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The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

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Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998;

1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001) References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

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ARTICLES

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Full Length Research Paper

Effect of beta-mannanase treatment on nutritive quality of palm kernel meal

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The aim of this study was to evaluate the nutritive quality of β-mannanase treated palm kernel meal (PKM). Beta-mannanase production was conducted using locust bean gum (LBG) as the sole carbon source; moisten with mineral salt solution, and enzyme activity determined by dinitrosalicylic acid. Crude β-mannanase was concentrated by ammonium sulphate. The chemical composition of enzyme treated PKM was determined according to standard chemical methods. The mineral composition of the enzyme treated PKM was determined using atomic absorption spectrophotometer method. The result obtained shows an increase in crude protein from 16.02±0.40 in non-enzyme treated PKM (NTPKM) to 23.26±0.13 in enzyme treated PKM (ETPKM). There were significant reductions (P>0.05) in ash and crude fibre contents in the ETPKM as compared to the control. The ETPKM showed a markedly reduction in crude fibre by 64.02%. The lignin, cellulose and hemicelluloses content decreased in ETPKM by 17.32, 76.85 and 11.74%, respectively. The phytate, tannin and cyanide contents of ETPKM had a reduction of 29.26, 6.99 and 58.44%, respectively. The mineral analysis of ETPKM showed that calcium, copper and potassium reduced in PKM after enzyme treatment. The percentage reduction of calcium, copper and potassium in ETPKM were 33.65, 81.28 and 29.12%, respectively. However, there was significant increase in zinc and phosphorus contents of ETPKM in comparison with the control. The treatment of PKM with β-mannanase resulted in decrease of complex fibre fractions in the PKM to increase its crude protein and certain minerals (zinc and phosphorus) contents.

Key words: Nutritive quality, palm kernel meal, β-mannanase, chemical composition, mineral composition.

INTRODUCTION

The utilization of agro-industrial wastes as feedstuff is one of the strategies involved in the reduction of cost of livestock production. Agro-industrial by-products in Nigeria vary from primary processing of farm produce wastes to wastes from agro allied industries. Some of these wastes are left unutilized, often causing environmental pollution and hazard. Those that are utilized do

not have their full potentials harnessed. Agro-industrial wastes can be of tremendous use in the livestock industry for feeding animals; they include brewers dried grain, palm kernel cake, maize offal, wheat offal, rice bran and cassava peels just to mention few. Microbial bioconversion and associated enzymes, especially fungal bioconversion of wastes seems to be a practical and

promising alternative for increasing their nutritional value, transforming them into animal feed and thus producing a value-added product (Villas-Boas et al., 2003; Agosin et al., 2006), fungal bioconversion of agro-industrial by-products is an environmentally friendly biotechnological process (Karunanandaa et al., 1995; Zhang et al., 2002; Mukherjee and Nandi, 2004). From an animal nutrition point of view, agro-industrial wastes are not suitable feed ingredients as they are deficient in digestible protein (Song et al., 2009), rich in β-mannan and anti-nutrient compounds (Khanongnuch et al., 2006).

Nigeria is one of the major producers of palm (*Elaeis guineensis*) oil in Africa. An important by-product gene-rated from palm oil industry is palm kernel meal (PKM) or palm kernel expeller (PKE) depending on the method used for the extraction of oil from the kernel 'with the latter normally containing slightly higher oil content'. Palm kernel expeller contains a moderate level of crude protein (14.5 to 19.6%) but a high level of fiber (13 to 20%) and poor amino acid profile (deficient in lysine, methionine and tryptophan) and thus it is considered to be a moderate quality feed ingredient for ruminant but not suitable for monogastric animals (Alimon, 2004; Saenphoom et al., 2011). The fiber of PKE is mainly hemicellulose consisting of 58% mannans (Saenphoom et al., 2011), moderate amounts of cellulose and small amount of other polysaccharides (Swe et al., 2004). Most mannan, making up between 25 to 32% of PKE consist of water insoluble glucomannan and small amount of water soluble galactosemannan thus making it resemble very much cellulose by being crystalline, hard and water insoluble (Knudsen, 1997; Sundu et al., 2006). Because of their complex chemical structure, the fiber of PKM and PKE require a combination of enzymes including man-nanases, galactosidases, glucosidases and xylanases to release the potential fermentable sugars to be of use for monogastric animals. As the use of PKM and PKE for monogastric animals such as poultry, pigs and fish is limited due to the lack of the appropriate enzymes in these animals to hydrolyze the fiber, two most widely used methods to overcome this limitation are: (i) the use of fungi in solid state fermentation (SSF) to breakdown and reduce the hemicelluloses, cellulose and lignin in PKM (Noraini et al., 2001), and (ii) supplementation of exogenous enzymes into the diet containing PKM in poultry (Chong et al., 2003).

In recent years, a β-mannanase produced from bacteria and fungi has been shown to improve feed conversion and performance of broilers, fish, turkeys and swine (Jackson et al., 1999). The important role of β-mannanase is hydrolyzing β-1,4-glycosidic linkages in β-mannan (Ooi and Kikuchi, 1995) and this can reverse the negative impact caused by β-mannan. For instant, Khanongnuch et al. (2006) reported an increase in the metabolizable energy (ME) and nutrient digestibility improvement of copra meal treated by β-mannanase. In addition, broiler chicks fed with enzymatic treated PKM

by commercial enzymes increased in weight gain, feed conversion efficiency, dry matter digestibility and nutrient digestibility, while the jejuna content viscosity was decreased (Sandu et al., 2006).

Manno-oligosaccharides (MOS), one of the major end products of β-mannan hydrolysis by β-mannanase was found to be a substance which could prevent the colonization of *Escherichia coli* and Salmonellae, leading to an improvement of animal growth performance (Ishihara et al., 2000; Khanongnuch et al., 2006). The aim of this study was to evaluate the nutritive quality of β-mannanase treated PKM.

MATERIALS AND METHODS

Microorganisms

Penicillium italicum (Akinyele et al., 2013) previously confirmed to posses mannolytic property was obtained from the Research Laboratory, Microbiology Department, Federal University of Technology Akure (FUTA), Ondo State, Nigeria. The authenticity of the culture was confirmed by the method of Pitt and Hocking (1997) on the bases of cultural characters (colour, shape of colony, surface and reverse pigmentation and texture of the colony) as well as microscopic structure (septate or nonseptate hyphae, structure of hyphae and conidia). The fungal isolate was maintained on Malt Extract Agar (MEA) and sub-cultured at regular intervals. They were incubated at 30 ±2°C until the entire plates were covered by active mycelium and stored at 4°C in refrigerator on agar slants.

Sample sources

PKM was procured from a reputable feed mill in Akure, Ondo State, Nigeria. The samples were stored in air tight transparent plastic containers to keep it moisture free until use. Locust bean gum (LBG) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Mannanase production

For the production of β-mannanase in solid state fermentation, the isolate was cultured at 30°C in 250 ml Erlenmeyer flasks containing 10 g LBG. The substrate was suspended in 33 ml Mandels and Weber's medium modified by El-Naggar et al. (2006). This medium (moistening agent) contained the following ingredients (g/L): Peptone 2, yeast extract 2, $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5 and $FeSO_4.7H_2O$ traces. After sterilization at 121°C for 15 min, it was cooled and inoculated with 2 discs of 8 mm diameter of the organism from MEA culture plate using sterile cup borer. The flask was incubated at 30°C for 5 days at static condition.

Enzyme extraction

The solid state cultures were prepared by adding 10-fold (v/w) 0.1 M phosphate buffer (pH 6.8) and shaking (180 rpm) at 30°C for 60 min. The fungal biomass was separated by centrifugation (Centurion Scientific Limited) (6000 rpm, 15 min at 4°C).

Enzyme assays

β-Mannanase activity was assayed in the reaction mixture

Table 1. Proximate composition of mannanase treated PKM (% dry weight).

Sample	Moisture	Ash	Crude fibre	Crude protein	Fat
PKM [a]	$10.33^b \pm 0.19$	$3.63^c \pm 0.08$	$40.00^c \pm 0.05$	$16.02^a \pm 0.40$	$5.90^a \pm 0.24$
[b]	$10.09^b \pm 0.02$	$3.29^b \pm 0.03$	$26.17^b \pm 0.08$	$22.34^b \pm 0.44$	$5.77^a \pm 0.33$
[c]	$9.77^a \pm 0.14$	$2.68^a \pm 0.09$	$14.39^a \pm 0.39$	$23.26^c \pm 0.13$	$5.48^a \pm 0.13$

[a] Untreated, [b] during treatment [c] after treatment. Values are means± S.E (n=3). Means with the same superscript letters down the same column are not significantly different (P>0.05).

composing of 0.5 ml of 1% LBG prepared in 50 mM potassium phosphate buffer pH 6.8 and 0.5 ml of enzyme solution at 45°C for 60 min (modified from El-Naggar et al. (2006)). Amount of reducing sugar released was determined by the dinitrosalicylic acid reagent (DNS) (Miller, 1959). One unit of mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

Preparation of enzymatic treated PKM (ETPKM)

Beta-mannanase was produced by *P. italicum* using LBG as a carbon source. The extracellular β-mannanase was harvested after 5 h of cultivation by centrifugation with 3.62×10^3 g for 20 min at 4°C and the supernatant was used as crude enzyme solution. The supernatant was brought to 30% ammonium sulphate concentration and centrifuged for 20 min. The supernatant collected was further brought to 70% ammonium sulphate concentration and re-centrifuged for 20 min (Adebiyi et al., 2008). The sediment, that is, the precipitate was taken as the enzyme. For every 100 ml of the solution that was spun in the centrifuge, the precipitate was suspended in 5 ml phosphate buffer at pH 6.8. The sample (PKM) was hydrolyzed with concentrated β-mannanase at 30°C for 60 h (36 g of PKM was suspended in 200 ml distilled water plus 12 ml of enzyme preparation with an activity of 69.36 U/ml) within a sealing system (modified method of Khanongnuch et al., 2006). After completion of the reaction, the product was completely dried by vacuum dryer at 65°C for 60 h to obtain the ETPKM (Khanongnuch et al., 2006).

Determination of proximate composition of ETPKM

The proximate composition of ETPKM and non-enzymatic treated PKM (NTPKM) were determined by standard methods according to AOAC (2005). Fiber compounds including acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) contents were measured sequentially with a fiber automatic analyzer (Fibertec System, M, Tecator, Hoganas, Sweden) (van Soest et al., 1991). Hemicellulose was calculated as NDF - ADF, cellulose as ADF - ADL, while lignin content is obtained by the subtraction of residue after extraction from ash. Phytate was determined through the extraction of the samples with hydrochloric acid and sodium sulphate and absorbance measured at 660 nm (De Boland et al., 1975). Tannin was determined using the method of vanillinhydrochloric acid and absorbance was measured at 500 nm (Price et al., 1978). Oxalate determination was done according to the standard method of Day and Underwood (1986), while cyanide content was evaluated by the method of Obadeni and Ochuko (2001).

Determination of mineral composition of ETPKM

The mineral composition of the enzyme treated PKM was determined using the atomic absorption spectrophotometer method as described by AOAC (2005).

Statistical analysis

The statistical analysis was performed using the general linear model function of Statistical Package for Social Science (SPSS), version 16.0. All data generated was subjected to one-way ANOVA while statistical differences of treatment were determined using Duncan's multiple range.

RESULTS AND DISCUSSION

Chemical composition of the control (NTPKM) and enzyme treated PKM (ETPKM) is shown in Table 1. As observed from the table, there was significant (P>0.05) increase in the crude protein of ETPKM when compare with control treatment (NTPKM). Crude protein increased from 16.02±0.40 in NTPKM to 23.26±0.13 in ETPKM. Liu and Baidoo (2005) reported that crude protein content of fungal fermented PKM increased nearly 2 folds (16.8 to 31.2%) while no significant increase in crude protein was detected in enzyme treated PKM. The authors suggest that the increased crude could be due to microbial protein synthesis during fermentation. Swe et al. (2004) also reported higher crude protein in fungal fermented PKM (29.4%) as compared to untreated material (16.9%), but the ratio of true protein (amino acids) to total crude protein for the fermented sample was much lower than the original PKM and further suggested that the increased crude protein in the fungal fermented PKE was due to non-protein nitrogen of the fungal cell wall which is non-digestible in poultry. The increase in crude protein value of the degraded PKC might partly be due to the ability of the enzyme to increase the bioavailability of the protein hitherto encapsulated by the cell walls (Ng et al., 2002). According to Bachtar (2005), the fungal enzymes have the potentials of improving not only the non starchy polysaccharides (NSPs) but also protein as well as other dietary components, such as fatty acids. Secretion of proteinase along side enzyme of interest by fungi invariably increases the protein content of feed materials. Many workers have reported similar increase in protein content. In his work, Iyayi and Aderolu (2004) reported increase in crude protein when *Aspergillus niger* was inoculated on sago fibre and cassava fibre resulting into 16.5 and 18.5% protein increase respectively. The author reported a 21.9% increase in the protein of cocoa shell when inoculated with *A. niger.* In their work, Ofuya and Nwajiuba (1990), reported increases in crude protein of 31, 36 and 41% with *A. niger*, 26, 33 and 38% with

Table 2. Fibre composition of mannanase treatment of *P. italicum* on PKM (% dry weight).

Sample	Lignin	Cellulose	Hemicelluloses
PKM [a]	10.64^b±0.50	62.11^c±0.49	12.91^b±0.64
[b]	10.38^b±0.13	24.01^b±0.24	12.42^b±0.33
[c]	8.80^a±0.20	14.38^a±0.37	11.40^a±0.14

[a] Untreated, [b] during treatment [c] after treatment. Values are means± S.E (n=3). Means with the same superscript letters down the same column are not significantly different (P>0.05).

Table 3. Anti-nutrient composition of mannanase treatment of *P. italicum* on PKM (mg/g dry) weight).

Sample	Phytate	Oxalate	Tannin	Cyanide
PKC [a]	15.63^c±0.83	0.36^a±0.01	0.14^b±0.01	9.84^c±0.02
[b]	12.24^b±0.07	0.36^a±0.00	0.14^{ab}±0.00	6.38^b±0.14
[c]	11.06^a±0.43	0.36^a±0.00	0.13^a±0.01	4.09^a±0.68

[a] Untreated, [b] during treatment [c] after treatment. Values are means± S.E (n=3). Means with the same superscript letters down the same column are not significantly different (P>0.05).

Aspergillus flavus and 27, 36 and 32% with *Penicillium* sp. in brewer's dried grain, maize offal and wheat offal, respectively after 14 days of their biodegradation. Similar results have been reported by Smith et al. (1996) when they cultured cassava peels with *Rhizopus* sp. The authors reported a 185% increase in the protein of the peels. Such high increase can be attributed to the fact that cassava peels are less fibrous than PKC. Results of other workers (Mikami et al., 1982; Balagopalan and Gregory, 1985; Manilal et al., 1985; Yokomizo, 2004) suggest the ability of fungi inoculated on low quality feed ingredients to increase the protein levels in such ingredients by the conversion of the carbon atom of the broken down carbohydrates into mycelia protein.

There was no significant decrease between the fat content of enzyme treated PKM and the control, however, enzyme treatment decreased fat content from 5.90±0.24 in untreated sample to 5.48±0.13 in ETPKM, which is in agreement with Swe et al. (2004) and Liu and and Baidoo (2005) who reported that crude fat content in enzyme and fungal treated PKM decreased by about 50% (from 6.82 to 3.36%) and 24% (from 6.82 to 5.15%), respectively. There were significant reductions (P>0.05) in ash and crude fibre contents in the ETPKM as compared to the control. The ETPKM showed a markedly reduction in crude fibre by 64.02%. The significant reduction in crude fibre content indicates that the enzyme is effective in breaking down mannan-hemicellulose, the main component of the fiber in PKM (Lawal et al., 2010; Saenphoom et al., 2011).

Lignin, cellulose and hemicelluloses (fiber compounds) contents decreased significantly (P>0.05) in ETPKM as

compared to the control (untreated PKM) (Table 2). The lignin, cellulose and hemicelluloses content decreased in ETPKM from 10.64±0.50 to 8.80±0.20, 62.11±0.49 to 14.38±0.37 and 12.91±0.64 to 11.40±0.14, respectively. The aforementioned result is in agreement with that of Albores et al. (2006) who found that the fungal enzyme treated PKC reduced lignin, hemicelluloses and cellulose contents resulting in increased crude protein and soluble sugar (glucose, fructose, galactose and sucrose) contents. The reduction in fibre compounds (lignin, cellulose and hemicelluloses) of ETPKM could be attributed to the ability of the fungi to secrete hydrolyzing and oxidizing enzymes, which could convert the recalcitrant compounds in the waste into utilizable compounds (Tanveer et al., 2000; Akinfemi, 2012).

The phytate, tannin and cyanide contents in the ETPKM were significantly lower than that of control treatment (Table 3). The phytate, tannin and cyanide contents of ETPKM had a reduction of 29.26, 6.99 and 58.44%, respectively. There was no significant different between the oxalate contents of the ETPKM and the control (NTPKM). The reduction in the anti-nutritional compounds in ETPKM could be due to the action of certain hydrolytic metabolites produced alongside the enzyme of interest (Nwafor and Ejukonemu, 2004; Cao et al., 2007; Safari, 2011). Reduction in the anti-nutrient compounds were reported by Ojokoh et al. (2012) for fermented groundnut and popcorn, fermented cassava tuber (Aro et al., 2008), fermented sorghum cultivars (Wedad et al., 2008) and fermented canola meal (Omid et al., 2012).

The mineral analysis of ETPKM and NTPKM are shown

Table 4. Mineral composition of mannanase treatment of *P. italicum* on PKM (ppm).

Sample	Calcium	Magnesium	Zinc	Phosphorus	Copper	Potassium
PKC [a]	1.15[c]±0.00	0.23[a]±0.00	0.08[a]±0.00	0.39[a]±0.01	0.39[c]±0.00	2.98[c]±0.00
[b]	0.91[b]±0.01	0.23[a]±0.01	0.08[a]±0.01	0.62[b]±0.05	0.33[b]±0.03	2.73[b]±0.02
[c]	0.76[a]±0.03	0.24[a]±0.02	0.12[b]±0.01	1.21[c]±0.03	0.07[a]±0.01	2.11[a]±0.14

[a] Untreated, [b] during treatment [c] after treatment. Values are means± S.E (n=3). Means with the same superscript letters down the same row are not significantly different (P>0.05).

in Table 4. It is shown in the table that calcium, copper and potassium reduced in PKM after enzyme treatment. The percentage reduction of calcium, copper and potassium in ETPKM were 33.65, 81.28 and 29.12%, respectively. The reason for decrease in certain minerals after enzyme treatment might be connected to the fact that some of these minerals could be utilized as co-factors for effective catalytic function of enzyme molecules. The reductions of certain minerals in fermented products have been documented (Aro, 2008; Akinyele et al., 2011; Ojokoh et al., 2012). The reduction in these minerals in fermented products could be as a result of their utilization by the fermenting micro-organisms as reported by Ojokoh et al. (2012). However, there was significant increase in zinc and phosphorus contents of ETPKM in comparison with the control. Similar observation was reported by Ojokoh et al. (2012) and Aro (2008) for fermented products, and it was attributed to the fact that some of these metals could be part of some biological macromolecules which were released into the solution from such structures during fermentation.

Conclusion

The treatment of PKM with β-mannanase resulted in decrease of complex fibre fractions in the PKM to increase its crude protein and certain minerals (zinc and phosphorus) contents. There was also reduction in the anti-nutrient compounds of enzyme treated PKM.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of Francis media for extended spectrum beta lactamase (ESBL) screening

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Francis media was developed for the differential screening of *Burkholderia pseudomallei*. It was later found to have additional function of selecting extended spectrum beta lactamase (ESBL) from *Enterobacteriaceae*. A total of 305 *Enterobacteriaceae* isolates from clinical specimens (*Klebsiella* spp. [191], *Escherichia coli* [96], *Enterobacter* spp. [9], *Citrobacter* spp. [3] and others [6]) were tested for the presence of ESBL.Out of 305, 135 were ESBL producing *Enterobacteriaceae* tested on Francis media for the presence of yellow colonies and haze after 24 h of incubation. Francis media revealed sensitivity and specificity of 89 and 99%, respectively in detecting ESBL producing *Enterobactericeae.*

Key words: Screening, extended spectrum beta lactamase (ESBL), Francis media.

INTRODUCTION

Extended spectrum beta lactamase (ESBL) is one of the multidrug resistant (MDR) organisms under constant surveillance in Malaysian hospitals. Due to its ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks and increase the length of hospitalization (Sturenburg and Mack, 2003; Kim et al., 2002; Paterson et al., 2001). Early detection and identification of ESBL is important to optimize anti-microbial therapy and to ensure timely introduction of appropriate infection control procedures (Cantón and Coque, 2006; Pfaller and Segreti, 2006; Ramphal and Ambrose, 2006).

Screening and confirmation of ESBLs are normally performed on isolated organisms following microscopic examination of Gram negative rods (GNR) and culture on MacConkey and antibiotic susceptibility testing. Most

times, there is a delay of 18-24 h before ESBL presence could be identified phenotypically from a primary culture. At this juncture, there is a need of rapid screening for carriage of ESBL producing *Enterobactericeae* among high-risk patients by using a highly selective media. Until date, there are few commercial selective ESBL media been evaluated and produced (Glupczynski et al., 2007; Huang et al., 2010; Paniagua et al., 2010; Reglier-Poupet et al., 2008; Saito et al., 2010).

Francis media was developed for the differential screening of *Burkholderia pseudomallei* and *Burkholderia cepacia* (Francis et al., 2006). This media which uses gentamicin as its inhibitory components, was later found to have additional function of selecting ESBL producing isolates. In this work, we reported an evaluation of Francis media in selecting ESBL producing

Figure 1. ESBL producing *Enterobactericeae* on Francis media.
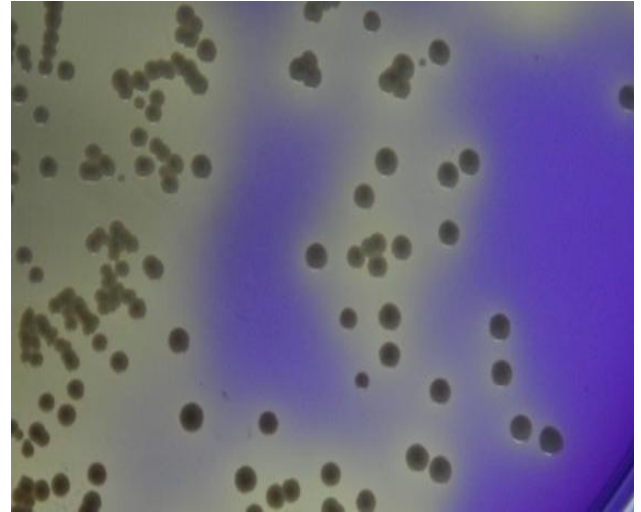
Figure 2. *K. pneumoniae* appearance of yellow colonies with yellow discoloration of Francis media.

Enterobactericeae from clinical isolates.

MATERIALS AND METHODS

A total of 305 clinical specimens, sent to our microbiology laboratory for culture and sensitivity were included in the study. These specimens included blood, pus (wound swabs or ear-nose-throat specimens), tissue, urine and stool, originating from 279 patients who had been hospitalized in various medical and surgical wards for more than 48 h. All blood specimens were isolated using the BD BACTEC™ blood culture system. Each specimen that was identified as Gram negative rods on microscopic examination was directly inoculated onto horseblood agar and MacConkey, (Oxoid, Basingstoke, United Kingdom) and incubated in air at 37°C for 18 to 24 h.

