma harzianum*. The powdered formulation of *T. harzianum* was mixed in SMC to obtain an initial inoculum (0 day) of $10^4$ cfu per gram of the substrate. Inoculated substrate was watered frequently to maintain 60-65 percent moisture and its turning was done at weekly interval. Colony forming units (cfu) of *T. harzianum* were estimated in the laboratory following serial dilution technique. One month old *Trichoderma* enriched button mushroom spent substrate (TEBMSS) was applied in root zone of Kinnow @ 25 kg/plant in the month of April (flowering/fruiting

stage). Uninoculated plants in normal intercropping system were treated as control. Fourteen replications were maintained for each treatment and observations were taken on the growth parameters and fruit yield of Kinnow mandarin.

## RESULTS AND DISCUSSION

Spent mushroom compost (SMC) favoured good growth of *T. harzianum* as evident in Fig.1. The cfu of *T. harzianum,* which was in the range of $10^4$/g substrate at starting (0 day), reached 8.72 x $10^6$/g substrate at the end of 4th week. It indicates the better growth and sporulation of *T. harzianum* in the SMC. The compost or substrate of many mushroom species has been reported to favour good growth of the competitive fungus *Trichoderma* spp. [4].

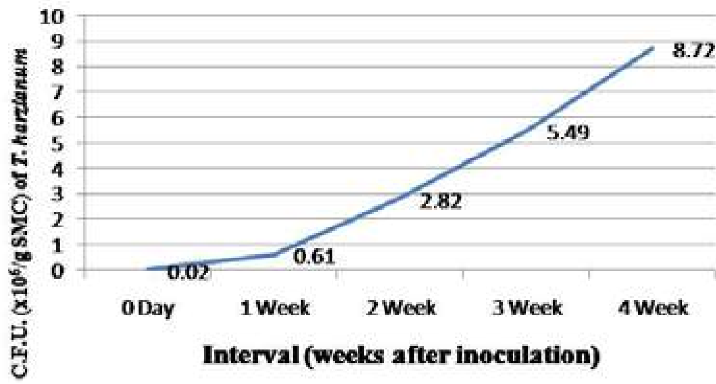


**Figure 1.** Growth of *T. harzianum* in SMC

There were encouraging results of TEBMSS application on the leaf growth and general canopy health in Kinnow mandarin (Table 1). Average leaf area in treated plants was 24.40 $cm^2$ against 14.23 $cm^2$ in control. The SPAD and NDVI values were also significantly higher in treated plants indicating increased greenness of leaves and better canopy coverage and this ultimately results in better accumulation of photosynthates and fruit yield. Use of nutrient rich spent mushroom substrate for enhancing yield and quality of some vegetables and fruits were also documented by Ahlawat [1] and Oei *et al.* [3]. In India, Punjab is number one in the production of spent mushroom substrate and Kinnow fruit as well and this opens the avenues for utilization of TEBMSS in Kinnow orchards for better yield. *T. harzianum* also possesses antifungal and anti-nematode properties which could also be beneficial for Citrus crops like Kinnow mandarin in long run.

**Table 1.** Effect of *Trichoderma* enriched button mushroom spent substrate (TEBMSS) on leaf growth parameters of Kinnow

| Treatment | Avg. Leaf area ($cm^2$) | Avg. SPAD value | Avg. NDVI value |
|---|---|---|---|
| 1. TEBMSS | 24.40 | 59.35 | 0.92 |
| 2.Control | 14.23 | 44.12 | 0.81 |
| CD ($p$=0.05) | 1.59 | 1.79 | 0.019 |

**Table 2.** Effect of *Trichoderma* enriched button mushroom spent substrate (TEBMSS) on fruit parameters of Kinnow mandarin

| Treatment | No. of fruits/ plant at 5 month of age | Avg. fruit drop/ plant in May-June | Avg. fruit drop/ plant till maturity | Avg. fruit circumference at 5 month of age (cm) | Avg. fruit wt. at 5 month of age (g) |
|---|---|---|---|---|---|
| 1. TEBMSS | 395 | 13.57 | 21.14 | 22.87 | 148.57 |
| 2.Control | 337 | 45.14 | 67.36 | 19.00 | 112.29 |
| CD ($p$=0.05) | 8.61 | 3.12 | 2.87 | 0.37 | 6.46 |

The number of fruits/plant (395), average fruit circumference (22.87 cm) and fruit weight (148.57 g) were significantly higher in treated plants as compared to control (Table 2). There was nearly 3 fold higher fruit dropping in control plants during May-June and September-October when Kinnow fruits are generally vulnerable for dropping. The positive effect of TEBMSS on fruit growth and negative effect on fruit dropping ultimately enhances the yield of Kinnow.

## CONCLUSION

Spent mushroom compost (SMC) supported a good growth of *T. harzianum* indicating its potential use for the mass production of this important bioagent. The increased leaf area, general greenness and canopy cover; increased fruit numbers, circumference and weight and decreased fruit dropping in TEBMSS treated Kinnow plants clearly exhibited the alternative use of SMC for multiplication of the fungal bioagent and its potential use in increasing the growth and yield of fruit crops like Kinnow mandarin.

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# NOTES