Gram negative colonies were inoculated unto Francis media (prepared as in Francis et al., 2006) and homogenized in 1 ml of sterile physiological saline (0.85%) into 0.5 McFarland suspension and plated spirally onto Muller Hinton agar with antibiotic disc (30 µgCefotaxime, 10 µg Amoxicillin-clavulanic acid and 30 µgCeftazidime placed 1.5 cm distance in a row) for ESBL screening and incubated at 35°C for 18 to 24 h. Growth on Francis agar is linked to the resistance to a specific antibiotic and signified by presence of yellow colonies and yellow discoloration of Francis agar were regarded as ESBL isolates (Figure 1). Positive screening of ESBL isolates on Muller Hinton agar were reflected by the presence of 'keyhole effect' between the discs after incubation and subjected to confirmation of ESBL-producing isolates by combined double disks (30 g ceftazidime and 30 g cefotaxime disks alone and the same antimicrobials with 10 g clavulanic acid) as recommended by CLSI guidelines (CLSI, 2011).

All culture plates were interpreted independently by two laboratory staff members.Pronounced yellow haze around the colonies on the Francis agar signified a presumptive ESBL organism (Figure 2). All types of colonies presenting different morphological aspects were identified by API 20NE and API 20E (bioMe´rieux, Marcy l'Etoile, France). The plates were incubated in ambient air at 37°C for 18 to 24 h. Genotypic characterization of resistance mechanisms was determined by PCR assays targeting bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes according to previously published methods (Sidjabat et al., 2009).

RESULTS AND DISCUSSION

Overall, 305 isolates were identified as Gram negative rods on microscopic examinations. They were screened for *Enterobacteriaceae* group by using simple TSI sugar sets. The isolates recovered included *Klebsiella* spp. [191], *Escherichia coli* [96], *Enterobacter* spp. [9], *Citrobacter* spp. [3] and other *Enterobacteriaceae* [6] (Table 1). Out of this total of 305 *Enterobacteriaceae* isolated, 135 were confirmed as ESBL isolates by the four disk method as mentioned earlier. These 135 isolates when streaked onto Francis media, 120 (89%) isolate yielded yellow colonies with yellow haze around the colonies after 24 h of incubation. However 11% (15/135) isolates did not produce yellow coloration on Francis agar after 24 h of incubation. Among the 15 false negative ESBL isolates, four *Klebsiella pneumonia* isolates yielded pale colored colonies, and the remaining 11 isolates (1 *Enterobacter aerogenes*, 1 *Enterobacter cloacae* and 9 *E. coli*) did not grow on the agar, as they were sensitive to gentamicin. Only two isolates of *K. pneumonia* showed false positive results, as they were resistant to gentamicin as shown by disk diffusion test for these isolates. They produced paleyellow colouration probably due to incomplete fermentation of the sugars present in the media.

Prolongation of incubation did not increase the sensitivity of detection of ESBL-producing organisms on the media and only marginally increased the growth of other Gram-negative isolates. Molecular typing of ten randomly selected isolates was done. The ESBLs was performed by PCR analysis for β-lactamase genes which showed all 5 isolates possessed the bla_{SHV}, bla_{TEM} and $bla_{CTX-M-15}$ cluster gene.

Overall, we showed that Francis media was a reliable culture media to screen for ESBL-producing *Enterobacteriaceae* directly from clinical samples. The

Table 1. Detection of ESBL and Non-ESBL organisms using Francis media as compared to CLSI recommended ESBL confirmatory disk diffusion method.

ESBL[±][^]	Francis	CLSI	Non-ESBL[±]	Francis	CLSI
	(n)	(n)		(n)	(n)
Enterobacter cloacae	0	1	*Acinetobacter baumannii*	1	1
Enterobacteraerogenes	2	3	*Citrobacter freundii*	2	2
Escherichia coli	6	15	*Enterobacter aerogenes*	1	1
Citrobacterfreundii	1	1	*Enterobacter agglomerans*	2	2
Klebsiellapneumoniae	110	114	*Enterobacter cloacae*	2	2
Klebsiellaoxytoca	1	1	*Escherichia coli*	81	81
			Flavobacterium spp.	1	1
			Klebsiella pneumoniae	69	71
			Klebsiella oxytoca	5	5
			Proteus mirabilis	2	2
			Proteus vulgaris	1	1
			Pseudomonas aeruginosa	1	1
Total	120	135	Total	168	170
Sensitivity (%)	89	NA	Specificity (%)	99	NA
PPV(%)	98	NA	NPV (%)	92	NA

[±]Number of ESBL isolates correctly screened by Francis media and confirmed by CLSI method;
[±]Number of non-ESBL isolates correctly screened by Francis media and confirmed by CLSI method;
[^]SHV, TEM and CTX-M-15 were found on these ESBLs isolated from Francis media.

main advantage of this media is its significant higher specificity (99%) after 24 h incubation. This was due to lower recovery of non-ESBL producing *Enterobacteriaceae* and less false-positive results due to non-*Enterobacteriaceae*. Thus, it reduces the need for further ESBL confirmation testing and unnecessary identification work when disregarding all colonies without a yellow colonies and yellowish discoloration of the agar. Yielding an excellent positive predictive value (98%), this media enable rapid identification of patients carrying ESBL-producing *Enterobacteriaceae*.

Most ESBL producing *Enterobacteriaceae* are noted to have co-resistance effect with aminoglycoside (Winokur et al., 2001; Obeng et al., 2013). This explains why in this study all 135 ESBL producing *Enterobacteriaceae* were able to grow in the Francis media which contains gentamicin. The *Enterobacteriaceae* carry out fermentation reaction on the complex sugars present causing the media to turn acid causing a profound yellow colouration on Francis media. The main limitation of Francis media was noted in ESBL detection in other isolates apart from *K. pneumonia* strains. This was noted in isolates with lower inoculum and particularly affected strains with pronounced substrate and supplement preference in the media. As ESBL enzymes have also become more prevalence among species with an inducible AmpC type β-lactamase such as *Enterobacter* spp. and *Citrobacter freundii,* interpretation of positive findings need to be performed cautiously as resistant phenotypes other than ESBL (the AmpC producers) may demonstrate similar phenotype as ESBL on Francis media.

From this study, Francis media showed high specificity and sensitivity as other reports of ESBL screening media (Hadziyannis et al., 2000; Huang et al., 2010; Sturenburg et al., 2005). However, the drawback in this study was limited strains of ESBL-producing *Enterobacteriaceae* as the samples were collected from one centre. A more comprehensive study is deemed important to evaluate its usefulness and to further improve its formulation as an ESBL screening media. Nevertheless, Francis media can be an alternative for a cheap routine ESBL screening to prompt the initiation of infection control measures.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Hyphomycetous fungi spore release induced by air currents and aqueous solution

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Hyphomycetous fungi originating from South Africa were morphologically characterised and ascribed to the genera *Acremonium*, *Aspergillus* and *Penicillium*, respectively. The primary means of spore dispersal employed by these isolates was investigated by quantifying colony forming units released into the air and into an aqueous solution. Measurement of spore liberation during humid aeration, revealed significant (P < 0.0001) differences among the hyphomycetous taxa investigated. Isolates of the genus *Penicillium* were more successful in releasing their spores than the Aspergilli and the *Acremonium* isolate. Spore liberation during desiccated aeration also showed a significant (P < 0.0001) difference between the respective isolates. Overall, isolates belonging to the genus *Penicillium* released more viable spores than *Aspergillus* spp., which in turn released more spores than *Acremonium*. In support of the theory that splashing rain may dislodge and disperse microfungal propagules, washing respective cultures with physiological salt solution resulted in an immediate massive spore release. However, the taxa investigated did not differ. These differences in airborne spore release that were observed between the hyphomycetous genera may be a result of different strategies to disperse their spores in nature. This phenomenon should be investigated further in future and the challenge now is to find correlations between the conidiophore morphology of each fungus and characteristics of their niche.

Key words: *Acremonium*, *Aspergillus*, Hyphomycetes, *Penicillium*, spore dissemination.

INTRODUCTION

Conidia are the means of asexual multiplication, dispersal, survival and their physical interactions have great importance in the life-cycle of fungi (Brown and Hovmoller, 2002; Sanderson, 2005; Elbert et al., 2007). Currently, we know that hyphomycetes may be considered as common airborne fungi occurring in both indoor and outdoor environments (Shelton et al., 2002;

de Ana et al., 2006). *Aspergillus* and *Penicillium* spores have been shown to occur commonly in "dry" air samples (Fogelmark et al., 1994; Shen et al., 2007). Some spores are inhaled by mammals; for example, humans and may be deposited into the respiratory tract. Inhaled spores are known to adhere to host plasma membrane once deposited, and attachment of conidia to host matrix

Table 1. Fungal isolates used in the experiment.

Isolate code	Species	Source
ABOACR	*Acremonium alternatum*	Culture collection
ABOAA	*Aspergillus aculeatus*	Culture collection
ABOAC	*A. carneus*	Culture collection
ABOAF	*A. fumigatus*	Culture collection
ABOAN	*A. niger*	Culture collection
ABOAT	*A. terreus*	Culture collection
ABO486	*Penicillium camemberti*	Culture collection
ABOPC	*P. candidum*	Culture collection
AB0272	*P. citrinum*	Culture collection
ABOR1	*P. citrinum*	Culture collection
ABOR2	*P. citrinum*	Culture collection
ABOF2	*P. commune*	Culture collection
ABOFG16	*P. commune*	Culture collection
ABOR4	*P. glabrum*	Culture collection
ABO268	*P. spinulosum*	Culture collection
ABO275	*P. sumatrense*	Culture collection
ABO487	*P. westlingii*	Culture collection

*Culture collection = Fungal Culture Collection of the Department of Microbiology, the University of Stellenbosch, South Africa.

surfaces has survival value and may be a requirement for colonisation (Peñalver et al., 1996; Kukreja et al., 2007; Tronchin et al., 2008). This deposition of spores may impact on health since these spores may result in respiratory tract infection (Supparatpinyo et al., 1994; Denning et al., 2006; Bellanger et al., 2009).

Hyphomycetous fungi have been known to liberate their hydrophobic conidia under desiccated environmental conditions, and also with the aid of rain drops by means of splash dispersal (Fitt and Nijman, 1983; Tadych et al., 2007). It was also suggested that the most effective means of spore dissemination among soil hyphomycetes is through the movement of rain water (Sutton et al., 1976; Horn et al., 2001). It was, therefore, essential to obtain knowledge of the environmental conditions that promote hyphomycetous spore release.

This study was undertaken to investigate the primary means of spore dispersal employed by representatives of the genera *Acremonium*, *Aspergillus* and *Penicillium*, isolated from Fynbos soil and indoor environments in the Western Cape, South Africa. Also, since all of these fungi contain different sporogenous structures, another objective of the study was to investigate whether the quantity of colony forming units released into the air and into an aqueous physiological saline solution differed among the isolates.

MATERIALS AND METHODS

Fungal isolates used

Hyphomycetous fungi were obtained from the fungal culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa (Table 1). The cultures in the culture collection originated from various sources in the Western Cape, South Africa, amongst others, Fynbos soil and interior of cellars. These cultures were subsequently used to inoculate 2% (w/v) malt extract agar (MEA) Petri-dish. The plates were incubated at 22°C and the resulting colonies were purified by consecutive transfer and incubation on MEA plates at 22°C.

Identification of isolates using morphological criteria

Single-spore cultures were prepared from the fungal cultures (Pitt, 1974). Each single spore culture was inoculated on differential media and subsequently incubated for seven days as required for the identification of these fungi. Following incubation, colonies were microscopically examined for characteristic features as described in the literature (Thom, 1930; Raper and Thom, 1949; Pitt, 1979; Domsch et al., 1993). In addition, microscopic and macroscopic characteristics such as conidial colour, presence of exudates, mycelial growth and coloration were used to identify the isolates according to the descriptions and keys in literature.

Measurement of spore liberation triggered by air

A comparative analysis of spore liberation of all isolates was conducted in an airflow cell as schematically illustrated (Figure 1). This airflow cell consisted of a horizontal, tubular growth chamber (450 mm in length, 70 mm in diameter). The growth chamber was aseptically filled with 100 ml MEA resulting in a surface area of 165 cm^2 after the MEA was allowed to solidify, whilst the growth chamber was positioned horizontally. A 10 ml conidial suspension (containing ca. 5 x 10^6 conidia/ml) of a week old culture was used to inoculate each chamber aseptically. Spore counts for the inoculum were determined microscopically using a haemocytometer. The chamber was incubated at 22°C for 48 h to allow conidial germination (colony establishment) and formation of fungal mycelia, whereafter it was connected at the one end to an air pump to
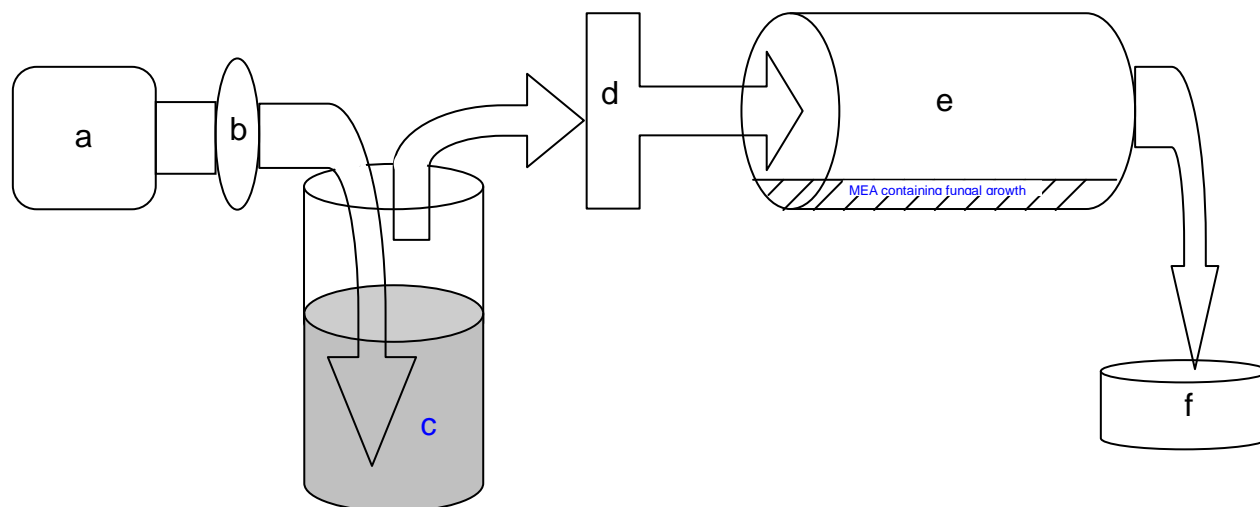
Figure 1. Schematical illustration of the airflow cell and accompanying componets. (a) fish-tank pump; (b) polytetrafluoroethylene (PTFE) 0.45 μm filter; (c) sterile water (or anhydrous $CaCl_2$) in flask; (d) airflow regulator; (e) airflow cell with fungal culture on MEA (surface area of 165 cm^2); and (f) outlet onto malt-extract agar (MEA) containing Petri dishes.

initiate the respective trials. Sterile air with a relative humidity of 36% was subsequently pumped through the chamber at a rate of $\pm$ 0.6 l/min (laminar flow rate of 16.3 cm/min) via a 0.45 μm Midistart® 2000 PTFE filter (Sartonet Sa. Cc), sterile water in a conical flask and a Gabler airflow regulator.

After two weeks, the water-containing flask was replaced with an anhydrous calcium chloride ($CaCl_2$) moisture trap in order to subject the fungal culture in the airflow cell to desiccated air for a further two-week period. Spore liberation was monitored by exposing a set of nine MEA containing Petri dishes consecutively to air at the flow cell's outlet for 15 min, respectively. The MEA plates were subsequently incubated for one week at 22°C and the number of colony forming units was counted. Spore release was monitored for five consecutive days per week over a four week incubation period.

Measurement of spore liberation into aqueous saline

After the four-week incubation period, the ability of the cultures to release spores into an aqueous physiological saline (isotonic salt) solution was determined. Pre-liminary investigations showed that 15 ml aqueous physiological salt solution (PSS) (0.85% (w/v) NaCl) was sufficient for this purpose. Therefore, 15 ml of PSS was gently transferred into each airflow cell. The number of spores in the resulting suspension was microscopically counted using a haemocytometer.

Statistical analysis

To test the effect of environmental conditions on spore liberation of isolates over time, a completely randomised experiment was conducted with the treatments in a 2 x 17 x 2 factorial with three random replications (Snedcor and Cochrane, 1967). The factors were two environmental conditions (humid and dry aeration); 17 isolates/strains which were grouped into three genera namely, *Acremonium* (ABOACR), *Aspergillus* (ABOAA, ABOAC, ABOAT, ABOAN, and ABOAF) and *Penicillium* (ABOF2, ABOR4, ABOFG16, ABOR1, ABOR2, ABO268, ABO272, ABO275, AB0486, ABO487 and ABOPC), and two time phases (weeks 1 and 2).

Total spore liberation was recorded and transformed by a Log_{10} (x + 1) transformation before being subjected to an appropriate factorial analysis of variance (ANOVA), using SAS statistical software (SAS Institute Inc., 1999). Shapiro-Wilk test was performed on the residuals to test for non-normality (Shapiro and Wilk, 1965). In order to compare the means of significant effects, the Student's t-least significant difference (LSD) was calculated at a 5% significance level. The means of the statistical analysis are presented in figures.

RESULTS

Identification of fungal isolates

Morphological characteristics revealed that the isolates were hyphomycetes that belonged to the genera *Acremonium*, *Aspergillus* and *Penicillium*, respectively (Pitt, 1979; Domsch et al., 1993; Klich, 2002) (Table 1).

Spore liberation into air

The Shapiro-Wilk test on spore liberation data revealed deviation from normality, subsequently outliers were removed until the residuals had a normal distribution or were symmetric (Glass et al., 1972). Significant three-factor interaction was found.

A comparative analysis of three factor interaction (time x isolate x environmental condition) showed significant (P < 0.0001) differences in spore liberation between the weeks, during humid and desiccated aeration, respectively. Thus, spore liberation during week 1 of incubation under humid conditions differed significantly from spore liberation during week 2 (Figure 2). Furthermore, intraspecific differences were observed, for example
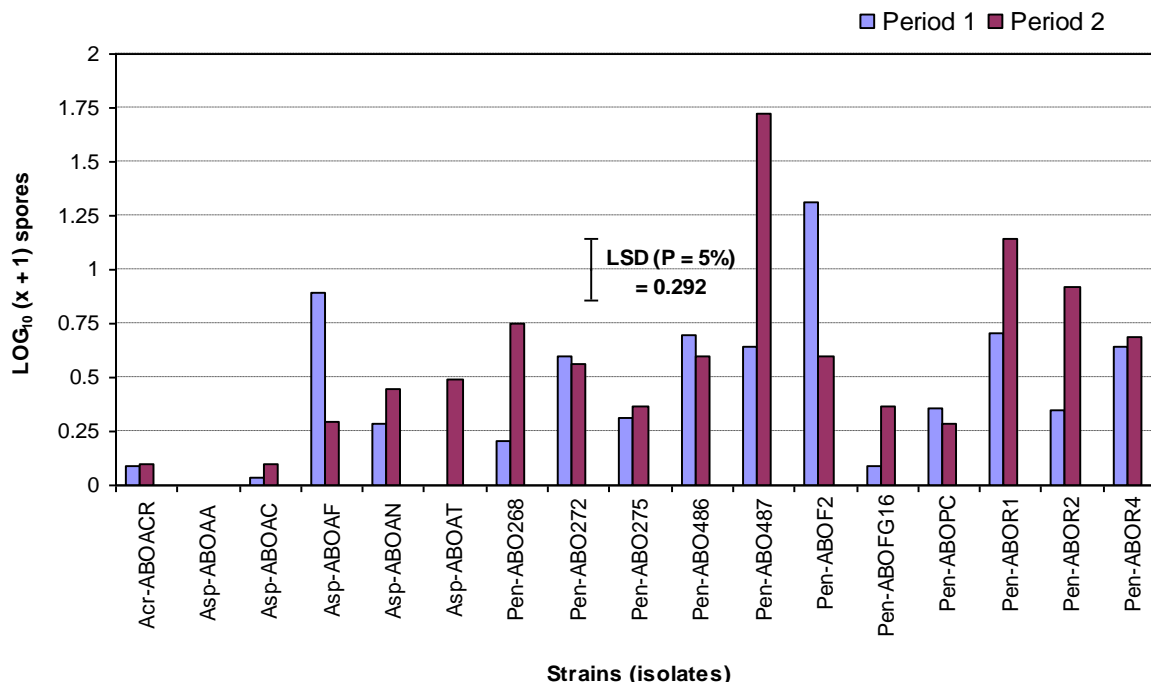
Figure 2. Three factor interaction (time x isolate x environmental condition) means of total spores liberated after weeks (period) 1 and 2 during humid aeration. The pre-fixes "Acr, Asp and Pen-" indicates the genera, *Acremonium*, *Aspergillus* and *Penicillium*, respectively.

Polytrichum commune ABOF2 liberated more spores during week 1 than week 2, whilst for ABOFG16 the spore liberation was reversed. *Penicillium citrinum* ABOR1 and ABOR2 liberated significantly more spores during week 2, whilst for ABO272 no significant differences was found. *Aspergillus aculeatus* ABOAA was the only *Aspergillus* isolate that released no spores in the presence of humid air, whilst *A. terreus* ABOAT only released spores during the second week. As was found for environmental condition 1, the comparative analysis for environmental condition 2 between week 1 and week 2 also showed a significant (P < 0.0001) difference between weeks (Figure 3).

Intraspecific similarities were observed; for example, *P. commune* ABOF2 and ABOFG16 liberated more spores during week 2 than week 1. However, intraspecific differences were also observed, for example, *Penicillium citrinum* ABOR1 and ABO272 liberated more spores during week 1, whilst ABOR2 showed a reversed spore liberation pattern compared to the former isolates. All aspergilli with the exception of *Aspergillus niger* ABOAN released significantly more spores during desiccated aeration as compared to humid aeration. Interestingly, *A. aculeatus* ABOAA released the most spores in the "dry" air, despite no spores being released during humid aeration.

Therefore, to investigate a possible correlation between fungal taxa and spore release, the isolates were grouped into genera and spore release under humid and desiccated conditions analysed. A comparative analysis of three factor interaction (time x genus x environmental condition) between weeks 1 and 2 for humid - and desiccated aeration, respectively, showed a significant (P < 0.012) difference in spore liberation between weeks.

In contrast to isolates of the genus *Penicillium*, members belonging to the genera *Acremonium* and *Aspergillus* did not differ between the two weeks (weeks 1 and 2) during humid aeration. Furthermore, spore liberation of the *Acremonium* isolate and organisms representing the genus *Aspergillus* did not differ significantly, but the *Penicillium* species were significantly more successful in dispersing their spores. *Aspergillus* isolates released significantly more spores under desiccated aeration during week 2 than week 1, whilst isolates of the other two genera, that is, *Acremonium* and *Penicillium*, did not differ. Also noteworthy is that *Penicillium* isolates differed significantly from the *Acremonium* isolate, but not from that of *Aspergillus*, with regard to the number of spores released.

Spore liberation into aqueous saline

Gently washing the culture with an isotonic salt solution in all cases resulted in an immediate massive release of colony forming units (Figure 4). However, no significant difference could be observed between cultures regarding the release of colony forming units into the aqueous
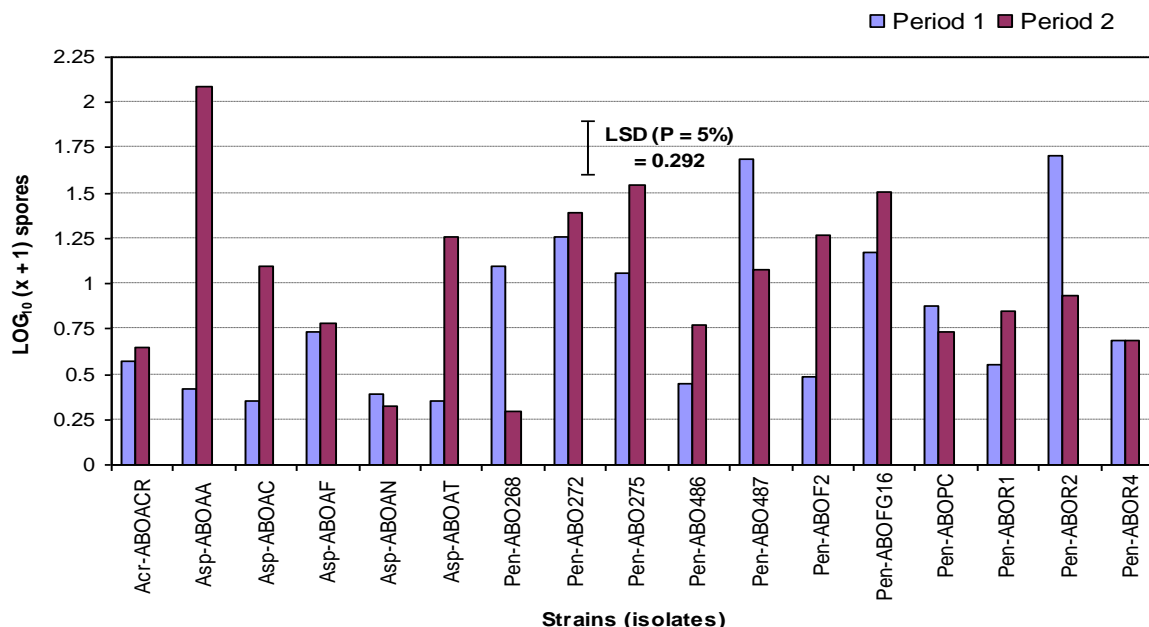
Figure 3. Three factor interaction (time x isolate x environmental condition) means of total spores liberated after weeks (period) 1 and 2 during desiccated aeration. The pre-fixes "Acr, Asp and Pen-" indicates the genera, *Acremonium*, *Aspergillus* and *Penicillium*, respectively.
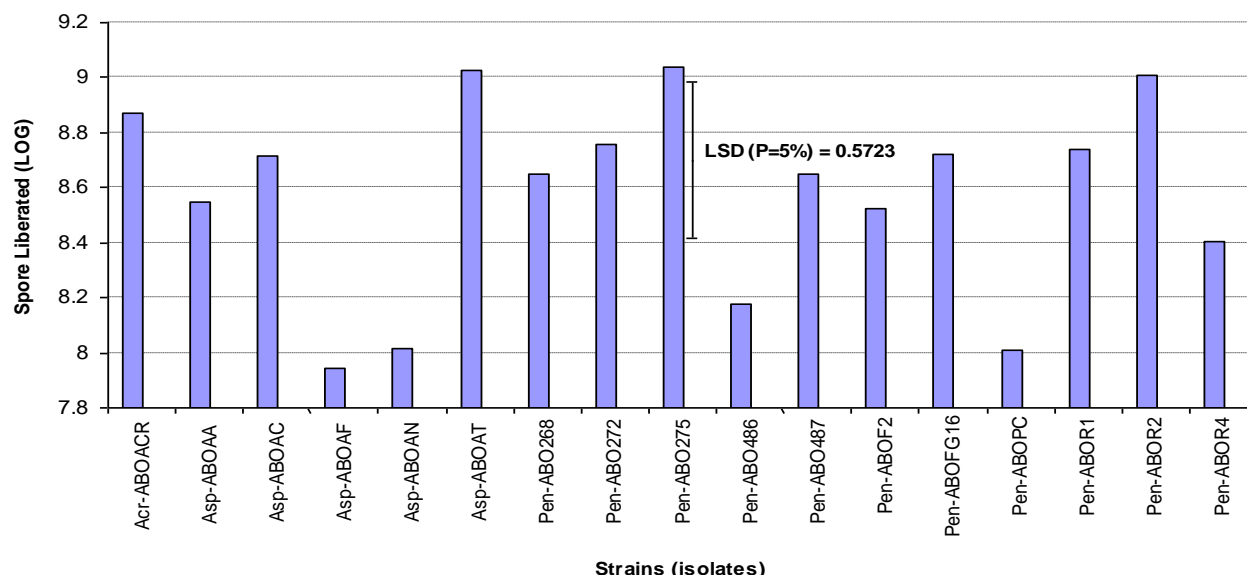
Figure 4. Means of total spores liberated when colonies were flushed with a saline solution. The pre-fixes "Acr, Asp and Pen-"represents the genera, *Acremonium*, *Aspergillus* and *Penicillium*, respectively.

saline solution (data not shown). The $\log_{10} (x + 1)$ of average number of *Acremonium*, *Aspergillus* and *Penicillium* spores released per cm^2 during the two fortnightly phases of airflow was 0.004, 0.006, and 0.009, respectively. Notably more spores were released when the cultures were washed with PSS, that is, 0.054, 0.052 and 0.051, respectively.

DISCUSSION

Fungal spores are the main mode of removing potential progeny from the direct vicinity of the parent mycelium (Ingold, 1953; Moore-Landecker, 1996; McGinnis, 2007). Spore disposal serve to minimise competition amongst siblings as a result of unfavourable nutritional conditions,

and thus promote the survival of the organism and increasing the habital range (Glenn et al., 2004; Gover, 2013). It has long been recognised that many hyphomycetous fungi liberate their spores in a passive manner (Ingold, 1971; Magyar, 2002; Tadych et al., 2007). During this process, millions of spores are released through the physical action of wind, rain and animals. This study was undertaken to obtain an indication of the primary means of spore dispersal employed by isolates of the genera *Acremonium*, *Aspergillus* and *Penicillium*, originating from the fungal culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. Also, since all these fungi contain different sporogenous structures, we were interested on whether the quantity of colony forming units, released into the air and into an aqueous isotonic salt solution, differed among the isolates.

Measurement of spore liberation during humid aeration, revealed significant ($P < 0.0001$) differences among the hyphomycetous taxa investigated regarding aerial spore release (Figure 2). Intraspecific differences were also observed as demonstrated by the results obtained for *P. citrinum* where two isolates, that is, ABOR1 and ABOR2 liberated more spores during week 2, whilst the other isolate, that is, ABO272 was consistent with regard to weeks 1 and 2. It can, therefore, tentatively be said that liberation of spores into humid air by some *Penicillium* isolates is dependant on the age of culture, whilst in spore liberation of other isolates, amongst others, ABO272 is not influenced by time and/or the age of the culture.

Intraspecific differences with regard to aerial conidial dispersal of *Penicillium* isolates have not been reported previously. Furthermore, a species known to thrive in humid conditions, *A. fumigatus* ABOAF (Wasylnka and Moore, 2000; Gamboa et al., 2005; Stark et al., 2006), released significantly more spores during week 1 than in week 2, whilst *A. aculeatus* ABOAA, a fungal species of the section *Nigri* that commonly occurs on vine fruit (Serra et al., 2006; Bufflier et al., 2007), did not release spores.

As was found for humid aeration, the comparative analysis for desiccated aeration between weeks 1 and 2 also showed a significant ($P < 0.0001$) difference in spore liberation by the respective isolates (Figure 3). Intraspecific differences were also observed as demonstrated by the results obtained for *P. citrinum* where one isolate liberated more spores during weeks 1 than 2, whilst the remainder did not differ between weeks. *A. aculeatus* ABOAA released most spores into the air amongst the aspergilli, despite no spores being released during humid aeration.

As a result of the intraspecific diversity observed, any conclusions on the possible correlation between species or morphology with the numbers of spores released should be made with caution. Although intraspecies differences were uncovered, this study demonstrated for

the genera investigated, that more spores are usually released as the culture matures, and in most cases older cultures released more spores under dessicated conditions. It was also found that the genera responded differently to differences in humidity regarding their aerial spore release. Under humid conditions, *Penicillium* isolates were more successful in releasing their spores than *Aspergillus* and *Acremonium* isolates.

Under desiccated conditions, the isolates representing *Aspergillus* took longer time to release their spores than the *Penicillium* isolates and the *Acremonium*. This may be as a result of the distinct morphology of the sporogenous structures of *Aspergillus*. In contrast to *Aspergillus*, the sporogenous structures of neither *Acremonium* nor *Penicillium* are characterised by a pronounced swollen vesicle at the tip of the conidiophore (Klich and Pitt, 1988; Larone, 1995). It can be speculated that this difference may be ascribed to longer time needed for the sporogenous structures of *Aspergillus* to reach the desiccated condition needed for increased spore release, than the time needed for the sporogenous structures of *Penicillium* and *Acremonium*.

Indications, therefore, are that the sporogenous structures of aspergilli are adapted for passive spore release upon changes in humidity and age of the culture. As only one isolate of the genus *Acremonium* was tested, results may have differed if more representatives of this genus were available for the investigation. Differences in spore liberation may also be tentatively ascribed to differences in the numbers of spores produced and to major differences in spore morphology. Also noteworthy is that *Aspergillus* and *Penicillium* spp. produce dry conidia that can be easily dispersed as opposed to that of *Acremonium* produced within a mucus (Davies et al., 2003; Summerbell et al., 2011).

The *Aspergillus* and *Penicillium* isolates used all produce countless globose to spheroidal conidia with a diameter of 2 to 5 µm (Eltem et al., 2004), while the *Acremonium* isolate formed a lesser number of ellipsoidal to fusiform conidia normally 3 µm wide and 8 µm in length (Schroers et al., 2005). Overall, *Penicillium* isolates, released more spores than *Aspergillus* isolates which in turn released more spores than the *Acremonium*. This observation can be ascribed to differences in sporogenous structure (condidiophore) morphology, as the genera *Aspergillus* and *Penicillium* have polyphialidic conidiophores bearing significantly more conidia than the monophialidic condiophore of *Acremonium* (Domsch, 2007; Sigler et al., 2010).

These findings support the results of others on the common occurrence of *Aspergillus* and *Penicillium* conidia in air samples (Shen et al., 2007; Spicer and Gangloff, 2008). It was also observed for some isolates that the airflow did not cause sufficient disturbance to dislodge and/or liberate a large proportion of the conidia (Tucker et al., 2007). Therefore, in support of the theory that splashing rain may dislodge and disperse microfungal

spores (Ntahimpera et al., 1998; Travadon et al., 2007), washing the culture with PSS resulted in all cases in an immediate massive release of colony forming units from the cultures (Figure 4). However, the taxa investigated did not differ from each other regarding the release of spores in PSS and it seems that water may act as an important dispersion agent for the isolates representing the genera *Acremonium*, *Aspergillus* and *Pencillium.* These findings support the views of others recorded in literature (Sutton et al., 1976; Horn et al., 2001).

Conclusion

It is known that species of hyphomycetous taxa such as *Acremonium*, *Aspergillus* and *Pencillium*, usually release more colony forming units into the air under dry conditions than under humid conditions. This study demonstrated that isolates of these genera released more spores after two weeks of incubation as compared to after one week. Also, noteworthy is that conidia are phialidically produced, and that appically positioned conidia are easiliy liberated by the slighest of air turbulence (air movement) after undergoing a maturation phase (Davies et al., 2003). It can be envisaged that younger conidia near the base of the phialide will remain attached until matured, but a stronger air flow will induce their forceful release. During humidified aeration *Penicillium* strains were more successful in releasing their spores than the strains representing *Aspergillus* and *Acremonium*, while during desiccated aeration, the *Aspergillus* took longer time to release their spores than representatives of *Acremonium* and *Penicillium.* Also, noteworthy is that *Aspergillus* spore release is influenced by conidial maturation. However, this phenomenon may be as a result of differences in the morphology of the sporogenous structures of these fungi. *Aspergillus,* for example is characterised by a swollen vesicle from which the metulae and conidiogenous cells arise. It is tempting to speculate that these vesicles may take longer time to dry and release conidia into the air than the more filamentous sporogenous structures of *Acremonium* and *Penicillium.*

Although not proven, the results also support the contention that an important dispersion agent for these filamentous fungi may be water, since the addition of physiological salt solution resulted in an immediate and massive release of spores from the colonies of strains representing *Acremonium*, *Aspergillus* and *Pencillium*. In general, it can be concluded that the filamentous fungal genera differed in their strategy to release airborne spores. Water however, may serve as the primary means of spore dispersal. The fact that the representatives of the different fungal genera differed in their strategy to release airborne spores, indicate that each of the genera occupy a different environmental niche. This pheno-menon should be investigated further in future and the

challenge now is to find correlations between the conidiophore morphology of each fungus and characteristics of its niche. A point of departure may be to study differences in spore dispersal within artificial ecosystems in which the abiotic and biotic components are manipulated. Microbiological culture techniques, electron microscopy, serological methods in combination with epi-fluorescense microscopy, as well as atomic force microscopy may then be used to monitor changes in dispersion, and subsequent adherence of the fungal propagules.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

Evaluation of efficacy of some botanicals and bioagents against stalk and ear rot pathogen of maize, *Fusarium verticillioides*

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The efficacy of some botanicals and bio-agents were assessed *in vitro* as the potential biofungicides against *Fusarium verticillioides*, the causal organism of stalk rot disease of maize plant. The botanicals used in this study were *Morinda lucida* (Oruwo) and *Tithonia diversifolia* (Mexican Sunflower) and the bio-agents used were *Trichoderma viride, Trichoderma harzianum, Trichoderma pseudokoningii* and *Bacillius subtilis.* All the plant extracts and antagonistic organisms significantly inhibited the growth of *Fusarium verticillioides*. However, out of the antagonists, *T. viride* provided the highest inhibitory effect followed by *T. pseudokoningii, T. harzianum*, and *B. subtilis* which was the lowest suppressive antagonist. The extract from *T. diversifolia*, provided the highest inhibitory effect followed by *M. lucida.*

Key words: Maize, ear rot, botanicals, bioagents, *Fusarium verticillioides.*

INTRODUCTION

Maize (*Zea mays L.*) is the third most important cereal crop in the tropical countries (CIMMYT, 2004). It belongs to the grass family *Poaceae*. It yields economically under the marginal production conditions of low fertility and management (FAO, 2005). Maize has been diversely used in several aspects including being food for human, animal feeds and industrial uses Fajemisin JM (1992).

All parts of the maize plant have economic values ranging from stalk, leaves and the cobs (Fajemisin, 1992). Maize can be eaten either boiled, roasted or processed into corn flakes or used as corn meal (Ubalua

and Oti, 2007; Iwena, 2008). It is also an important raw material in pharmaceutical, baby food, soft drink and brewing industries. Oil obtained from maize is used to make soap and glycerine or is refined for cooking and salad making (Adesimi, 1982).

The production of maize in the whole world at large including Nigeria is being threatened by various constraints including its susceptibility to both pests and diseases that causes pre and post harvest losses which cause great economic losses in Nigeria (Agoda et al., 2011). Moreover, the limiting factors that reduce the yield

of maize plants are pathogen infection. *Fusarium* ear and stalk rot of maize is among the destructive diseases in many areas of Nigeria. This maize fungus produces mycotoxin (fumonisins) which is associated with serious animal and human diseases. Ear rot reduces maize production by destroying the grains and, causing food contamination which is poisonous to human and livestock. Stalk rot causes stalk breakage and lodging thereby making harvesting difficult and consequently reduce yield. Ear and stalk rot of maize is caused by *Fusarium verticillioides.* The fungus *F. verticilliodes* lives in soil, penetrate stalks and root directly or spread systemically in the plant after infection that originated from seed-borne inoculums (Soonthornpoct et al., 2000). Also *F. verticillioides* produces phytotoxin fusaric acid (FA) in naturally infected plants (Nadubinska et al., 2002). Maize seeds infected by *F. verticillioides* enter the food chain of animal and human with the potential of pro-ducing a mycotoxin that adversely affect the consumers. The mycotoxin occurs anywhere this fungus proliferates, causing chemical pollution of biological origin and setting (Bacon et al., 1992a, b, c; Merill et al., 1996a, b). The level of damages caused by this pathogen to maize production calls for the control measures that are cheaper, economically sound and environmentally safe to eliminate or reduce the incidence of this pathogen.

In Nigeria, the public is generally unaware of the hazard posed by this fungus on any infected seed. There is little information on how to reduce or eradicate its presence in maize either at pre - and post - harvest. The approaches to control pathogens have taken various from such as the use of chemicals, cultural control strategies and integrated methods (Macdonald and Chapman, 1997). The control measures sometimes have various setbacks even at the farmers' level of operation in Nigeria. This has raised the interest in developing alternative method of control from nature. This of course requires little or no skill; it is a cheap and readily available control measure, an environmentally friendly strategy for disease management which could inactivate the pathogen on maize.

Nature has a lot of plants and micro-organisms that can be used for disease control which is cleaner and safer in the environment, this is the use of botanicals and antagonistic organisms. In recent year, much attention has been given to non-chemical production against plant diseases due to environment pollution and health hazards caused by these chemicals, and various set-backs at farmers' level. This has raised the interest in developing alternative methods of control from nature. Moreover, some plant materials are found to be biocidal (Gurib-Fakim et al.,1996) among which are *Azadirachta indica* (Neem) and *Vernonia amygdalina* for the control of *Fusarium moniliforme* (now *F.verticillioides)* (Macdonald and Chapman,1997; Owolade et al., 2002). The abundance of plant on the earth surface has led to an increasing interest in the investigation of different extracts

obtained from traditional medicinal plants as potential sources of new antimicrobial agents (Bonar and Forrokha, 2004). This will help in reducing the health hazards and contaminations due to the use of synthetic fungicides. Efforts has been made to investigate the method, time of application of the botanicals and antagonistic microorganisms on maize stalk, as well as the toxin inactivation in maize using this control strategy especially in Nigeria (Sobowale *et al.*, 2007). It was reported that the extracts prepared from plants have variety of insecticidal properties against pests such as toxicity to nematode, mites and other agricultural pests. They also have antifungal, antiviral and antibacterial properties against pathogens (Charleston, 2002; Bushra and Genga, 2003).

However, since most farmers do not have resource to purchase nor apply expensive synthetic pesticides, using biological control in form of locally natural enemies together with the preparation of plant extracts from tree growing in the surrounding area, have little to no cost and are therefore uniquely suited for integrated pest management (Charleston, 2002).

The chemical analysis of extract from leaf of *Tithonia* showed that they contain sequiterpene lactones (Achakzai, 2006; Gu, 2002), diversifolia methyl ether and Tirotundin as active component against inflammatorty activity (Rungeler et al., 1998; Kuo and Chen, 1997). Methanol extracts of *M. lucida* showed anagelsic antipyretic effects and potentiated phenobarbitone sleeping time (Awe et al., 1998).

Trichoderma species are fungi that are present in nearly all soils; they frequently are the most prevalent cultivable fungi (Kubreek et al., 1998, Ubalua and Oti 2007). They are common inhabitants of the rhizosphere and are well recognized as biological agents of soil-borne plant pathogens (Chet, 1987). The efficient use of rhizosphere micro-organisms to plant pathogens has been reported worldwide in different plants (Cook, 1993). *Bacillus subtilis* is used as soil inoculants in horticulture and agriculture, it is used as a model organism for laboratory studies (Baker et al., 1983), and it has a natural fungicidal activity (Enzeby, 2008; Mazza, 1994). Due to these potentials of these plants and microorganisms, their efficiencies were also tested on *F. verticillioides* pathogen of maize. The objective of this work was to evaluate the antifungal potentials of two (2) botanicals and antagonistic of four (4) microbial agents on *Fusarium* stalk rot pathogen of maize.

MATERIALS AND METHODS

Isolation of *F. verticillioides* and antagonistic organisms

Maize sample that was infected with *F. verticillioides* was collected from farm and was taken to the laboratory. The infected samples were cut into pieces and surface sterilized for 2 min in 10% hypochlorite solution and rinsed in five changes of sterilized distilled water before planting them on PDA in Petri plates. Morphological

Table 1. Effect of antagonistic organisms on the growth of *Fusarium verticillioides.*

Treatment	Mean growth (cm)	Inhibition of the pathogen (%)
B. subtilis	2.22b	0.89
T. pseudokoningii	2.14c	4.46
T. harzianum	2.22b	0.89
T. viride	2.29a	-2.23
Control	2.24ab	

Mean with the same letter are not significant at (P<0.05).

identification of the pathogen, *F. verticillioides* was done using the criteria of Gerlach and Nirenberg (1982) and Leslie and Summerell (2006). Methods employed in isolating antagonistic organisms were the soil plate method (Warcup, 1950) and soil dilution plate method (Tuite, 1969). Identifications were performed by using the keys provided by Rifai (1969) and Bissett (1991) for the *Trichoderma* species, and *B. subtilis* was identified using Zheng et al. (2008) method.

The effect of antagonistic microorganisms on target organism was done by pairing the antagonists with the target organism. Cork borer of 5 mm diameter was used to make 5 holes in the solidified PDA, where the target pathogen was placed in four holes at the side of the plate and antagonist was placed at the middle hole. Also, all the materials were incubated at room temperature. The measurement of rate of growth of the fungus was taken at 24 h interval until there was no further growth.

Preparation of plant extracts

The leaves of *T. diversifolia* and *M. lucida* were oven dried at 60°C for 48 h and after which they were blended, using Binatone Blender. Twenty grams of each blended leaves were measured and poured in Elemeyer flasks. Sterile distilled water of 200 ml was added to each of flask, soaked for 2 h, and then filtered with a white muslin cloth into a 250 ml beaker. 5 and 3 ml of the filtrate were released into the plate with the molten 10 and 12 ml of PDA respectively, and allowed to solidify.

In vitro study of *F. verticillioides*' growth suppression

The potency of two plant extracts on *F. verticillioides* growth and inhibition was investigated. Inoculating needle was used to take *F. verticillioides* into the extract-containing (poisoned) PDA in Petri dishes to check for its growth and PDA without extract served as the control. The extracts were then incorporated as 5 and 3 ml into the agar. Each treatment was replicated 3 times.

All plates were incubated at room temperature (28 ±2°C) and the measurement of the growth of the fungus was taken with a metre ruler, at every 24 h interval until there was no further growth. The fungal growth rate on extract incorporated agar plates (using a metre rule to measure the radial growth) were compared with the control that is non extract incorporated agar plates. The difference in growth rate represented the activities of the extract in inhibiting the growth of the fungus. Laid out in a 2 x 5 factorial experiment in a Completely Randomized Design (CRD) for the antagonists (2 locations and 5 treatments: 4 antagonistic organisms + control) and 2 locations x 2 botanicals, factorial experiment for the botanicals were used. Data collected was subjected to statistical analysis using SAS, 2001 and means were separated by least significant difference (LSD).

RESULTS AND DISCUSSION

Effect of treatments and location on the growth of *F. verticillioides*

The mycelium of *F. verticillioides* appeared pinkish in colour with white fluffy at the top. The antagonistic organisms such as *B. subtillis, T. harzianum, T. pseudokoningii, T. viride* were inoculated at the middle and at the side of the media culture. *B. subtilis* was inoculated at the middle of the growth medium and the growth of *F. verticillioides* was 0.44 cm on the second day and increased to 1.85 cm on the seventh day. The observation showed that the *B. subtilis* grew slowly compared with the growth on the control plate (Table 1); had 0.89% inhibition on the growth of the pathogen, which is similar in action to the effect of *T. harzianum*, *T. harzianum* that had 0.89% growth inhibition of *F. verticillioides*. *T. pseudokoningii* had 4.46% inhibition of the pathogen.

The growth of target organism, *F. verticillioides* was inhibited by *T. pseudokoningii*. The mycelium growth of *F. verticillioides* paired with *T. pseudokoningii* on the second day was 1.23 cm and on the seventh day it grew to 2.48 cm, which was significantly different from the control plates. The mycelium of *T. pseudokoningii* appeared greenish in colour and fully occupies the plate, covering up the pathogen. (Table 1)

For *T. harzianum,* the mycelium appeared green with white fluffy parches, and suppressed the growth of *F. verticillioides.* The mycelium growth of *F. verticillioides* was 1.50 cm second day and increased to 2.47 cm seventh day. There was significant difference compared with the control plate which showed inhibition of *F. verticillioides'* growth at these times (Table 1).

For *T. viride,* the mycelium appeared in ring shape, greenish at the tip and white at the centre. *T. viride* fully occupy the plate at 7 days of incubation. It inhibited the growth of target organism, *F. verticillioides.* The growth of *F. verticillioides* on the second day was 1.81 cm and seventh day 2.46 cm; there were significant differences when compared with the control plate, which had higher growth of the pathogen (Table 1).

There is significant difference in the location effect irrespective of the antagonistic organisms paired with the

Table 2. Effect of location pairing on the *Fusarium verticillioides* with the antagonistic organisms.

Location	Mean growth (cm)
Centre	1.29[b]
Side	2.75[a]

Mean with the same letter are not significant at (P<0.05)

Table 3. Effect of location and bioagents on growth of *Fusarium verticillioides* at 120 hof incubation.

Location	Treatment	Mean growth (cm)	Percentage inhibition (%)
	B. subtilis	1.83	63.27
	T. pseudokoningii	0.72	68.14
Centre	*T. harzianum*	0.81	64.15
	T. viride	0.83	63.27
	Control	2.26	
	B. subtilis	0.61	73.01
	T. pseudokoningii	3.56	13.27
Side	*T. harzianum*	3.64	16.81
	T. viride	3.75	21.68
	Control	2.26	
L X T		*	

L, location; T, treatment; *, significant at P = 0.05.

pathogen (Table 2). When *F. verticillioides* was placed at the centre of the antagonistic organism, there was significant difference compared with when it was placed at the edge of the plate. The mean value for centre and side were 1.29 and 2.75 cm respectively. The centre pairing had higher inhibitory effect on *F. verticillioides* than the side pairing (Table 2).

The mean for location by the treatment, had significant difference between location and treatment (Table 3) at 120 h of incubation. When *T. harzianum* and *T. viride* were placed at the centre of the plates paired with *F. verticillioides*, the mean growth were 0.81 and 0.83 cm of *F. verticillioides* respectively which gave percentage inhibition of 64.15 and 63.27% respectively while *B. subtilis* and *T. viride* were not significantly different from each other. When *F. verticillioides* was placed at the side of the plates, paired with the antagonists, its growth was more than when placed at the centre. *B. subtilis* at this location gave the 73.01% highest inhibitory effect on the pathogen of all the antagonists tested and the least effective was *T. pseudokoningii* with 13.27% inhibition. The effect of time shows that there was significant difference at the times the observation was done in all the treatments. When the targeted organism, *F. verticillioides* was at the centre of the plate, *T. pseudokoningii* had the

highest inhibitory effect and *B. subtilis* had the lowest inhibitory effect. Also, when the *F. verticillioides* was placed at the side *B. subtilis* have lowest growth (0.60 cm) and *T. viride* had highest growth of 3.75 cm.

The result shows that there was significant difference between treatment and the time taken (Table 4). *B. subtilis* had the mean value of 0.44 and 1.85 cm during the second and the seventh days. *F. verticillioides* alone had 1.05 and 3.39 cm and *T. pseudokoningii*, *T. harzianum* and *T. viride* had 1.05 and 3.39 cm, 1.23 and 2.48 cm, 1.50 and 2.47 cm, 1.81 and 2.46 cm respectively during the second and seventh days.

Effects of plant extracts on *F. verticillioides*

Different concentrations of extract of *M. lucida* and *T. diversifolia* were incorporated into agar at different volumes of 3 and 5 ml and the poisoned agar was inoculated with 0.5 cm of *F. verticillioides.* After two days of inoculation, the growth of the organism on the concentrations of *M. lucida* extract were 1.93 and 1.80 cm respectively. By the fifth day, the growth were 3.26 and 3.16 cm respectively. The percentage inhibition of *F. verticillioides* due to the 3 and 5 ml of *M. lucida* extract

Table 4. Effect of location, time and bioagents on the growth of *Fusarium verticillioides.*

Location	Time	*T. harzianum*	*T. pseudokoningii*	*T. viride*	*B. subtilis*	Control
	48	7.58	9.00	5.83	5.20	10.50
	72	9.25	10.25	6.66	11.00	13.33
	96	9.83	9.25	8.16	15.62	19.33
Centre	120	8.41	8.16	7.91	18.62	23.75
	144	7.25	7.33	7.41	22.62	26.83
	168	7.16	7.08	7.58	25.62	31.08
	192	7.16	7.08	7.33	30.00	33.91
	48	22.41	27.33	18.91	3.62	10.50
	72	24.41	31.41	21.58	5.75	13.33
	96	39.33	36.16	40.25	6.25	19.33
Side	120	41.75	41.41	41.83	6.25	20.00
	144	41.91	41.83	42.25	6.75	26.83
	168	42.25	42.25	42.41	7.00	31.08
	192	42.25	42.25	42.41	7.00	33.91
Sign.		S	S	S	S	S

S, Significant at 5%.

Table 5. Growth of *F. verticillioides* on PDA incorporated with two different concentrations of two plant extracts at 144 h of incubation.

Treatment	Concentration (ml)	48 h	72 h	96 h	120 h	Inhibition (%) at 120 h
M. lucida	3	1.93[d]	2.46[d]	2.90[d]	3.26[c]	56.14
	5	1.80[d]	3.06[c]	3.13[c]	3.16[c]	57.49
T. diversifolia	3	2.53[d]	2.96[d]	3.20[c]	3.90[c]	47.65
	5	2.76[d]	3.63[c]	3.63[c]	3.63[c]	51.24
Control	-	3.35[c]	4.20[b]	4.90[b]	7.45[a]	

Mean (in cm) with the same letters are not significantly different P<0.05.

incorporated into the growth media were 56.14 and 57.49% respectively at 5 days of incubation.

This observation shows that the growth of target organism on poisoned agar plate was reduced compared with the growth on the control plates which were not poison with extract of *M. lucida* (Table 5). There was significant difference in the growth of *F. verticillioides* on different concentration of *M. lucida* extracts. The 5 ml was observed to be the most effective and 3 ml was the least effective (Table 5).

After 48 h of inoculation (two days), the mycelium growth of the organism on different concentration of 3 and 5ml of *T. diversifolia* were 2.53 and 2.76 cm respectively. By the fifth day, the growths of target organism on the concentrations of *T. diversifolia* were 3.90 and 3.63 cm respectively. The percentage inhibition of *F. verticillioides* due to the 3 and 5 ml of *T. diversifolia* extract incorporated into the growth media; were 47.65 and 51.24% respectively at 5 days of incubation.

This shows that the growth of target organism was reduced when compared with the growth on the control plate which was not poisoned with extract of *T. diversifolia*. In past researches, some of the bioactive compounds that have been isolated from the leaves of *T. diversifolia* included sesquiterpenes, saponins and alkaloids (Rurgler, 1998; Tona et al., 2000; Liasu and Achakzai, 2000) and this made *T. diversifolia* active in inhibiting mycelia growth of *C. lunata*. Methanol extracts of *M. lucida* shows analgesic, antipyretic effects and potentiated phenobarbitone sleeping time (Awe et al., 1998). Kemabonta and Okogbue (2000) reported efficacy of *M. lucida* in causing mortality of adult *C. maculatus* and reduction of oviposition and first filia generation emergence. The major constituents of *M. lucida* as reported by Nweze et al. (2004) and Akinyemi et al. (2005) are anthraquinones and anthraquinols. The most effective of the plant extracts was *T. diversifolia,* followed by extract of *M. lucida*. Extract of *M. lucida* also had inhibitory effect but not as much as the extracts of *T. diversifolia*.

The aim of finding alternative to synthetic fungicide use in Agriculture has led to the search for plant materials with biocidal properties and the use of antagonistic organisms. Therefore, the results of this investigation indicated that antagonistic organisms such as *T. viride, T. harzianum, T. pseudokoningii* and *B. subtilis* are toxic to *F. verticillioides.* The extract of *M. lucida* and *T. diversifolia* are also effective in the control of *Fusarium verticillioides* by inhibiting its growth *in vitro.* These plant extracts and the antagonistic organisms caused significant differences on the growth of *F. verticillioides.*

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Optimizing fermentation conditions for fructosyltransferase enzyme production by *Lactobacillus plantarum*

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Enzymatic production of fructooligosaccharides (FOS) from sucrose using β-D fructosyltransferase is commonly employed in commercial scale. FOS are low calorie sweetners with prebiotic property widely used as functional food material. In the present study, a strain of *Lactobacillus plantarum* (LABF 16) was found to produce bioactive oligosugars such as kestose and nystose exhibiting fructosyltransferase (FTase) enzyme activity. Further maximization of FTase production by the particular isolate was also attempted. The fermentation parameters *viz.*, pH, temperature and sucrose concentration, which was found to influence the fructosyltransferase yield, were optimized by response surface methodology. The optimized conditions for the FTase production were pH 6.33, temperature of 32°C, sucrose concentration of 20.74 g/l, which resulted in an enzymatic activity of 129.62 U/ml/min.

Key words: Fructooligosaccharides, *Lactobacillus*, response surface methodology.

INTRODUCTION

Bioactive oligosaccharides are gaining outstanding popularity because of their excellent functionalities such as being low calorific, non-carcinogenicity and acting as a growth factor for useful microorganism in the intestinal microflora (Nadeau, 2000; Urgell and Orleans, 2001; Patel and Goyal, 2010). Among commercially available dietary sugars, fructooligosaccharides (FOS) possess extraordinary importance as functional food ingredients owing to their prebiotic properties by beneficially affecting the health of the host by selectively stimulating the growth and/or activity of certain gastrointestinal bacteria. Furthermore, their sweet taste is similar to that of sucrose, a traditional sweetener (Huebner et al., 2007). The health benefits associated with FOS includes

activation of the human immune system, resistance to infection, enhanced mineral absorption in the gastrointestinal tract, synthesis of B-complex vitamins, lowering of serum cholesterol, suppressing prevalence to diarrhea and preventing carcinogenic tumours (Cummings and Roberfroid, 1997). FOS has received Generally Recorded As Safe (GRAS) status from the Food and Drug Administration (FDA)-U.S. (Godshall, 2007).

The main commercial production of FOS is carried out by enzymatic transformation of sucrose by the microbial enzyme fructosyltransferase (FTase). Two classes of enzymes are particularly useful for FOS' production at industrial scale: FTases (EC 2.4.1.9) and

Table 1. Range of values for the response surface method.

Independent variable	Level				
	-α	-1	0	+1	+α
Sucrose concentration (g/l)	11.59	15	20	25	28.40
Temperature (°C)	26	28	30	32	33
pH	4.3	5	6	7	7.6

fructofuranosidases, also called invertases (EC 3.2.1.26). FTases possess higher transferring activity than fructofuranosidases (Antosova and Polakovic, 2001). Many lactobacilli exhibit extracellular FTase activity on sucrose (Korakli et al., 2003).

Recent research efforts have focused on optimization of process for improved FTase enzyme production. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of various factors and searching for the optimum conditions. RSM has been successfully used in the optimization of media components in various bioprocesses such as of protease (Adinarayana and Ellaiah, 2002), xylanase (Park et al., 2002), mycophenolic acid (Sadhukhan et al., 1999) and lactic acid production (Hujanen et al., 2001).

The present study was aimed to identify the influential fermentative parameters to increase fructosyltransferase enzyme production by *Lactobacillus plantarum* (LABF 16) isolated from fermented maize product. RSM was used for further optimization of influential process variables to enhance the yield of enzyme.

MATERIALS AND METHODS

Organism and culture condition

The high fructosyltransferase activity exhibiting strain *L. plantarum* (LABF 16) was isolated from fermented maize product. Microorganism was cultured at 37°C in MRS agar medium (De man et al., 1960) containing the following components per liter of distilled water: peptone (10 g), yeast extract (4g), beef extract (8 g), glucose (10 g), $K_2HPO_4.3H_2O$ (2 g), sodium acetate (5 g), tri ammonium citrate (2 g), $MgSO_4.2H_2O$ (0.2 g), $MnSO_4.H_2O$ (0.5 g), Tween 80 (1 ml). Culture was incubated at 37°C in an incubator for 48 h.

Oligosaccharide identification

For the detection of oligosaccharides, the end products of enzyme reaction (1 µl) were spotted on a thin layer chromatography (TLC) ready foil. The TLC foils were run in choloroform : acetic acid : water (6:7:1) and the sugars were specifically stained with diphenyl amine (1.8% w/v) and aniline in phosphoric acid. Sigma grade 1- kestose (GF_2), 1-nystose (GF_3), glucose and sucrose were used as reference sugars.

Assay for fructosyltransferase

The reaction mixture for the assay of FTase activity consisted of 1.5 ml of 60% (w/v) sucrose in 0.1 M citrate buffer (pH 5.5) and 0.5 ml

crude enzyme-culture fluid. The reaction was carried out at 55 ± 1°C for 1 h using a water bath. The reaction was terminated by keeping the reaction mixture in boiling water bath for 15 min. Glucose released at the end of the reaction was estimated using dinitrosalicylic acid (DNS) method (Debois et al., 1956). One unit of FTase activity was defined as the amount of enzyme required to release 1 µmol of glucose released per ml per minute under the above mentioned reaction conditions (Park et al., 2001; Sangeetha et al., 2004).

Effect of different sucrose concentration on FTase production

Sucrose was added to the MRS broth in the range of 10 to 60 g/l. Culture was inoculated and incubated for 48 h at 32°C, 200 rpm in a rotary shaking incubator. The cell was removed from culture broth by centrifugation at 6000 rpm for 10 min and the cell-free culture fluid serve as a crude enzyme source. The enzyme activity was determined by dinitrosalicylic acid (DNS) method (Debois et al., 1956).

Effect of pH and temperature on FTase production

Fifty milliliters of MRS broth containing optimized concentration of sucrose was prepared in 250 ml Erlenmeyer flasks. The pH of MRS media was adjusted to values ranging from 4 to 7 with 4 N NaOH or HCl. The optimum temperature was determined by incubating the inoculated flasks at temperature range from 28-37°C for 48 h. FTase activity was determined by the procedure as described earlier.

Experimental design and statistical analysis

A set of 20 experiments was performed to optimize fermentative parameters for FTase production. Three independent variables, including sucrose concentration, temperature and pH were studied at five different levels (Table 1). One of the response surface methodologies, central composite design was employed for experimental design (Design Expert version 7.0, State-Ease inc., Minneapolis, U.S.A.). The regression and graphical analysis of the data was obtained. The variables were coded according to the Equation 1:

$$x_i = \frac{X_i - \overline{X}_i}{\Delta x_j}, \qquad (i = 1, 2, 3, \ldots\ldots\ldots, k) \tag{1}$$

Where, x_i = Dimensionless value of independent variables, X_i = real value of independent variable, $\overline{X}_i$ = real value of the independent variable at the centre point, Δx_3 = the step change.

The behavior of the system was explained by following the second degree polynomial Equation 2:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{23} X_2 X_3 + b_{13} X_1 X_3 \tag{2}$$

Where, Y is predicted response, X_1, X_2, X_3 are independent variables, b_0 is offset term, b_1, b_2, b_3 are linear effect, b_{11}, b_{23}, b_{33} are squared effects and b_{12}, b_{13}, b_{33} are interaction terms.

RESULTS AND DISCUSSION

Plant associated lactic acid bacteria produce a large
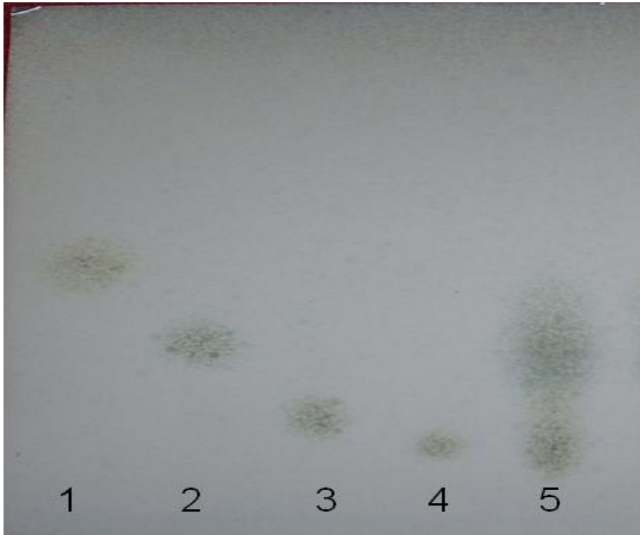
Figure 1. Oligosaccharide detection by thin layer chromotography (TLC). 1 - glucose, 2 - sucrose, 3 - kestose, 4 - nystose, 5 - enzymatic product of *L. plantarum* (LABF 16).
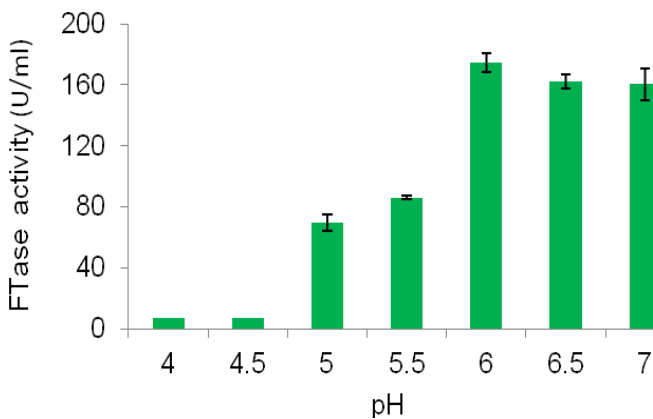
Figure 3. Effect of pH on FTase production by *Lactobacillus plantarum* (LABF 16). Values represent mean (± SE) of three replicates. (n=3). Values are significantly different at p=0.05.

variety of exopolysaccharide (EPS) and oligosaccharide from sucrose through the activity of glycosyltransferases (Tieking and Ganzle, 2005). The *L. plantarum* (LABF 16) used in this study was found to exhibit FTase enzyme activity and produce bioactive kestose and nystose sugars.

Oligosaccharides detection

Bacterial fructansucrase enzyme by catalase transfructosylation reactions with sucrose, resulted in the synthesis of fructooligosaccharide and/or a fructan polymer and this enzyme belongs to the glycosidic hydrolase
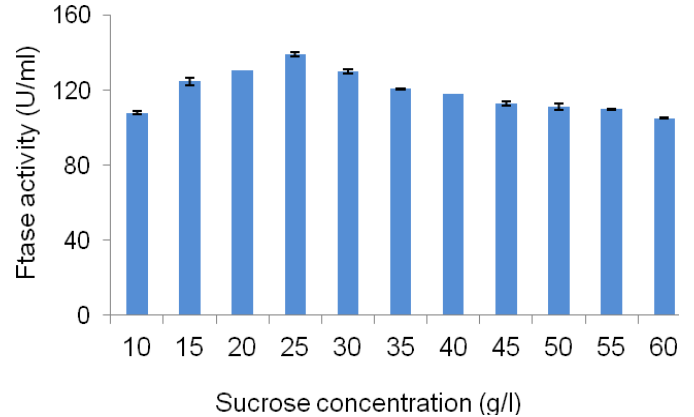
Figure 2. Effect of sucrose concentration on FTase production *by Lactobacillus plantarum* (LABF 16). Values represent mean (± SE) of three replicates (n=3). Values are significantly different at p=0.05.

family (Ozimek et al., 2006). The fructan transferring reaction of FTase enables the production of beneficial oligosaccharides, and also maintains a similar sweetness level as well as preventing unfavorable polymer synthesis. TLC analysis showed evidence for the accumulation of short chain oligosaccharides or FOS namely kestose and nystose at the application site (Figure 1).

Effect of sucrose concentration on FTase enzyme production

Of the various concentrations of sucrose tested with crude enzyme, the maximum enzyme activity of 130.32 ± 0.04 and 139.23 ± 1.03 U/ml was observed at 25 g/l sucrose concentration (Figure 2). At high sucrose concentration, FTase activity was suppressed in lactic acid bacterial isolate, possibly due to substrate repression (Vandamme and Deryeke, 1983; Singh and Gill, 2006).

The effect of pH and temperature on fructosyltransferase enzyme production

The pH of medium plays an important role in the FTase production and microbial growth. The influential pH for supporting higher FTase production of present study was pH 6 (Figure 3).

The influence of temperature on FTase activity was investigated by measuring activity in the temperature range of 28 to 37°C and maximum activity (160.54±12.6 U/ml) was exhibited at 32°C (Figure 4).

Optimization of fermentative parameters using statistical analysis

Many studies have investigated the effect of culture
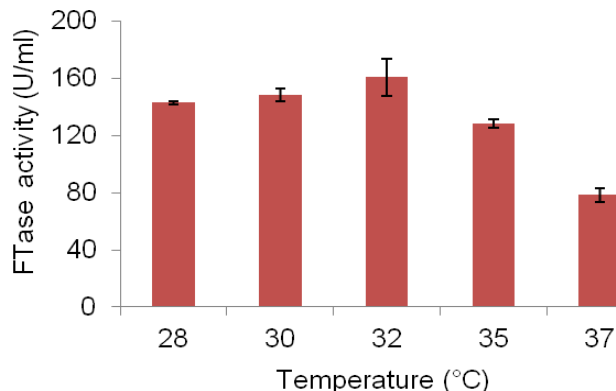
Figure 4. Effect of temperature on FTase production by *Lactobacillus plantarum* (LABF16). Values represent mean (± SE) of three replicates. (n=3). Values are significantly different at p=0.05.

Table 2. Central composite design matrix of independent variables with corresponding experimental and predicted values of FTase enzyme activity for influencing fermentative parameters.

Run order	Initial pH		Temperature		Sucrose concentration		Enzyme activity (U/ml)*	
	X_1	code x_1	X_2 (°C)	code x_2	X_3 (g/l)	code x_3	Experimental	Predicted
1	5	-1	28	-1	15	-1	66.92	61.23
2	7	+1	28	-1	15	-1	53.34	51.22
3	5	-1	32	+1	15	-1	100.86	100.33
4	7	+1	32	+1	15	-1	116.19	115.41
5	5	-1	28	-1	25	+1	71.246	63.59
6	7	+1	28	-1	25	+1	78.03	70.13
7	5	-1	32	+1	25	+1	96.814	90.50
8	7	+1	32	+1	25	+1	124.85	122.11
9	4.32	-1.682	30	0	20	0	40.77	48.72
10	7.68	+1.682	30	0	20	0	62.90	66.89
11	6	0	26.64	-1.682	20	0	46.27	56.11
12	6	0	33.36	+1.682	20	0	130.60	132.71
13	6	0	30	0	11.59	-1.682	100.00	101.46
14	6	0	30	0	28.41	+1.682	98.50	109.08
15	6	0	30	0	20	0	114.19	110.78
16	6	0	30	0	20	0	102.96	110.78
17	6	0	30	0	20	0	112.46	110.78
18	6	0	30	0	20	0	113.74	110.78
19	6	0	30	0	20	0	102.87	110.78
20	6	0	30	0	20	0	120.49	110.78

*μmol of glucose released/ml /min.

conditions and medium components on cell growth, enzyme production and rheological properties during fermentation using RSM (Kristo et al., 2003; Muralidhar et al., 2003). In our experiment, to obtain maximum production of FTase, the best combination of pH, temperature and sucrose concentration were determined by using RSM. The central points for this design were selected based on results obtained from the above experimental results. The design matrix explaining the experimental plan with coded and uncoded value of the independent variables are shown in Table 2.

A second order regression equation shows the

Table 3. Analysis of variance (ANOVA) for the selected model.

Source	Sum of squares	Degrees of freedom	Mean square	F value	P>F
Model	13357.90	9	1484.21	20.01	0.0001
Error	741.64	10	74.16		
Corrected total	14099.55	19			

Coefficient of variation = 7.0%; Coefficient of determination (R^2) = 0.9474, correlation coefficient (R) = 0.9733.

Table 4. The least-squares fit and the parameter estimates (significant of regression coefficient).

Model term	Parameter estimate	Computed t	P- value
Intercept	110.78	3.51	<0.0001*
x_1	2.27	2.33	0.3539
x_2	22.77	2.33	<0.0001*
x_3	5.40	2.33	0.0429
x_1x_2	-3.05	3.04	0.3400
x_2x_3	6.27	3.04	0.0665
x_1x_3	4.13	3.09	0.2044
x_1^2	-1.95	2.27	0.4105
x_2^2	-5.79	2.27	0.0288
x_3^2	-18.73	2.27	<0.0001*

dependency of output responses as function of pH, temperature and sucrose concentration. The parameters of the equation were obtained by multiple regression analysis on the experimental data.

The following second order polynomial equation explains the experimental data:

$$Y = -1799.8 + 7.76\ x_1 + 85.52\ x_2 + 119.564\ x_3 - 0.077\ x_1^2 - 1.44x_2^2 - 18.72x_3^2 - 0.30\ x_1\ x_2 + 3.13x_2\ x_3 + 0.82\ x_1x_3$$

Where, x_1, x_2, x_3 are the coded values of sucrose concentration, temperature, initial pH of medium respectively. Analysis of variance for the selected model is shown in Table 3. The coefficient of correlation (R) was 0.9733 which indicates a good agreement between experimental and predicted values. The coefficient of determination (R^2=0.9474) is also very high, making the model very significant. The parameter estimated and the corresponding p values (Table 4) suggest that sucrose concentration and temperature are the main factors affecting FTase production.

Results obtained from experimental design showed that increased sucrose concentration was the main consideration of process parameters. This is in agreement with many reports for transferase activity from filamentous fungi (Rehm et al., 1998), yeasts (Boon et al., 2000) and bacteria (Rabiu et al., 2001). The strong effect of substrate concentration observed in our experiments suggested that FTase production could be substantially increased by increasing the sucrose concentration. Moreover, transfructosylating activity was exhibited only

under higher sucrose concentration in *Aspergillus* sp. (Chien et al., 2001; Cuervo et al., 2004). While the strain of *L. plantarum* (LABF 16) used in our study exhibited fructosyltransferse activity only upto 25 g/l of sucrose concentration. The concentration of sucrose above 25 g/l resulted in reduction of enzyme production. Lower cell growth observed for this culture when it is grown in the MRS medium containing more than 25 g/l sucrose might have resulted in lowered enzyme production for this culture.

The initial pH of medium plays a key role in enzyme production and in utilization of media constituents and growth of the microorganism (Kim et al., 2000; Bonnin and Thibault, 1996). Although we did not find pH as main effect, it was involved in one significant interaction. To keep both pH and temperature at high levels was influential to the enzyme production. In addition, the positive effect of sucrose concentration on the FTase production could be further enhanced by increased temperature due to the interaction between sucrose concentration and temperature (Figure 5). By adopting the RSM, the optimal set of conditions for maximum FTase production was as follows: sucrose concentration of 20.74 g/l, pH 6.33 and temperature of 32°C. At the optimal conditions, a maximum FTase activity of 129.62 U/ml was obtained in batch fermentation process (Table 5).

Conflict of Interests

The author(s) have not declared any conflict of interests.
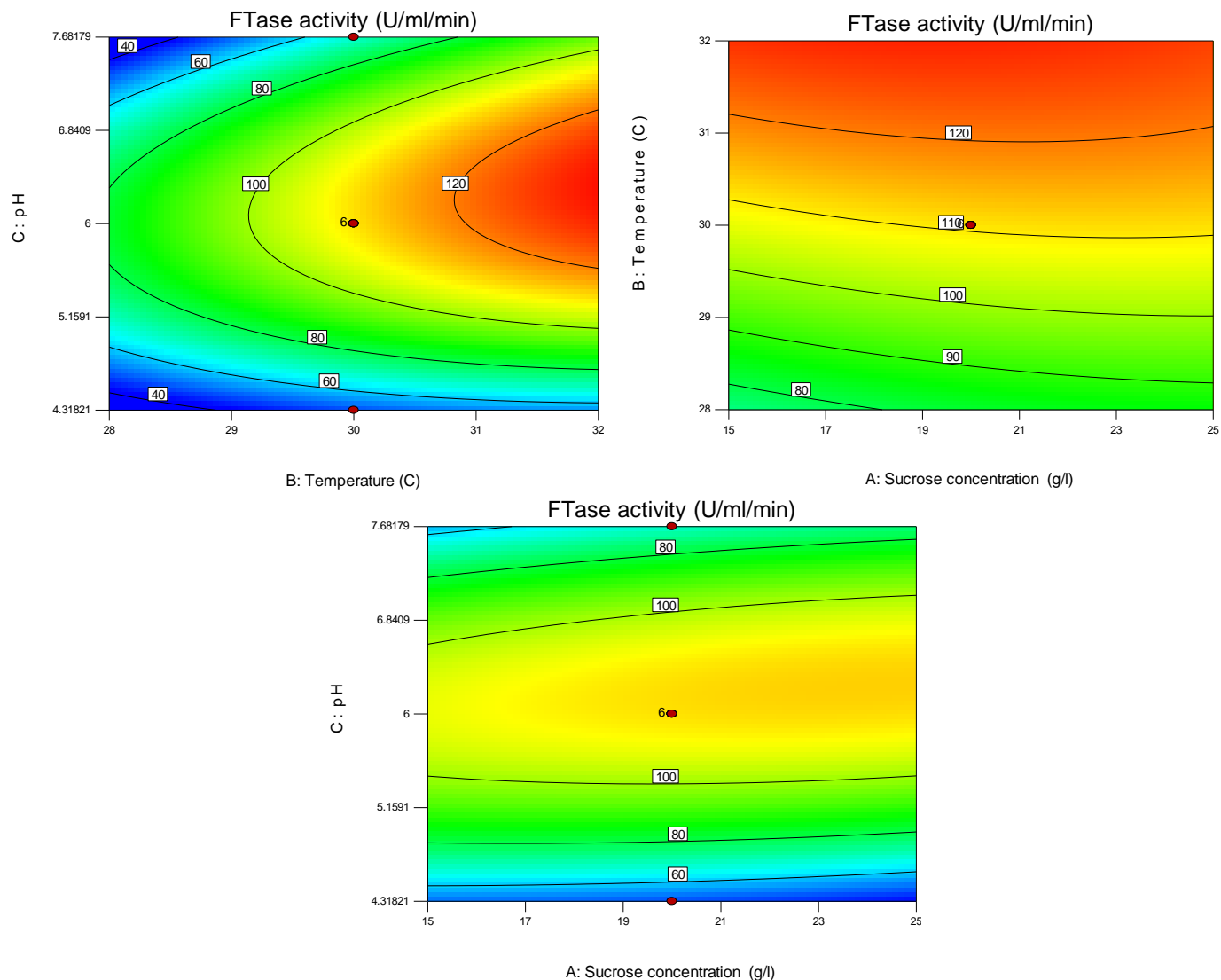
Figure 5. Isoresponse contour plots showing the effect of sucrose concentration, pH and temperature on fructosyltransferase enzyme production by *L. plantarum* (LABF 16)

Table 5. Predicted influential fermentative parameters for maximum fructosyltransferase enzyme activity.

Dependent variable	Independent variable			Enzyme activity (U/ml)*
	pH	Temperature	Sucrose concentration (g/l)	
Fructosyltransferase enzyme activity (U/ml)	6.33	32	20.74	129.62

*µmol of glucose released/ml /min.

ACKNOWLEDGEMENT

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Full Length Research paper

Growth enhancement in vegetable crops by multifunctional resident plant growth promoting rhizobacteria under tropical Island Ecosystem

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Plant growth promoting rhizobacteria have been used to improve crop production. A total of 114 bacterial isolates were recovered from the rhizosphere soils of healthy plantation and vegetable crops grown in Andaman Islands. These isolates were evaluated *in vitro* for plant growth promoting traits, hydrolytic enzyme production and antagonistic activity. Among the isolates, NPB6 and MNB1 were positive to 8 out of 10 properties tested followed by NFB3, MKP3, NNB4 and NTB2. In the dual culture assay, NFB3 showed highest inhibition against the plant pathogen *Macrophomina* spp (33.3%) and *S. rolfsii* (23%). Six most promising isolates were selected and identified as *Bacillus* and *Pseudomonas* spp. on the basis of Microbial Identification System and 16S rDNA. These isolates significantly increased the seed germination, vigor index, radical and plumule length however, the individual isolates effect varies with crop. All the six isolates enhanced the root and shoot length of brinjal, chilli and okra seedlings while *Bacillus cereus* (NPB6) in brinjal, *B. stratosphericus* (NFB3) in chilli and *Pseudomonas fluorescens* (NNB4) in okra were most effective. The promising isolates can be used in combination with other beneficial native microbes for plant growth promotion even under abiotic stresses and promotion of organic agriculture.

Key words: Biolog, plant growth promotion, rhizobacteria, vegetables.

INTRODUCTION

Soil micro-organisms belonging to different groups are known to play prominent role in plant-soil interactions at the rhizosphere (Mantelin and Touraine, 2004). Among them bacteria that colonize the rhizosphere (rhizobacteria) are very important and beneficial for plant health. They are referred to as plant growth promoting bacteria (PGPB) or plant-growth promoting rhizobacteria (PGPR) (Kloepper, 1993). The principal mechanisms of growth

promotion include production of growth stimulating phyto-hormones, solubilization and mobilization of phosphate and siderophore production whereas antibiosis, inhibition of plant ethylene synthesis and induction of plant systemic resistance against pathogens are favourable health effects (Gutierrez-Manero et al., 2001; Whipps, 2001; Idris et al., 2007; (Gutierrez-Manero et al., 2001; Whipps, 2001; Idris et al., 2007; Richardson et al., 2009). However, only 1–2% of rhizobacteria are known to promote plant growth, which are associated with many plant species and commonly present in varied environments (Antoun and Kloepper, 2001). Some of the most promising genera reported to exhibit plant growth promotion are *Bacillus*, *Pseudomonas, Azospirillum*, *Azotobacter*, *Enterobacter* and *Serratia* (Hurek and Reinhold-Hurek, 2003).

The interactions of rhizobacteria in the rhizosphere soil play a pivotal role in transformation, mobilization, solubi-lization of nutrient, from the nutrient pool in the soil and subsequent uptake by the crops. As a result, PGPR increases germination rate, root length, leaf area, chloro-phyll content, protein content, nitrogen content, tolerance to drought, shoot and root weight and delayed leaf senes-cence (Cakmakci, 2005; Babalola et al., 2006). Therefore, soil microorganisms including PGPR play prominent role in the maintenance of soil health (Lucy et al., 2004).

A significant increase in growth and yield of cowpea and other important crops in response to seed bacterization with PGPRs have been reported by many workers (Amara and Dahdoh, 1997; Asghar et al., 2002; Minaxi et al., 2012). This considerably reduces the input cost on fertilizers and pesticides. In the tropical Islands of Andaman and Nicobar brinjal, tomato, chilli and okra are the major vegetable crops cultivated and consumed. Out of the total consumption of fertilizers and pesticides, a significant amount is used for these crops (DES, 2011). Keeping this in view, field survey was conducted to collect rhizosphere soil samples from different locations followed by *in vitro* and green house experiment was conducted to find out the promising native PGPR strains with multifunctional properties, which can be used for increasing the productivity of vegetable crops in these islands, in particular and other parts of the world, in general.

MATERIALS AND METHODS

Sampling site

The tropical island of Andaman and Nicobar is located in the Bay of Bengal 1200 km off the eastern coast of main land India. It experiences annual mean temperature of 32°C, annual rainfall of 3180 mm and high evaporation during the summer months. The rhizosphere soil samples from vegetable and plantation crops were collected from across North (13° 22'02.48''N-92° 58'05.61''E) and Middle Andaman Islands (12° 30'16.21''N-92° 55'34.13''E) and their cropping history were recorded. The soil samples were brought to the laboratory in an icebox and processed further for bacterial isolation.

Isolation of bacteria

Bacteria from the rhizosphere soil samples were isolated using serial dilution method on King's B agar and Nutrient agar medium. This was incubated at 28°C for 48 h. A total of 114 colonies were recovered on King's B agar and nutrient agar, which were purified with repeated culturing and maintained in 20% glycerol at -20°C. These isolates were used to study its plant growth promoting properties and antagonistic activities.

Assessment of plant growth promoting traits

Indole 3-Acetic Acid (IAA) production by the isolates was estimated by qualitative method (Naik et al., 2008) and it was further confirmed by quantitative estimation (Benizri et al., 1998). Bacteria were cultured overnight in Luria-Bertani broth in the dark at 30°C. The bacterial cells were removed from the culture medium by centrifugation at 8,000 *g* for 10 min. 1 ml of supernatant was mixed vigorously with 2 ml of Salkowski's reagent and incubated at room temperature in the dark for 30 min. The absorbance at 535 nm was measured and the concentration of IAA produced was estimated from a standard IAA graph and expressed as micrograms per milliliter (Patten and Glick, 1996).

Two days old pure bacterial culture grown in nutrient agar was streaked on King's B medium amended with an indicator dye. Change of blue color of the medium to yellow halo surrounding the bacterial growth indicated the production of siderophore. The reaction of each bacterial strain was scored either positive or negative to the assay (Schwyn and Neilands, 1987).

All bacterial isolates were screened for inorganic phosphate solubilization according to the method suggested by Verma et al. (2001). A loopful of fresh bacterial culture was streaked onto Pikovaskaya's medium amended with inorganic phosphate and the plates were incubated at 28±2°C for 4 days. A clear halo around the bacterial colony indicated solubilization of mineral phosphate.

Analysis of extracellular enzyme activity

Bacterial isolates were analyzed for production of six enzymes *viz.* amylase, cellulase, chitinase, lipase, protease and pectinase by plate method. Proteolytic activities of the cultures were screened qualitatively in a medium containing skimmed milk (HiMedia, Mumbai). Zones of precipitation of paracasein around the colonies appearing over the next 48 h were taken as evidence of proteolytic activity. For cellulase activity, mineral-salt agar plate containing 0.4% $(NH_4)_2SO_4$, 0.6% NaCl, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.01% $CaCl_2$ with 0.5% carboxy methyl cellulose (CMC) and 2% agar (Hi-Media) were surface inoculated. Iodine solution was used to detect cellulase activity as described by Kasama et al. (2008). The clear zone formation around the growing colony was considered as positive. The lipase activity of bacterial isolates was determined according to the diffusion agar methods in which nutrient agar medium was supplemented with $CaCl_2.H_2O$ 0.01%. Tween 80 was sterilized for 20 min at 121°C and added to the molten agar medium at 45°C to give a final concentration of 1%. The medium was shaken until the Tween 80 had dissolved completely and then poured onto Petri dishes. For positive test, an opaque halo that occurred around the colonies was considered as positive.

Antagonistic activities against plant pathogenic fungi

The antagonistic effects of bacterial isolates were tested against

two fungal plant pathogens namely: *Sclerotium rolfsii* and *Macrophomina* sp. For this, the bacterial isolates were streaked at a distance of 3.5 cm from rim of individual Petri plate containing potato dextrose agar (PDA) medium. 6 mm mycelial disc from a 7-day old PDA culture of fungal pathogens were then placed on the other side of the Petri dish and the plates were incubated at 28°C for 4 days. The percent inhibition was calculated by using the formula:

I = (C – T)/C * 100

Where, I is percent inhibition of mycelial growth over the control, C is mycelial growth of fungal pathogen in control plate and T is mycelial growth of fungus in bacteria inoculated plate. The experiment was carried out in three independent replicates.

Identification of the selected isolates with Biolog system

Six potential bacterial isolates were selected and tested for preliminary biochemical characterization as per standard methodologies (Collins and Lyne, 1980). The identities of the bacterial isolates were revealed on the basis of Biolog carbon source utilization. Bacterial suspensions were inoculated into Biolog GENIII Micro plates and incubated at 30°C for 24 h. The results were interpreted with Biolog Micro Log^{TM}, Release 4 software (Biolog, Hayward, CA).

16S rDNA amplification and phylogenetic analysis

Genomic DNA was extracted using the method described by Chen and Kuo (1993). The extracted DNA was dissolved in 20 µl TE buffer and used as a template for the Polymerase Chain Reaction (PCR). The PCR mix included 3 U of *Taq* DNA polymerase, 10X Taq buffer A, 2.5 mM concentrations of each dNTP (Bangalore GeNel, Bangalore India), 1 µl each of pA 5'-AGAGTTTGATCCTGGCTCAG-3' and pH 5'-AAGGAGGTGATCCAGCCGCA-3' primers (Edwards et al., 1989) and 20 ng of the template DNA in a total volume of 50 µl. Amplification of 16S rDNA was performed in a Gene Amp-PCR system 9700 (Applied Biosystems) under the following conditions: 130 s of denaturation at 92°C followed by 35 cycles of amplification with a 60 s denaturation at 92°C, 30 s of annealing at 48°C and 130 s of extension at 72°C. An extra extension step of 360 s at 72°C was added after completion of the 35 cycles. Amplified PCR products were sequenced and were aligned with 16S rDNA gene sequences available by the BLASTN search in the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). Phylogenetic tree was generated after performing multiple alignments (CLUSTAL W version). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances, phylogenetic dendrogram was constructed by the neighbour-joining method and the tree topologies were evaluated by performing bootstrap analysis of 1,000 dataset using MEGA 3.1 software (Kumar et al., 2004). The sequence obtained in this study was deposited in the GenBank nucleotide sequence database under the accession number JX885487, JX960423-26 and JX9604230 (6 sequences).

Seed germination rate and seedling vigour index

Brinjal (CARI Brinjal-1), chilli (Cv. K2) and okra (Cv. Kashi Lalima) seeds were surface-sterilized with 70% ethanol for 2 min and in 2%

sodium hypochlorite for 2 min followed by ten times washing in sterile distilled water. The surface sterilized seeds were inoculated by soaking in the respective rhizobacterial suspension (10^8 cfu/ml) for 45 min at 28 ± 2°C. The coated seeds were incubated in petri dishes lined with moist filter paper and incubated at 28 ± 2°C. Seeds immersed in sterilized distilled water served as control. The seed germination rate and vigor index were calculated after five days of incubation using the formula given below (Zucconi et al., 1981).

$$\text{Germination rate (\%)} = \frac{\text{Number of germinated seed}}{\text{Number of total seed tested}} \times 100\%$$

Plume and radicle lengths were recorded for the calculation of vigor index

Vigor index = (mean of plumule + radical lengths) x germination rate (%).

Pot experiment

Soil analysis

Physico-chemical characterization of experimental soil collected from the vegetable block of Central Agricultural Research Institute, Port Blair was carried out before filling the pots. The soil pH, EC, available potassium, phosphorous and nitrogen were determined as per the standard procedure (Jackson, 1973). Briefly, pH and EC was measured both in supernatant and in the suspension using pH and Electrical Conductivity meter. The soil was medium in 0.51% organic carbon content, available N (base hydrolysable N), available phosphorus (P) (Bray 1-P, 0.03 mol L^{-1} NH$_4$F + 0.025 mol l^{-1} HCl extractable), available potassium (K) (1 mol L^{-1} NH$_4$OAc extractable) were determined.

Preparation of inoculum and seed bacterization

Pure colony of each multi trait plant growth promoting (PGP) bacteria was inoculated in 100 ml of Nutrient Broth and incubated at 30 ± 2°C in an orbital incubator shaker for 3 days at 130 rpm. Seed bacterization was carried as per standard procedure of Silva et al. (2003). Three different varieties of vegetable crops *viz.*, brinjal, chilli and okra were used in this study. Seeds of brinjal, chilli and okra were surface-sterilized as mentioned above. Approximately, 500 seeds from each vegetable crop were immersed in appropriate inoculum of PGP bacterial for 1 h at a concentration of 10^8 cfu/ml in sterile saline water (0.85%). Bacterized seeds were then air dried in laminar air flow hood and sown immediately.

Experimental design

The treatments were arranged in a Completely Randomized Block Design (CRBD) and were placed on a platform in the greenhouse. The treatments were as follows: T1, control (seeds coated with media); T2, seeds coated with NFB3; T3, NPB6; T4, MKP3; T5, MNB1; T6, NNB4 and T7- NTB2. Ten seeds of each brinjal, chilli and okra were sown in each plastic pots of 15 cm diameter containing 1 kg of sterile field soil. The seedlings were grown at a temperature of 28-32°C and 85% relative humidity in a greenhouse under a day-night cycle of 11–13 h natural light. The soil was

moistened to 60% water-holding capacity and was maintained at this moisture content by watering to weight every day. Uninoculated seeds sown in pots served as control. In each of the treatment, the plants were harvested 3 weeks after the emergence of the seedlings. The morphological characteristics of each plant were recorded *viz.* root length, shoot length and total number of secondary root of each plant.

Statistical analysis

Data of morphological characteristics of seedlings were statistically analyzed using the general linear model software Agres (3.01) and Agdata. The means were compared using the least significant difference (LSD) method at P ≤ 0.05.

RESULTS

Distribution of rhizobacteria

There was a significant variation in the distribution of rhizobacteria in the soils samples of different crops. The total bacterial count in Middle Andaman samples ranged from 0.6 to 77 x 10^3 cfu ml^{-1} and most of the samples belong to the rhizosphere of spices. In North Andaman samples, the value ranged from 1.6 to 52 x 10^3 cfu/ml wherein most of the samples were collected from the rhizosphere of vegetable crops. The number of cfu was more in coconut + vegetables with regular supply of organic manures whereas regular use of only inorganic fertilizers in rice-vegetable resulted in the reduction of microbial count. A total of 114 bacterial isolates were isolated from the rhizosphere soils from Middle and North district of Andaman and Nicobar Islands, India (Table 1). In this study, all the isolates were screened for *in vitro* PGP, extracellular enzyme and antagonistic activity.

Traits of rhizobacterial isolates

Out of 114 rhizobacteria, it was found that 110, 107, 67 and 59 isolates produced siderophore, ammonia, P-solubilization and IAA, respectively, whereas none of the isolates produced HCN. In addition, some isolates produced extracellular enzyme such as protease (92), amylase (73), cellulase (58), chitinase (37), lipase (24) and pectinase (14). Six most promising isolates (NFB3, NPB6, MKP3, MNB1, NNB4 and NTB2) were selected on the basis of having multi-functional properties. The PGP, extracellular enzyme and antagonistic activity of the selected isolates are shown in Table 2. All the isolates utilized a significant amount of iron in siderophore production showing a yellow zone on the CAS agar medium plate, solubilized P in the plate-based assay as evidenced by the formation of a clear halo zone around the colony and tested positive for ammonia production. All the promising

isolates exhibited IAA production in qualitative assay. Further the quantitative determination revealed that bacterial strain NTB2 showed maximum significant concentration of IAA followed by NFB3, NNB4, NPB6, MNB1 and MKP3.

It was observed that five isolates except MKP3 were found positive for protease production, four isolates except NNB4 and NTB2 produced cellulase, three isolates except NFB3, NTB2 and NNB4 produced chitinase and three isolates except NFB3, NTB2 and NNB4 produced amylase. However, only one isolates (NFB3) was found positive for pectinase production. In contrast, none of them were found to be positive for lipase production. Among all NPB6 and MNB1 were positive for maximum number of hydrolytic enzymes. Antagonistic activity of the bacterial isolates was evaluated in terms of inhibition zone diameter as an indicator of the reduction in growth of pathogenic fungi. The all selected isolates exhibited most obvious antagonistic activity *in vitro* against the tested pathogens and showed significant growth inhibition activity against *S. rolfsii* and *Macrophomina* sp. In the dual culture assay, NFB3 showed highest inhibition against the plant pathogen *Macrophomina* spp. (33.3%) and *S. rolfsii* (23%). Among the isolates, NPB6 and MNB1 were positive to many of the properties tested followed by NFB3, MKP3, NNB4 and NTB2 in PGP and extracellular enzyme activity.

Identification of isolates using Biolog system

The selected isolates were examined for their ability to oxidize different carbon sources using Biolog's automated identification system in which all the potential isolates were revealed as *Bacillus* and *Pseudomonas* species. There were large differences in the C utilizations among the *Bacillus* species, which are arranged according to the order of best C sources utilized (Table 3). Among the multi-trait isolates, the dominant ones were *B. cereus* (3 strains) followed by *Pseudomonas tolaasii* (2) and one strain of *B. pumilus*. Among the isolates *B. pumilus* (NFB3) and *P. tolaasii* (NNB4) utilized highest numbers (55) of substrates followed by NTB2 (*P. tolaasii*). Three strains of *Bacillus cereus* named NPB6, MKP3 and MNB1 utilized 14, 15 and 22 common carbon. All the strains utilized 1% sodium lactate and sodium chloride up to 4% (w/v) have the ability to grow at pH 5.0.

Sequence analysis of partial 16SrDNA and phylogenetic analysis

About 1.5 kb fragment of 16Sr RNA gene of six selected bacterial isolates were amplified by PCR using universal primers pA and pH. The PCR products were purified and sequenced. The sequence obtained was analyzed using a BLAST search in which six bacterial strains were placed into two genus viz. *Bacillus* and *Pseudomonas*.

Table 1. Effect of crop and cropping history on the CFU in rhizosphere soil samples.

Location	Cropping history	Inputs	Crop rhizosphere	CFU
Middle Andaman				
Panchwati	More than 30 years of coconut crop mixed with spices	Only manure recycling	Pepper	$10\text{-}35 \times 10^3$
			Cinnamon	$0.6\text{-}26 \times 10^3$
			Clove	$1.9\text{-}32 \times 10^3$
			Nutmeg	$5.2\text{-}77 \times 10^3$
Citrakut	More than 30 years of coconut + arecanut plantation mixed with cinnamon, pepper and recent cultivation of vegetable	FYM and compost only to vegetables	Ladies finger	$33\text{-}63 \times 10^3$
			Paper	$24\text{-}48 \times 10^3$
			Cinnamon	$4.2\text{-}12 \times 10^3$
			Clove	$4.2\text{-}22 \times 10^3$
Wavi	New plantation + pepper with intercropping of vegetable crops	Organic manures and compost only to vegetables	Pepper	$21\text{-}35 \times 10^3$
Nimbudara	Vegetable crop rotation mainly of cucurbits	FYM and inorganic fertilizers	Cucumber	$17\text{-}39 \times 10^3$
			Bitter gourd	$18\text{-}42 \times 10^3$
North Andaman				
Kudirampur	Intensive vegetable cultivation with irrigation	Compost only	Bitter gourd	$4.8\text{-}26 \times 10^3$
Keralapurm	10 year old coconut + arecanut plantation with young spices	Organic recycling and occasional FYM	Nutmeg	$2.3\text{-}15 \times 10^3$
DB gram	10 years old coconut + arecanut plantation with vegetable cultivation in the slopes	Occasional FYM and regular inorganic fertilizers	French bean	$32\text{-}52 \times 10^3$
			Pumpkin	$1.6\text{-}25 \times 10^3$
			Bitter gourd	$16\text{-}23 \times 10^3$
			Ladies finger	$6.3\text{-}18 \times 10^3$
			Chilli	$6.3\text{-}43 \times 10^3$
RK gram	Rice followed by irrigated vegetable crops	Regular use of inorganic fertilizers with FYM	Pumpkin	$5.8\text{-}12 \times 10^3$
			Cauliflower	$4.8\text{-}25 \times 10^3$
Kalipur	Rice followed by irrigated vegetable crops	Regular use of inorganic fertilizers	Tomato	$4.2\text{-}18 \times 10^3$

Table 2. Determination of substrates utilization as carbon sources by selected strains.

Substrate Group	Substrate	Isolates					
		NFB3	NPB6	MKP3	MNB1	NNB4	NTB2
Carbohydrates (28)	D-Maltose	-	-	±	±	-	-
	D-Trehalose	+	-	-	±	+	+
	D-Cellobiose	+	-	-	-	-	-
	Gentiobiose	+	-	-	-	-	-
	Sucrose	+	-	-	-	-	-
	D-Turanose	-	-	-	-	-	-
	Stachyose	+	-	-	-	-	-
	D-Raffinose	+	-	-	-	-	-
	α-D-Lactose	-	-	-	-	-	-
	D-Mellibiose	+	-	-	-	-	-
	β-Methyl-D-glucoside	+	-	±	±	-	-
	D-Salicin	+	-	-	-	-	-
	N-Acetyl-D-glucosamine	+	±	±	±	+	+

Table 2. Contd.

	N-Acetyl-β-D-mannosamine	±	-	-	±	-	-
	N-Acetyl-D-galactosamine	-	-	-	-	-	-
	α-D-Glucose	+	-	-	±	+	+
	D-Mannose	+	-	-	-	+	+
	D-Fructose	+	±	±	±	+	+
	D-Galactose	+	-	-	-	+	+
	3-Methyl glucose	±	-	-	-	-	-
	D-Flucose	+	±	-	±	±	+
	L-Fucose	±	±	-	±	±	±
	L-Rhamnose	-	-	-	-	-	-
	D-Sorbitol	-	-	-	-	+	+
	D-Mannitol	+	-	-	±	+	+
	D-Arabitol	-	-	-	±	+	+
	myo-Inositol	-	-	±	±	+	+
Polymer (2)	Dextrin	+	±	-	+	-	-
	Tween 40	±	-	-	-	-	-
	B-Hydroxy-D,L- butyric acid	-	-	±	-	+	+
	D-Galacturonic acid	+	-	-	±	+	+
	L-Galactomic acid lactone	-	-	-	-	-	-
	D-Gluconic acid	+	±	±	±	+	+
	D-Glucuronic acid	±	-	-	-	+	+
	D-Lactic acid methyl ester	-	-	-	±	-	-
	p-Hydroxy-pheyl acetic acid	-	-	-	-	+	+
	N-Acetyl neuraminic acid	-	-	-	-	-	-
	γ-Amino-butyric acid	+	-	-	-	+	+
	α-Hydroxy-butyric acid	-	-	-	-	±	±
	α-Keto-butyric acid	-	-	-	-	±	±
	Acetoacetic acid	+	±	±	±	±	±
	Propionic acid	±	-	-	±	+	+
Carboxylic acids (26)	Methyl pyruvate	-	-	±	±	±	±
	Acetic acid	+	±	±	+	+	+
	Fusidic acid	-	-	-	-	+	+
	Formic acid	-	±	±	+	-	-
	L-Lactic acid	-	±	±	+	+	+
	Citric acid	+	-	-	-	+	+
	α-Keto-glutaric acid	+	-	-	-	+	+
	Bromo succinic acid	+	±	±	±	±	±
	Nalidixic acid	+	-	-	-	+	+
	D-saccharic acid	-	-	-	-	+	+
	D-Malic acid	-	-	-	-	-	-
	L-Malic acid	+	±	±	±	+	+
	Quinic acid	+	-	-	-	+	+
	Mucic acid	+	-	-	-	+	+
Amino acids (10)	L-Arginine	+	-	±	±	+	+
	L-Aspartic acid	+	±	±	±	+	+
	L-Glutamic acid	+	±	±	+	+	+
	L-Histidine	+	±	±	+	+	+
	L-Pyroglutamic acid	+	-	-	±	+	+

Table 2. Contd.

	L-Serine	+	±	+	+	+	+
	D-Aspartic acid	+	-	-	-		
	D-Serine	-	±	±	+	+	+
	Glycyl-L-proline	-	-	-	±	-	-
	L-Alanine	+	-	-	±	+	+
	Troleandomycin	-	-	-	-	+	+
	Rifamycin SV	-	-	+	+	+	+
Antibiotics (5)	Minocycline	-	+	+	+	-	-
	Vancomycin	-	-	-	-	+	+
	Lincomycin	-	-	-	-	+	+
Amides (1)	Gluccuronamide	+	-	-	-	+	+
	Inosine	+	±	±	±	+	+
	Glycerol	+	±	±	±	+	+
	1% Sodium lactate	+	+	+	+	+	+
	Gelatin	+	+	+	+	-	-
	D-Glucose-6-PO_4	+	±	±	+	-	-
	D-Fructose-6-PO_4	+	±	±	+	+	+
	Pectin	+	-	-	-	-	-
	Nilaproof 4	-	-	-	-	+	+
	Tetrazolium violet	-	-	-	-	+	+
	Tetrazolium blue	-	-	-	-	±	+
Miscellaneo-us (21)	Lithium chloride	+	+	+	+	-	-
	Guanidine HCL	+	+	+	+	±	+
	Potassium tellurite	+	+	+	±	+	+
	Aztreonam	+	+	+	+	-	+
	Sodium butyrate	+	+	+	+	-	-
	Sodium bromate	-	+	+	-	±	-
	pH 6	+	+	+	+	+	+
	pH 5	+	+	±	-	+	+
	1% NaCl	+	+	+	+	+	+
	4% NaCl	+	+	+	+	+	+
	8% NaCl	+	+	+	+	-	-

-, negative reaction; ±, moderate; +, positive reaction.

Among six strains, four strains MNB1, NPB6 and MKP3 were identified as *Bacillus cereus,* NFB3 was *Bacillus stratosphericus*, NNB4 was *Pseudomonas fluorescens* and NTB2 was *Pseudomonas simiae* (Table 4). Phylogenetic analyses of the strains based on the neighbor joining method resulted into three major clusters (Figure 1). Cluster I formed with *Bacillus cereus*, cluster II formed with *Bacillus stratosphericus* and cluster III formed with *P. fluorescens* and *P. simiae.*

Effect of rhizobacteria on growth parameters of vegetables

In *in vitro* seed germination test of inoculated and un-

inoculated seeds, the growth performance of the seedlings were measured and the results are presented in Table 5. Inoculation of rhizobacteria such as *B. stratosphericus* and *B. cereus* had resulted in significantly higher seed germination rate in brinjal and okra whereas *P. simiae* produced similar effect in chilli. In both cases, there was 20% higher seed germination over the control. Radical length was significantly higher due to seed inoculation of all the six selected rhizobacteria and more conspicuous effect was seen in *B. cereus* in okra (4.06 cm) and *P. simiae* in brinjal (2.11 cm) and chilli (1.03). Significantly higher plumule length of 2.43, 1.44 and 1.24 cm was observed in brinjal, chilli and okra, respectively in seed bacterization with *B. cereus.* Seed bacterization also

Table 3. Characterization of selected bacterial isolates for plant growth promoting and antagonistic traits.

Properties	Isolate name					
	NFB3	NPB6	MKP3	MNB1	NNB4	NTB2
PGP Properties						
IAA Production	+++	++	+	+	++	+++
IAA Production (µg/ml)	34.91	31.79	21.6	23.19	32.68	35.26
P-Solubilization	++	+	++	+	+++	+++
Ammonia Production	+	+	+	+	+	+
Siderophore	++	+++	+++	+++	+++	++
Hydrolytic enzymes						
Protease	++	+++	-	+	+++	+++
Cellulase	+	+	+	+	-	-
Lipase	-	-	-	-	-	-
Pectinase	+++	-	-	-	-	-
Chitinase	-	++	++	++	-	-
Amylase	-	++	++	++	-	-
Antagonistic activity (% inhibition)						
Macrophomina sp	33.3	14.1	23.0	23.7	23	23.7
Sclerotium rolfsii	23.0	14.1	18.5	18.5	21.5	22.2

-, No activity; + 0.3-0.5 cm; ++, 0.6-1.0 cm; +++, >1.0 cm.

Table 4. Identification of bacterial isolates using carbon source utilization and 16SrDNA partial sequences.

Isolates	Identified organism		Accession number based on 16S rDNA
	Biolog	16S rDNA	
NFB3	*Bacillus pumilus*	*Bacillus stratosphericus*	JX960424
NPB6	*Bacillus cereus*	*Bacillus cereus*	JX960425
MKP3	*Bacillus cereus*	*Bacillus cereus*	JX960430
MNB1	*Bacillus cereus*	*Bacillus cereus*	JX960426
NNB4	*Pseudomonas tolaasii*	*Pseudomonas fluorescens*	JX960423
NTB2	*Pseudomonas tolaasii*	*Pseudomonas simiae*	JX885487

produced similar effect on the vigor index as that of radian length. *P. simiae* gave higher vigor index in brinjal (408%) and chilli (235%) whereas in the case of okra *B. cereus* performed better (510%).

Six promising IAA producing isolates with multi-functional properties were tested for their influence on growth parameters on the brinjal, chilli and okra (Figures 2, 3 and 4). In brinjal, isolates NPB6 (75.04 and 58.15%), MKP3 (61.5 and 68.08%) and NTB2 (58.6 and 53.9%) have increased shoot and root length respectively, over control. In chilli, three isolates namely: *B. stratosphericus*-NFB3, *B. cereus*-MNB1 and *P. simiae*-NTB2 showed increased shoot (37.4, 32.2 and 39.4%) and root length (52.6, 42.8 and 38.4%) over untreated controls. In okra, *P. fluorescens*- NNB4, *B. cereus* -MKP3 and *B. cereus*- NPB6 showed significant plant growth promotion with respect to increase in root and shoot

length. There was no clear trend in increasing secondary root number by all the isolates. It was found that seed bacterisation of *P. simiae*-NTB2 significantly increased the number of secondary root in chilli followed by NPB6 in brinjal and NNB4 in okra over control. Among theisolates, the overall performance of *B. cereus* - NPB6 for root length in brinjal, *P. fluorescens* - NNB4 for shoot length in okra and *P. simiae*-NTB2 for secondary root number in chilli were highly significant.

DISCUSSION

The PGPR isolates promote plant growth and induce resistance in different crops (Podile and Kishore, 2006). PGPR or potential biological control strains isolated from one region may not produce similar results in other soils
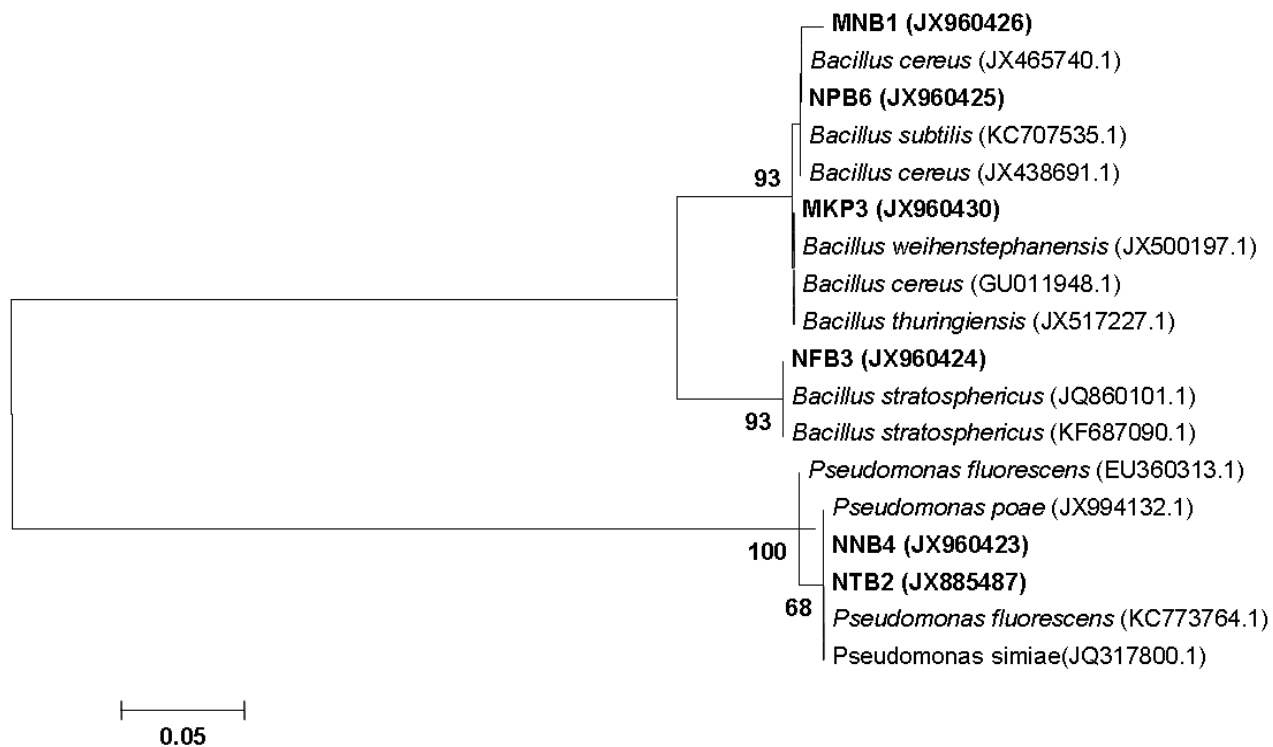
MNB1 (JX960426)
Bacillus cereus (JX465740.1)
NPB6 (JX960425)
Bacillus subtilis (KC707535.1)
Bacillus cereus (JX438691.1)
MKP3 (JX960430)
Bacillus weihenstephanensis (JX500197.1)
Bacillus cereus (GU011948.1)
Bacillus thuringiensis (JX517227.1)
NFB3 (JX960424)
Bacillus stratosphericus (JQ860101.1)
Bacillus stratosphericus (KF687090.1)
Pseudomonas fluorescens (EU360313.1)
Pseudomonas poae (JX994132.1)
NNB4 (JX960423)
NTB2 (JX885487)
Pseudomonas fluorescens (KC773764.1)
Pseudomonas simiae(JQ317800.1)

93
93
100
68

0.05

Figure 1. Neighbour-joining phylogenetic tree-based 16S rDNA sequences and their closest phylogenetic neighbours. Bootstrap values are indicated at nodes. Scale bar represents observed number of changes per nucleotide position.

Table 5. Effect of rhizobacteria on growth parameters of brinjal, chilli and okra seeds.

Isolate	Germination rate (%)			Radical length (cm)			Plumule length (cm)			Vigor Index (%)		
	B	C	O	B	C	O	B	C	O	B	C	O
B. stratosphericus- NFB3	100^c	80^a	100^b	1.05^b	0.65^c	3.51^d	1.30^b	0.63^a	1.04^b	234.0^b	102.4^b	456.0^d
B. cereus- NPB6	100^c	80^a	100^b	1.42^{bc}	0.96^d	2.62^b	2.01^d	0.97^b	1.15^b	308.7^{cd}	154.4^c	402.0^c
B. cereus- MKP3	90^b	90^b	100^b	1.43^{bc}	0.67^c	3.48^d	1.88^{cd}	1.44^c	1.24^c	298.8^c	192.6^e	482.0^e
B. cereus- MNB1	90^b	90^b	100^b	1.09^b	0.68^c	4.06^e	1.58^{bc}	1.32^c	1.04^b	240.3^b	180.0^d	510.0^f
P. fluorescens-NNB4	80^a	80^a	80^a	1.83^{cd}	0.49^b	3.69^c	2.17^{de}	0.83^b	1.22^b	320.0^d	106.4^b	392.8^c
P. simiae - NTB2	90^b	100^c	90^{ab}	2.11^d	1.03^e	3.09^c	2.43^c	1.32^c	1.03^b	408.6^e	235.0^f	367.6^b
Control	80^a	80^a	90^{ab}	0.73^a	0.44^a	2.09^a	0.80^a	0.50^a	0.74^a	122.4^a	76.00^a	254.1^a
CD (p< 0.05)	9.50	6.1	12.27	0.54	0.06	0.21	0.37	0.18	0.19	12.68	5.03	12.28

Results obtained were of mean of triplicates. Data were analyzed using one-way analysis of variance and treatment means were compared (p ≤ 0.05). B = Brinjal; C = Chilli; O = Okra. Column with different letters are significant, with similar letter non significant at p<0.05

and climatic conditions as in the case of its original habitat (Duffy et al., 1997). Therefore, isolation of resident microbial strains and their utilization constitute the best alternate strategy. This is more pertinent to Andaman and Nicobar Islands, which is a repository of biodiversity by virtue of its nature and location. In the present study, six potential PGPR isolates were selected on the basis of

their performance with multi-functional properties such as production of IAA, siderophore, ammonia, P-Solubilization, extracellular enzyme and antagonistic activity. Among the bacterial isolates, *P. simiae* (NTB2) showed significant concentration of IAA followed by *B. stratosphericus* (NFB3), *P. fluorescens* (NNB4), *B. cereus* (NPB6), *B. cereus* (MNB1) and *B. cereus* (MKP3) sugges-ting that these
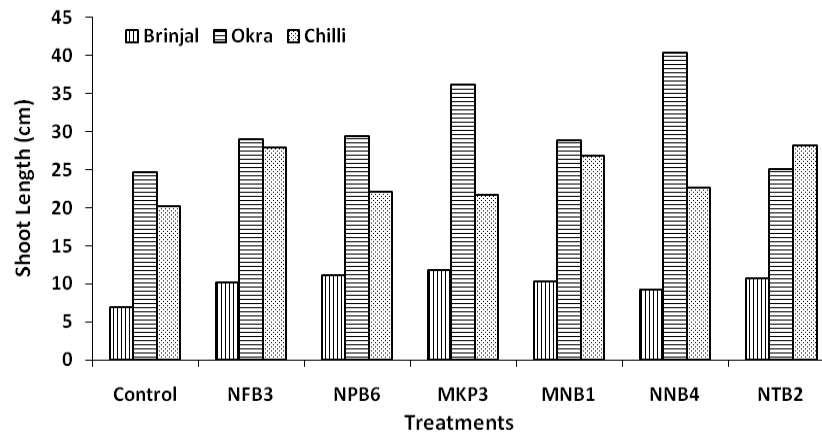
Figure 2. Inoculation effect of PGPR strains on shoot length of seedlings after one month.
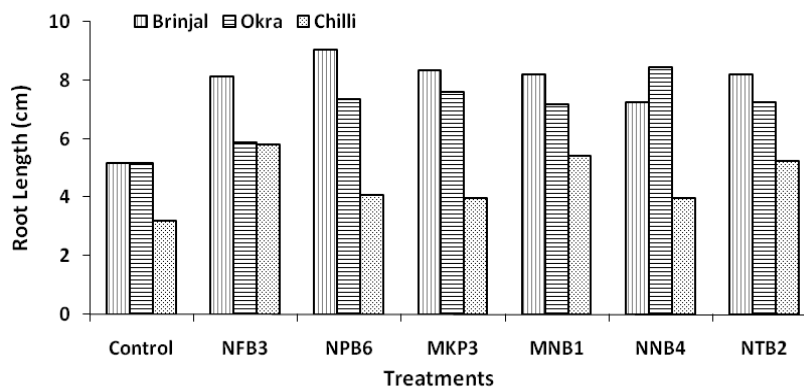

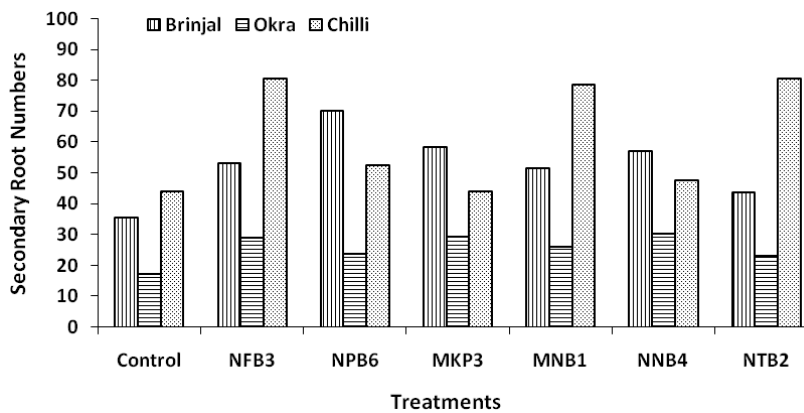**Figure 3.** Inoculation effect of PGPR strains on root length of seedlings after one month.

Figure 4. Inoculation effect of PGPR strains on secondary root numbers seedlings after one month.

isolates could be used for plant growth promotion. A higher amount of IAA was produced in the presence of L-tryptophan by *P. simiae* (NTB2) because L-tryptophan acts as suitable precursor for IAA biosyn-thesis. Similarly, higher level of IAA production in the presence of L-tryptophan by *Pseudomonas* and *Bacillus* sp. was reported by Xie et al. (1996) and Srinivasan et al. (1996).

All isolates showed solubilization zone on Pikovskaya medium, indicating its potential role as a P-solubilizer. The phosphate solubilization by the isolate could increase the availability of phosphorous in the rhizospheric region. It is a well established fact that improved phosphorous nutrition results in the overall plant growth and root development (Jones and Darrah, 1994). All the selected isolates utilized a significant amount of iron by siderophore production, which is indicative of their ability to suppress fungal pathogens in the rhizosphere by chelating iron. It also exhibited production of ammonia, which could be taken up by plants as a source of nitrogen for their growth (Ahmad et al., 2008).

It was also shown that rhizobacteria inhibits phytopatho-gens by producing chitinase, β-1, 3-glucanase and pectinase, which degrade the fungal cell wall (Friedlander et al., 1993; Bloemberg and Lugtenberg, 2001; Persello-Cartieaux et al., 2003). In the present study five isolates were found positive for protease production followed by four isolates for cellulase, three each for chitinase and amylase and only one isolate produced pectinase whereas, none of the isolates produced lipase. This could be a principal reason for the observed antagonistic activity against *S. rolfsii* and *Macrophomina* sp. Charac-terization of the isolates on the basis of carbon source utilization in Biolog system revealed that they are gram-positive *Bacillus* and gram-negative *Pseudomonas* sp. Compared to other bacterial groups, *Bacillus* group were found to have the ability to survive in hot humid climate of Andaman and Nicobar Islands because of its ubiquitous nature like multilayered cell wall, stress resistant, endo-spore formation, secretion of peptide antibiotics, peptide signal molecules and extracellular enzyme productions. Among the identified isolates, three isolates of *Bacillus* sp. followed by two isolates of *Pseudomonas* sp. were found to exhibit multi-trait properties. There were large differences in the C-utilizations among the *Bacillus* sp., which are arranged according to the order of best C sources utilized. The data obtained showed a greater abundance of Gram-positive bacteria in the tropical soils of Andaman and Nicobar Islands. This was in agreement with the previous studies (Garbeva et al., 2003; Rau et al., 2009; Kumar et al., 2011; Amaresan et al., 2012) that showed a higher level of Gram-positive *Bacillus* and *Paenibacillus* species in the rhizosphere soils of cultivated vegetable crops and wild grass. GEN III micro-plates contain some substrates that commonly occur in root exudates, which represent a primary source of

carbon and energy and are likely to favor fast-growing microbes in the rhizosphere (Alisi et al., 2005). Thus, the ability to metabolize root exudates at high rates has been often related to the colonization of roots by bacteria (Baudoin et al., 2003).

Bacteria are known to produce different metabolites like IAA, gibberellins and cytokinin like substances, which can exert positive effect on seed germination and radicle length (Tien et al., 1979). In the present study, under laboratory conditions, seed treatment with the test strain improved seed germination and seedling emergence of brinjal, okra and chilli over the control, which might be due to the production of IAA by the rhizobacteria (Mirza et al., 2001). Similar improvement of seed germination parameters by rhizobacteria was reported in rice seedlings, cowpea seedlings (Minaxi et al., 2011) and sorghum (Raju et al., 1999) due to increased synthesis of gibberellins, which triggered the activity of specific enzymes such as α-amylase that promoted early germination by increasing the availability of starch assimilation.

IAA-producing bacteria are known to promote root elongation and plant growth (Patten and Glick, 2002). The significant increase on the root and shoot length of brinjal, chilli and okra crops could be attributed to the production of growth promoting substances by the inoculated bacteria that carry out an important role in the stem expansion process (Burd et al., 2000). In a similar study, Adesemoye et al. (2008) reported that inoculation of tomato, okra and African spinach with *Bacillus subtilis* and *P. aeruginosa* enhanced percent emergence and growth of seedlings. In addition, the isolates significantly increased the secondary roots production prominently. *B. stratosphericus* (NFB3) showed increase in secondary roots in all the crops.

As these isolates were observed to produce significant amount of IAA, which positively influenced the root growth and development thereby enhancing nutrient uptake (Khalid et al., 2004). In addition, healthy plants may exhibit greater resistance to pathogens than that which are under stress. Among the isolates, *B. cereus* (NPB6) in brinjal, *B. stratosphericus* (NFB3) in chilli and *P. fluorescens* (NNB4) in okra significantly improved the growth parameters.

These IAA producing multi-functional *Bacillus* and *Pseudomonas* sp. with broad range of carbon sources has the potential to be used as bio-inoculants to attain the desired plant growth promotion in brinjal, okra and chilli seedling. Also, it showed the usefulness of PGPR-inoculated treatment for improving crop productivity of tropical crops growing in hot humid climate of Andaman and Nicobar Islands, which help to minimize the uptake of fertilizers, reduce environmental pollution and promote sustainable agriculture. These isolates having the property to tolerate 8% NaCl stress and pH 5, can also

be used as a bio-inoculants in alkaline and acidic agriculture soil conditions of Andaman and Nicobar Islands.

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Full Length Research Paper

Preliminary investigation on anthelmintic activity and phytochemical screening of leaf crude extracts of *Tithonia diversifolia* and *Tephrosia vogelii*

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This investigation lies within the framework of studying the phytochemistry of two medicinal plants *Tithonia diversifolia* and *Tephrosia vogelii* and assessing their potentials in controlling the helminthiasis in goats. The crude extracts were obtained from the leaves by Soxhlet methodology and phytochemical screening, fecal eggs reduction and the anthelmintic activities were tested in various laboratories in Butare. The results from this investigation showed that both *T. diversifolia* and *T. vogelii* have lots of active principles grouped in alkaloids, tannins, flavonoids, terpenoids,and sterols and exhibit anthelmintic activities in goats. The percentages of reduction of fecal eggs are 98, 97, 96 and 95% with methanol crude extract of *T. vogelii*, infusion of *T. vogelii*, methanol crude extract of *T. diversifolia* and infusion of *T. diversifolia*, respectively. This investigation showed the efficacy of both *T. vogelii* and *T. diversifolia* against gastrointestinal nematodes in goats and therefore their potentials in assuring more the animal health care in Rwanda by for treating the parasitic nematodes in goats by plant-based drugs instead of crude extracts.

Key words: Anthelmintic activity, feacal egg, gastro-intestinal nematodes, goats, phytochemical screening traditional medicine, *Tithonia diversifolia*, *Tephrosia vogelii*, medicinal-plant, Rwanda.

INTRODUCTION

In Rwanda and elsewhere, the medicinal plants have been used by Man from the prehistoric times to present days and in all civilizations. By trial and error, he distinguished between the beneficial and poisonous

Figure 1. Flowering plant of *T. diversifolia.*

plants. Man has also observed that in large quantities medicinal plants may be poisonous, and learned about the usefulness of plants by observing sick animals that use some plants that they usually ignore. Today, the scientists took in this way to isolate active compounds from the medicinal plants in order to provide the way of formulating various plant-based drugs (Kabera et al., 2013). The medicinal value of plants is directly connected to the vast array of chemical compounds, known as secondary plants products or secondary metabolites, manufactured by their various biochemical pathways. Among them, some have been conformed to be active against microbes and parasites. They are usually classified in different groups namely alkaloids, flavonoids, tannins, quinones, terpenoids and saponins (Margaret and Wink, 1998; Levetin and McMahon, 1999; Njoroge and Bussmann, 2006). Researches show that the drugs from plants are better accepted by the body than synthetic substance (Bodeker et al., 2005). Although some plants may have toxic properties associated with their curative power, this does not mean necessarily to desert directly their uses. In all the ways, further knowledge on plants chemical constituents and their uses are however needed to valorize them and to see if they can be used for one or other purpose. In this way, many plants came under examination leading to extraction and characterization of their actives ingredients, where obtained results make pharmacological studies and synthesis of more potent drugs with reduced toxicity (Levetin and McMahon , 1999; Bodeker et al., 2005).

The availability of food and products of animal origin are still being the basic needs of man as they have been

since the dawn of his creation. In the most developing African countries, like Rwanda and regional countries, the animal's breeding is the one of the sectors which contributes to maintain food security and provide the income for population from rural areas (Van, 2011). In spite of these important needs of animals by their nationals, the diseases have always negatively affected their production and directly or indirectly affect the nutritional as well as economical sector. Consequently, the constant challenge of nature to animal health is often also a challenge to man's health. For this reason, the maintenance of high standards of animal health is essential to the public health of any country of the third world (Vaarst et al., 2008).

In our country Rwanda, most of animals breeders have not access to modern therapy provided by modern veterinary; reason why they have recourse to the traditional medicine to treat different diseases of their animals by using two main plants namely "Kimbazi" and "Umuruku" scientifically named *Tithonia diversifolia* and *Tephrosia vogelii*, respectively. Even if the efficacy of these medicinal plants is not undisputable, more investigation should be done in many fields of science in order to know exactly their chemical composition, the diseases they treat and their posology particularly in Rwanda. It is within this framework that we conduct a survey on the phytochemical and anthelmintic activities of *T. diversifolia* and *Tephrosia vogelii*, mostly used by Rwandan traditional healers in treating their flock of small animals.

T. diversifolia (locally known as Kimbazi or Icyicamahirwe) is an impressive flowering plant belonging to the Eudicots class, *Asterale* order and in the Asteraceae family (Schilling and Panero, 1996). *T. diversifolia* is used for different purposes such as ornamental purpose because of its characteristic bitter taste. It has been also used to induce a fever, to help fight poisoning, although not used for direct medicinal purposes (Schilling and Panero, 1996; Jama et al., 2000). In traditional medicine, it has been used to treat some ailments such as throat and liver ailments, stomach upset, and diarrhea in livestock. It is also used as an anti-diabetic, anti-malaria, anti-inflammatory, antibacterial, antimicrobial and potential cancer chemopreventive (Touqeer et al., 2013; Hoffmann and Fnimh, 2003; Adebayo et al., 2009) (Figure 1).

*Tephrosia vogelii (*locally named *Umuruku)* is a soft woody branching herb with dense foliage reaching up to 0.5-4 m tall and belongs into the class of Magnoliopsida, order of Fabales and in the family of Fabaceae (Gadzirayi et al., 2009). It is used in various applications in human daily life. It is used by the farmers to improve the soil fertility because of the nitrogen found in their leaves and seeds. It is also used as firewood, as an insecticide against storage pests and mites on plants, as piscicidal although this last use is now illegal in many countries

Figure 2. Blooming plant of *T. vogelii* grown in Cyarwa cy'Imana (Butare, Rwanda).

Figure 3. Location of Butare, the sampling site of this investigation.

because of the rotenone obtained in the leaves and seeds). In traditional medicine, *T. vogelii* is used to treat many animal ailments such as skin diseases and intestinal worms. It has antibacterial activities against *Staphylococcus aureus* and *Bacillus subtilis*. It is also used to treat ectoparasites and endoparasites in cattle (Blommaert, 1950; Hoffman, 2003; Hammond et al., 1997; Colin, 2011) (Figure 2).

MATERIALS AND METHODS

Plants materials and animals fecal collection

After the dew was removed by the morning sun, the fresh and

mature leaves of *T. diversifolia* were harvested from the arboretum of the University of Rwanda (UR) the leaves of *Tephrosia vogelii* leaves were collected from "Cyarwa cy'Imana" (near Agateme centre) during wet season. The collected leaves were then dried under weak sunlight and ground into powder that was stored in cool place for further oil extraction. The feaces samples were collected from a flock of 30 goats of the School of Agri-Veterinary of Kabutare (EAVK) and stored for further tests. These sampling sites are located in Butare, Eastern Province with geographic coordinates 2°36′S 29°45′E (Figure 3).

Extraction and phytochemical screening

The plant crude extracts were obtained by using the Soxhlet extractor and n-hexane and methanol as solvents. The obtained crude extracts were used in evaluation of anthelmintic activity of both plants and the phytochemical screening method was carried out in the standard procedures (Table 1 and Figure 3).

Reduction of eggs per gram of feaces (EPG) after treatment with crude extract and powder infusion

The fecal samples collected from the goats of EAV Kabutare; were examined under microscopy by using fecal flotation methods based on principle that parasites are less dense than the fluid flotation medium; this method helped to know the goats infected by parasites. McMaster method using McMaster eggs-counting slide and microscope was employed to quantify the eggs per gram of feaces (Margaret et al, 1994).

Assessment of anthelmintic activity of extracts

To evaluate the anthelmintic activity, McMaster Method was used before and after the treatment of the goats by using methanol crude extracts and powder infusion of these two plants studied here in order to know the reduction of eggs after treatment. The number of eggs per gram was calculated by using the formula below.

$$EPG = Y \times 100$$

Where, EPG: number of eggs per gram
Y: Number of egg counted in both chambers of McMaster egg-counting slide.

To qualify the efficacy of solutions from both plants (*T. diversifolia* and *T. vogelii*) against goats' helminthes, the test of reduction of EPG was used to calculate the percentage of reduction of eggs in feaces is calculated by using the formula below.

$$P = 100 \left[1 - \left(\frac{\overline{X_t}}{\overline{X_C}} \right) \right]$$

Where, P: percentage of reduction of EPG
$\overline{X}_C$: Arithmetic mean of EPG of the control group of goats (group of untreated goats) between 7 days and 14 days of treatment.
$\overline{X}_t$: Arithmetic mean of EPG of group of treated goats between 7 days and 14 days of treatment.
If the percentage reduction is greater or equal to 95%, the solution is effective and the solution is ineffective if the percentage

Table 1. Phytochemical screening of *T. diversifolia.*

Group of active principles	Reagents	Initial colour	Observed colour	Colour expected	Test results
Alkaloids	Mayer	Brown- black	Brownish white	Cream formation, yellowish white	++
	Wagner	Brown- black	Reddish brown	Reddish brown(orange)	++
	Dragendorff	Brown- black	Reddish brown	Reddish brown	++
Tannins	Saline gelatin + NaCl and H_2O	Brown- black	White precipitate	White precipitate or trouble	++
	$CuSO_4$	Brown- black	White precipitate	White precipitate	++
	$FeCl_3$	Brown- black	Browinish green precipitate	Brownish green or blue- black	++
Flavonoids	HCl, CH_3OH, H_2O, Mg	Brown- black	Reddish brown for flavones	Reddish brown for flavones, red-celise for flavonols, redish violet for flavonones	++
Terpenoids-Sterols	Chloroform, H_2SO_4	Green- precipitate	Reddish brown coloration	Reddish brown coloration	++
	Chloroform, anhydride acetic,H_2SO_4	Green precipitate	Blue- brown	Blue -brown coloration	++
Saponins	-	Brown- black	Persistent Froth	persistent Frothing	++
Quinone	Ether-chloroform, HCl, ethanol, NaOH	Green precipitate	Purplish	Red or purplish	+
Leucoanthocyans	HCl_2N	Brown- black	Brownish red precipitate	Reddish violate precipitate	+ -
Anthocyans	HCl	Brown- black	Reddish brown	Red	+
	NH_3	Brown- black	Brownish blue	Blue	+
Anthraquinones	KOH	Brown- black	Reddish black	Red	+ -

Caption: ++: abundant, +: present, ±: no decision, -: absent

reduction is less than 95.

RESULTS AND DISCUSSION

Phytochemical screening of *T. diversifolia* showed that alkaloids, tannins, flavonoids including their derivatives such as the rotenoids, saponins and terpenoids- steroids are in abundance in this plant. There is no decision for Anthraquinones and leucoanthocyanins because the observed colour is not very similar to expected colour. These results led us to the same observations that were made by previous research on this plant (Fasuyi et al.,

2010; Kalume et al., 2012; Ezeonwumelu, 2012; Siamba et al., 2007). The Table 2 shows that alkaloids, tannins, flavonoids and terpenoids-steroids are abundant in the leaves of *Tephrosia vogelii* while the compounds of saponins group are absent. The same situation was also observed in previous phytochemical screening of this plant which was carried out in the past by Dzenda and his collaborators (2012). There is no decision for anthraquinones and anthocyanins because the colour observed for anthraquinone is not very similar to the expected one while for anthocyanins the colour expected in acidic medium is observed in basic medium and the colour to be observed in acidic medium was absent.

Table 2. Phytochemical screening of *T. Vogelii.*

Groups of active principles	Reagents	Initial colour	Colour observed	Colour expected	Test results
Alkaloids	Mayer	Yellowish brown(purplish yellow)	Yellowish white	Yellowish white	++
	Wagner	Yellowish brown	Reddish brown	Reddish brown(orange)	++
	Dragendorff	Yellowish brown	Reddish brown	Reddish brown(orange)	++
Tannins	Saline gelatin, NaCl, H_2O	Yellowish brown	White precipitate	White precipitate or trouble	++
	$CuSO_4$	Yellowish brown	White precipitate	White precipitate	++
	$FeCl_3$	Yellowish brown	Blue-black precipitate	Brownish green or blue- black	++
Flavonoids	HCl,CHOH,H2O, Mg	Yellowish brown	Reddish brown (flavones)	Red-celise for flavonol, Reddish brown for flavones, Reddish violet for flavonone	++
	Chloroform, H_2SO_4	-	Reddish brown	Reddish brown	+
	Chloroform, Anhydride acetic, H_2SO_4	-	Blue - brown	Blue-brown	++
Saponins	-	Yellowish brown	-	Persistent frothing	-
	Ether-chloroform, HCl, ethanol, NaOH	Green precipitate	Red	Red	+
Quinones	HCl_2N	Yellow	Reddish violet	Reddish violet	+
Leucoanthocians	HCl_2N	Yellow	Reddish violet	Reddish violet	+
Anthocians	HCl	Yellow	Whitish yellow	Red	+
	NH_3	Yellowish brown	Red	Blue	+
					-
Anthraquinones	KOH	Yellowish brown	Reddish yellow	Red	+
					-

Caption: ++: abundant, +: present, ±: no decision, -: absent

From what we learn from Fluck (1955), the climate has influence in one way or another to the active compounds of medicinal plants. Therefore, the abundance of some of the aforementioned active compounds should also be explained by the good quality of the Rwandan climate under which the plants have grown and leaves were harvested.

Phytochemical screening of both *T. diversifolia* and *T. vogelii* showed that these plants have nearly the same chemical compositions; therefore it will not be surprising if both plants have anthelmintic activity. The results obtained from Soxhlet extraction show that there is a lot of crude extract with methanol extraction more than with n-hexane as solvents (Figure 4). Usually, the methanol is a high polar solvent and dissolves the polar compounds present in plant tissues while and n-hexane which is non polar solvent dissolves the non polar compounds present in the plant tissues. Therefore, these two statements of this investigation led to conclude that these plants contain more polar compounds than non polar compounds in their leaves.

The histograms (Figures 5, 6, 7 and 8) generated by the analysis of the results obtained from the tests of reduction of EPG were by Microsoft Excel show clearly
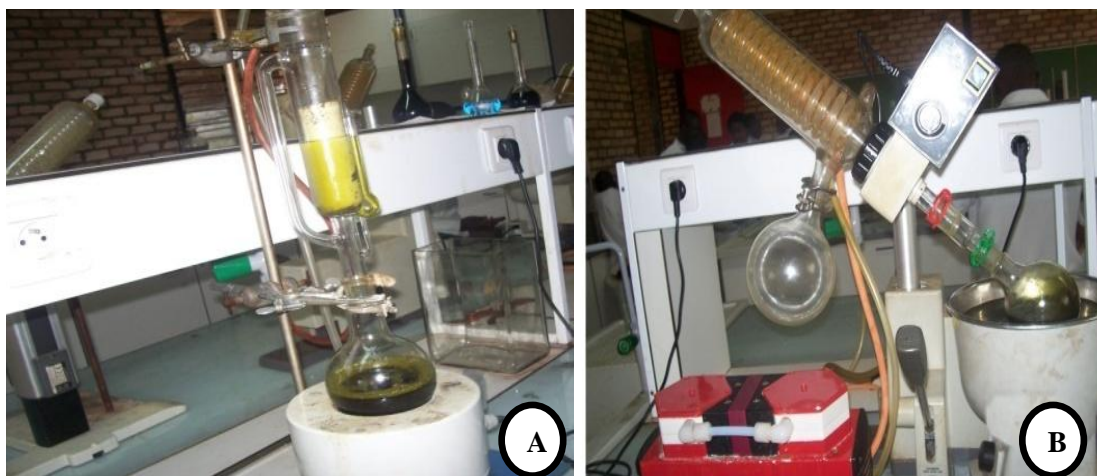
Figure 4. Soxhlet extraction assembly of (A) plant crude extracts and (B) solvents recovery.
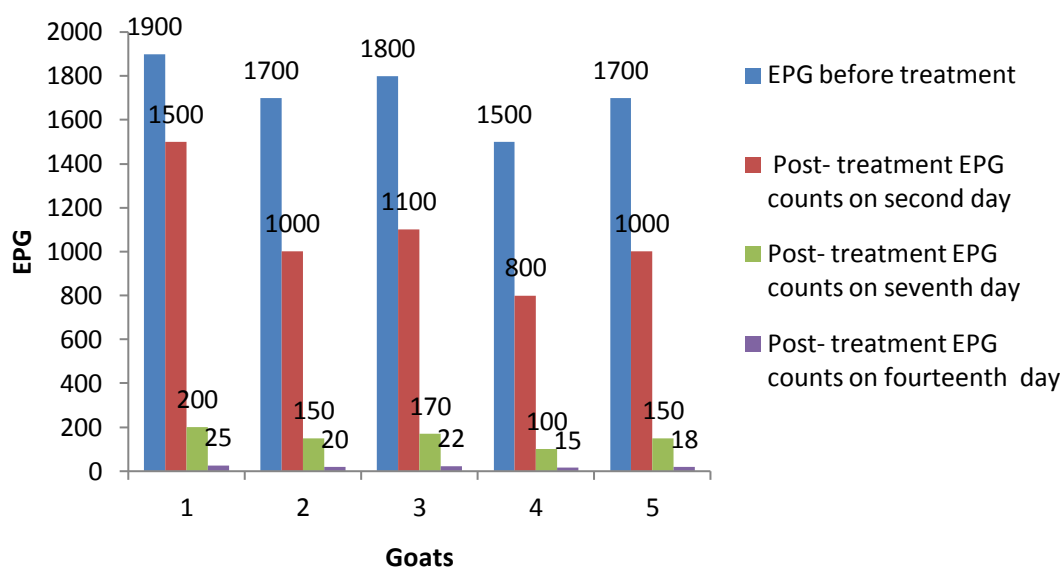

Figure 5. Number of eggs per gram after the treatment with crude extract of *T. vogelii.*

how the number of eggs per gram of feaces decreased after treatment with crud extracts of these medicinal plants. The high reduction rate was observed between seventh day and fourteenth day of the treatment with methanol crude extracts and powder infusions from both plants.

Given that the percentages obtained in this study is above 95 everywhere (Table 3), the solution is effective and therefore, the extracts from the leaves of both *T. diversifolia* and *T. vogelii* are effective against gastro-helminthes in goats. In other worlds, the bioactivity of phytochemicals varied significantly, the same observations with Langat and his collaborators (2012). In

additions, these results show that the methanol crude extracts are more effective than the powder infusion and so the crude extracts should contain more active principles than the powder. From these percentages of EPG reduction, it has been observed that *T. vogelii* is more effective against helminthes for goats than *T. diversifolia* though the different is not *significant.*

In conclusion, this present investigation revealed that both *T. vogelii* and *T. diversifolia* exhibit a remarkable anthelmintic activity in goats of Rwanda. The results of this investigation are supported by previous findings from the studies carried out on these plants (Edeki, 1997). The use of these plants in making the plant-based drugs
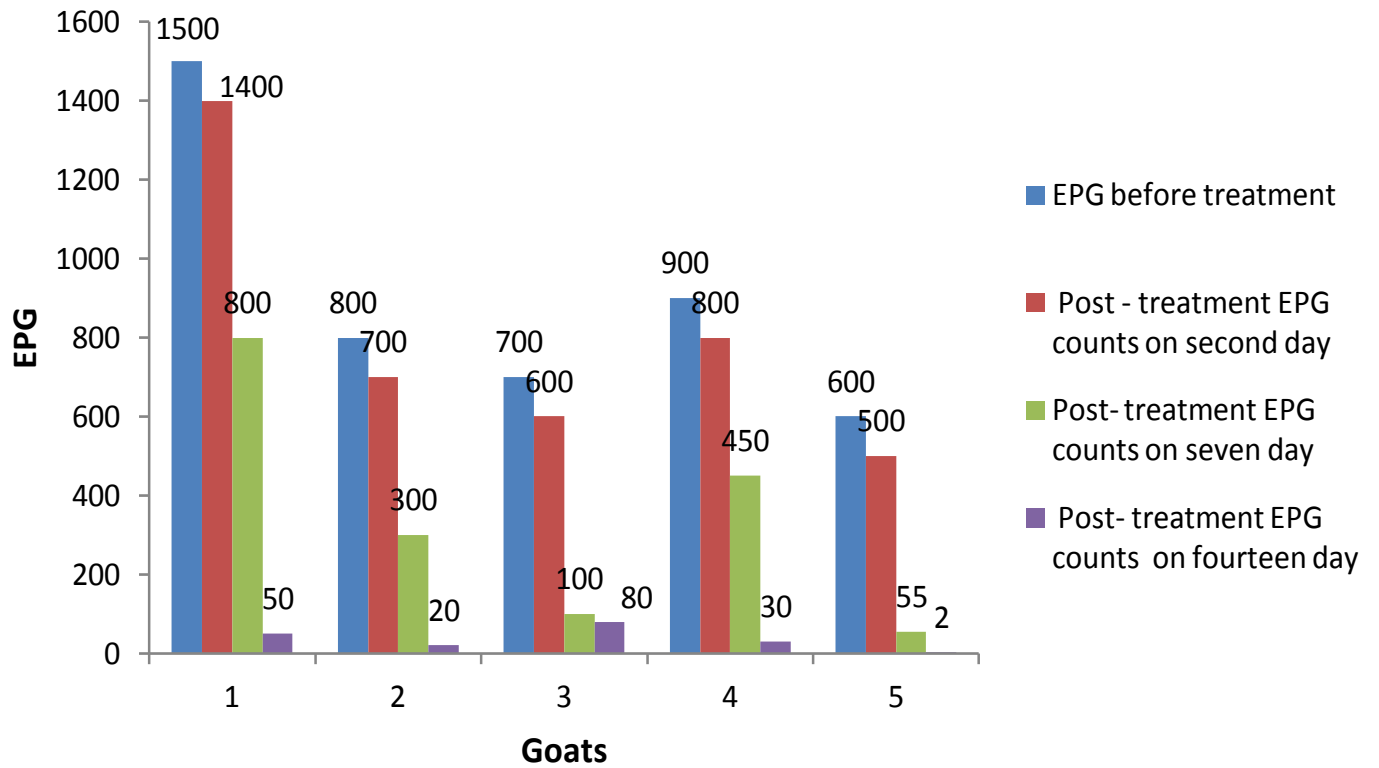
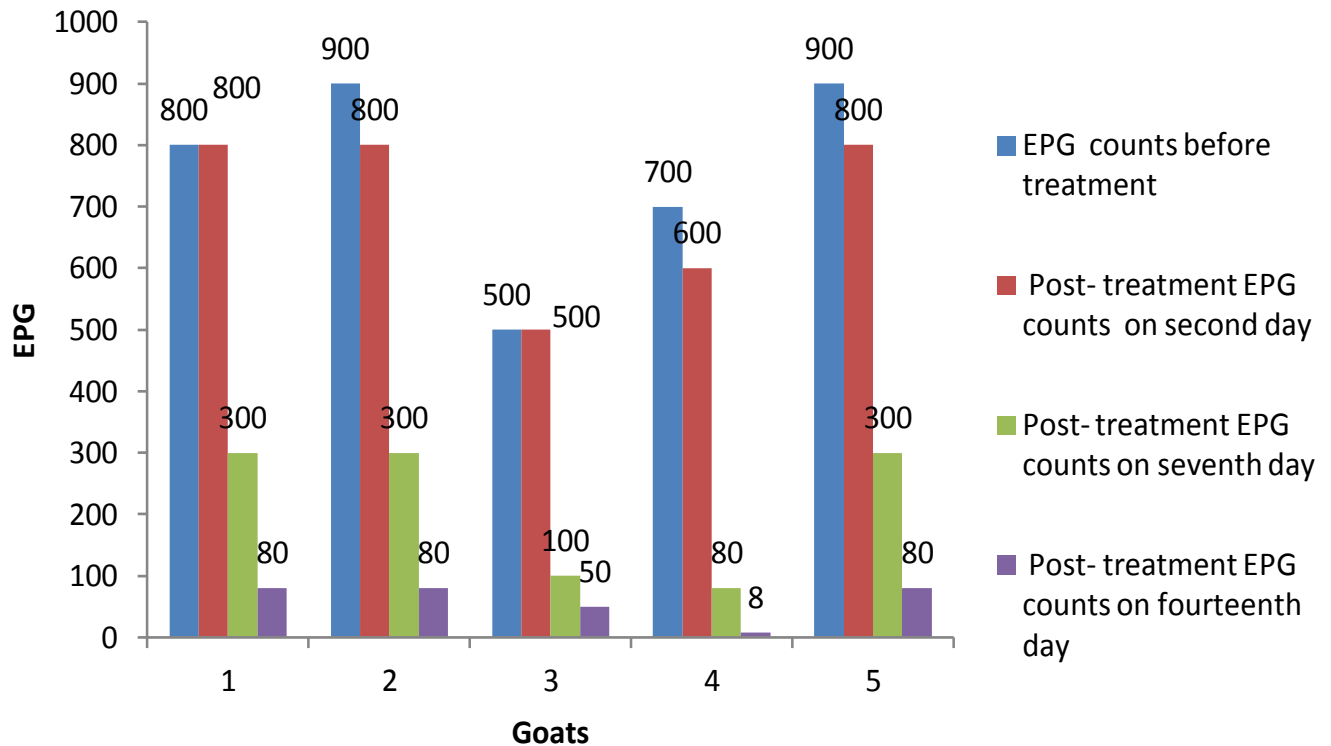**Figure 6.** Number of eggs per gram after the treatment with powder infusion of *T. vogelii*

Figure 7. Number of eggs per gram after treatment by with crude extract from *T. diversifolia.*
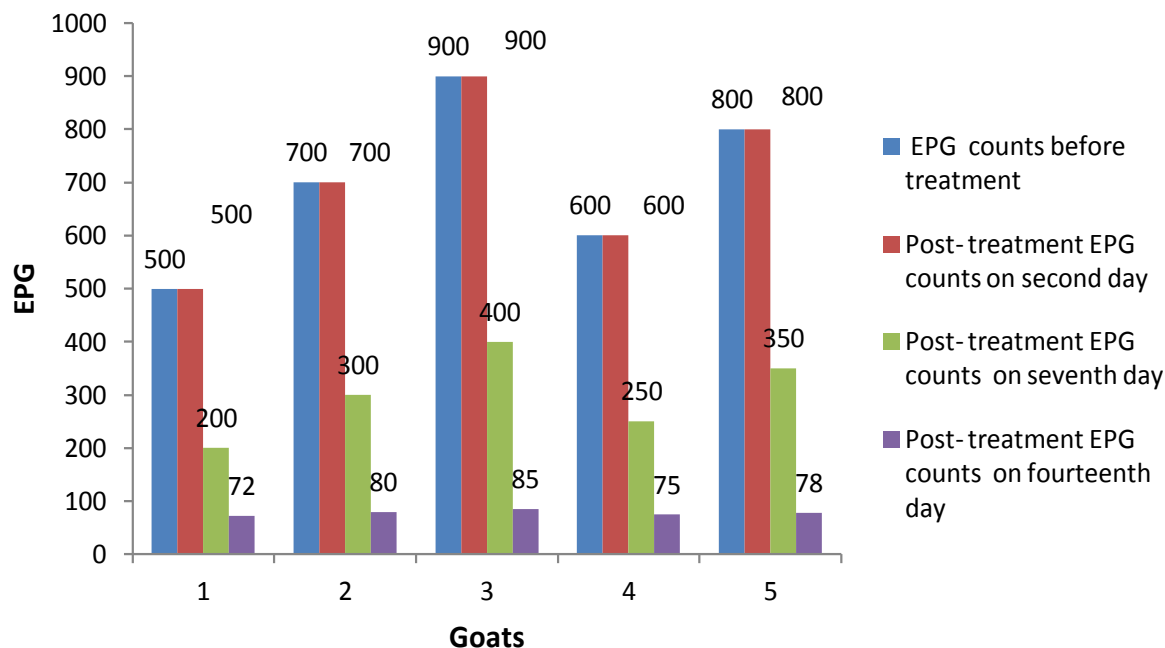
Figure 8. Number of eggs per gram after the treatment of goats with powder infusion of *T. diversifolia.*

Table 3. Results of the calculation of the percentage of EPG reduction.

Type of treatment	% of EPG reduction
Methanolic crude extract from *T. vogelii* leaves	98
Infusion of powder from *T. vogelii* leaves	97
Methanolic crude extract from *T. diversifolia* leaves	96
Infusion of powder from *T. diversifolia* leaves	95

against animals's parasites should be the positive alternative to the use of synthetic anthelmintics such as Thiabendazole, Phenothiazine and Levamizole which have often raised objections.

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Full Length Research Paper

Antimicrobial activity of *Anacardium occidentale* L. leaves and barks extracts on pathogenic bacteria

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The aim of this study was to investigate the antibacterial activity of aqueous, ethanol, ethyl acetate and dichloromethane extracts of two different cashew tree parts (leaf and bark). The susceptibility of strains to the different extracts was evaluated *in vitro* by disc diffusion method on ten reference strains and nine foods strains. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined respectively by macro-dilution method and seeding on solid medium. Among the four types of extracts, only the ethanol and ethyl acetate extracts of the two organs inhibited the growth of both bacteria and yeast. The MIC varies respectively between 0.039 and 0.625 mg/ml for reference strains and from 0.078 to 2.5 mg/ml for food isolated strains. Concerning MBC, it varies from 0.313 to 20 mg/ml for food isolated strains and from 0.078 to 20 mg/ml for reference strains. The ethanol and acetyl acetate extracts of the two cashew organs displays both a bacteriostatic and bactericidal effects on tested microorganisms. Our results suggest that extracts from cashew leaves may provide novel precursors for antimicrobial drug development research.

Key words: Antibacterial activity, *Anacardium occidentale* L., *Staphylococcus*, Benin.

INTRODUCTION

African flora in general and Benin in particular, have an important reserve of aromatic, food and medicinal plants. It was demonstrated that medicinal plants play an important role in the African pharmacopoeia (Badiaga,

2011). Indeed, according to WHO (2002), about 80% of Africans have recourse to traditional medicine that involves the use of plants' active principles, to treat most of diseases. Thus, a medicinal plant is defined as all plant

that one or more of its part including a substance can be used for therapeutic purposes or as a precursor of the synthetic antimicrobials (Sofowora,1984).

In Benin, several ethnobotanical studies have focused on identifying medicinal plants species (Sopkon and Ouinsavi, 2002; Biecke, 2004, etc.). Among these plant species, *Anacardium occidentale* L. has an important place. Its leaves, bark, roots and stem are traditionally used for the treatment of numerous diseases such as, allergy, cough, stomach ache, diarrhea, skin infections, etc. (Chabi Sika et al., 2013). Besides these medicinal uses, cashew plays several other important roles. Its wood is used mainly in carpentry, as firewood or turned into charcoal (Akinwale, 2000) whereas the resins are used in the manufacture of plastics and natural insecticides (Cavalcante et al., 2003).

In contrast to conventional medicine which seeks the origin and causes of diseases and infections, traditional medicine goes directly to the illness. The scientific study of the use of plants allows establishing a link between the two medicines and at term could eventually relieve populations (Badiaga, 2011). Nowadays, infectious diseases are responsible for 45% of deaths in low-income countries and 50% of premature death worldwide (Gangoue, 2007). In addition, among the death caused by microorganisms, bacterial infections account for 70% of cases (Walsh, 2003). To control these pathogens, antibiotics are frequently used. Effective use of antibiotics in the control of pathogens has raised hopes for eradicating infectious diseases. Unfortunately, the emergence of antibiotic-resistant bacteria has put an end to this wave of optimism (Adejuwon et al., 2011).

To face this increasing inefficiency observed with available antibacterial, it is essential to seek new wide spectrum action substances with more effective action. For this kind of research, there are many options. Among the possible options, we can cite the exploration of natural resources which contains a numerous active substances (Bocanegra-Garcia et al., 2009). The aim of this work was to study the antibacterial activity of cashew barks and leaves on 10 reference strains and nine *Staphylococcus* species isolated from three meat products.

MATERIALS AND METHODS

Tested microorganisms

The microorganisms tested include both bacteria (Gram positive and negative) and yeast. These microorganisms are composed of ten (10) reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* T22695, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilus* A24974, *Micrococcus luteus*, *Proteus vulgaris* A25015, *Streptococcus oralis* , *Enterococcus faecalis* ATCC 29212,*Candida albicans* MHMR) obtained from the National Laboratory for Quality Control of Medicines and Medical Consumables (LNCQ) and nine (9) species of *Staphylococcus* isolated from three different meat products in Ivory Coast (Attien et al., 2013).

Two parts (barks and leaves) of *Anacardium occidentale* L. were collected in plantations at Savè (Department Zou- Colline; Benin) in April 2013. After harvest, these parts are washed with clean water and then dried in the laboratory at room temperature (25°C) for about two weeks. After drying, the plant samples were then ground with a Retschmachine (SM 2000/1430/Upm/Smf) grinding. The obtained powders were used for the further extractions.

Preparation of extracts

Aqueous extracts

The aqueous extracts were obtained by using an adaptation of the method developed by Guede-Guina et al. (1995). Briefly, 50 g of leaf or bark powder of *A. occidentale* obtained above was macerated in 500 ml of distilled water on a magnetic agitator for 72 h at room temperature. The homogenate was then filtered two times on absorbent cotton and once on Whatman N° 1 paper. This filtrate was dried in the oven at 40°C; the obtained powder is considered as the total aqueous extract ready to use for antimicrobial tests (Adebo et al., 2008).

Ethanol, acetyl acetate and dichloromethane extracts

For these extractions, the methodology used was an adaptation of the protocol described by Sanogo et al (2006) and N'Guessan et al. (2007). Briefly, 50 g of *A. occidentale* powders were macerated in 500 ml of solvent (96% ethanol, ethyl acetate or dichloromethane) on magnetic agitator for 72 h. After two successive filtrations on hydrophilic cotton and Wattman N° 1 paper, the filtrate was concentrated in a Rotavaporvacuum packed at 50°C. After concentration, the filtrates were dried in an oven at 50°C. After drying, the powders were stored in tightly sterile bottles at 4°C until used.

Evaluation of the plant extracts'antibacterial activity

Sensitivity test

The antibacterial activities of the different extracts obtained were determined by employing the agar diffusion method of Anani et al. (2000). Four to five sterile discs with 5 mm as diameter were lodged, under aseptic conditions, in a Petri dish previously flooded of the appropriate bacterial culture. The discs were aseptically impregnated with 30 µl of the float of the crude plant extract stock solution, prepared 24 h before handling from 20 mg crude extract dissolved in 1 ml of sterile distilled water (SDW). The extracts were allowed to diffuse (15-30 mm) into the medium at room temperature and the plates were incubated at 37°C for 24 h (Adesokan et al., 2007). The zones of inhibition were measured using a scale (Doughari et al., 2007) after incubation time of 24 and 48 h.

Determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The minimum inhibitory concentrations of the plant extracts were determined using the tube dilution method test with visual assessment of growth of microorganisms (Delarras, 1998).

To achieve the range of concentrations, 1 ml of plant extract of known concentration (20 mg/ml) was added to 1 ml of the tube T1 SDW. After homogenization, 1 ml of the mixture (sample + distilled water) was taken for the tube T1, for the tube T2. For the remaining range, 1 ml of the mixture taken from the tube T2 was transferred into the tube T3. This procedure was repeated until T9 tube; 1 ml of
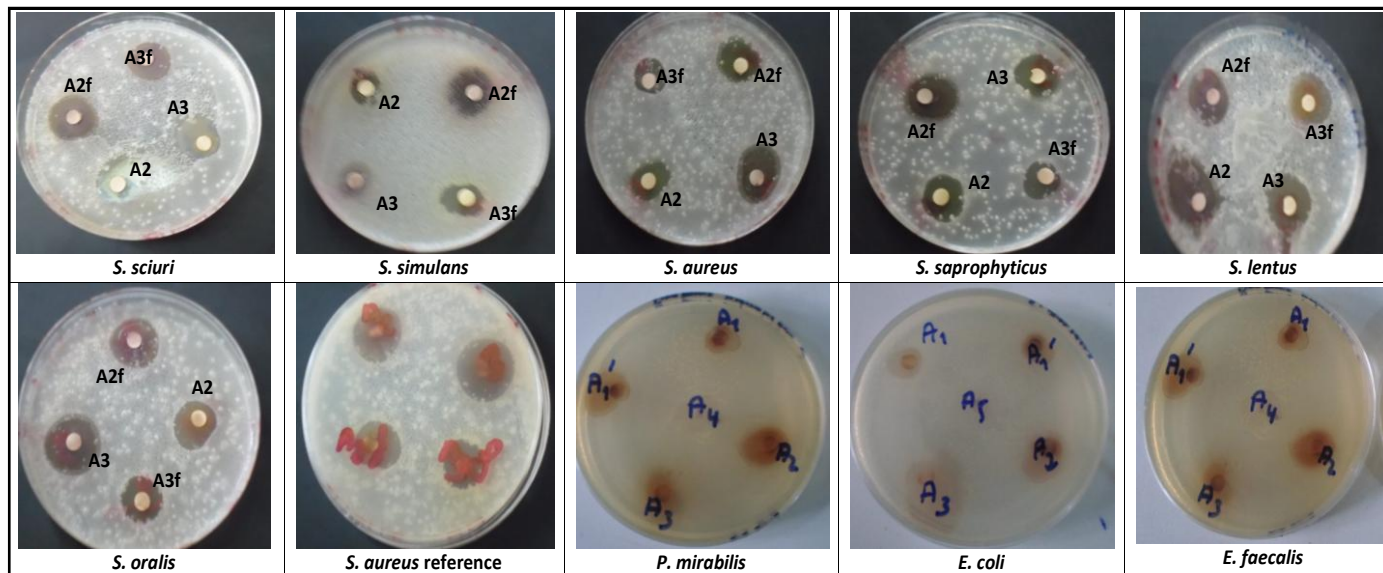
Figure 1. Sensitivity test realized on the reference and isolated food strains using the diffusion method. A1: aqueous bark extract, A2: ethanol bark extract, A3: acetyl acetate bark extract, A2f: ethanol leave extract, A3f: acetyl acetate leave extract, A4: dichloromethane bark extract, A1': control (ethanol), A5: control (acetyl acetate).

the content of this tube (T9) was rejected. Note that the control tube T0 receives only 1 ml extract with the concentration of 20 mg/ml.

The inoculum for each bacterial strain was made from a pre-culture. In brief, 1 ml of the respective bacteria inoculum was added to experimental (T1 to T9) and control (T0 and T10) tubes. After 24 h of incubation, the bacterial growth which leads to a turbidity was examined in each tube. The MICs of an extract against a given strain is the smallest concentration showing no visible growth on the naked eye.

The materials from each test tube used in the minimum inhibitory concentration assay that showed no growth after incubation, were streaked onto a solid nutrient agar plate and then incubated at 37°C for 24 h. The lowest concentration of the extract that showed no growth on the plate after 24 h taken as the minimum bactericidal concentration (MBC) following Alade and Irobi (1993).

Data analysis

The data obtained were subjected to analysis of variance (ANOVA) using SAS 9.2 software. Duncan test is used to compare the average difference at a significance level of 0.05.

RESULTS

Antimicrobial activity of cashew's leaf and bark

The results of sensitivity tests realized on the references strains and isolated food strains are summarized in Figure 1. By observing these results, it appears that the ethanol and acetyl acetate extracts have inhibited the growth of several microorganisms resulting in observation of the inhibition zone (Figure 1).The aqueous and dichloromethane extracts have no effect on the growth of studied strains. Thus, later on, only ethanol and acetyl

acetate extracts will be used to determine susceptibility parameters.

Susceptibility of studied strains in the presence of ethanol and acetyl acetate extracts of cashew's leaves and backs

Figure 2 shows the antimicrobial activities of different cashew extracts on the reference and food strains. In total, 30% (3/10) of reference strains were sensitive to ethanol and acetyl acetate bark extracts. Considering the isolated food strains, they were sensitive respectively at 90 (8/9) and 78% (7/9) for ethanol and acetyl acetate bark extracts. The isolated food strains were more sensitive to these two extracts than the reference strains. The same observations were made with ethanol and acetyl acetate extracts of the leaves with higher percentages. From these results, it is possible to conclude that extracts are more efficient on food isolates strains than on reference strains. Also, leaves extracts are more active than bark extracts independently of the origin of the strains.

Susceptibility of reference strains in the presence of cashew's ethanol and acetyl acetate extracts

The diameters of inhibition zones of the four extracts on the reference strains are shown in Figure 3. The susceptibility of reference strains varied depending on the type of extract but their effect was statistically different
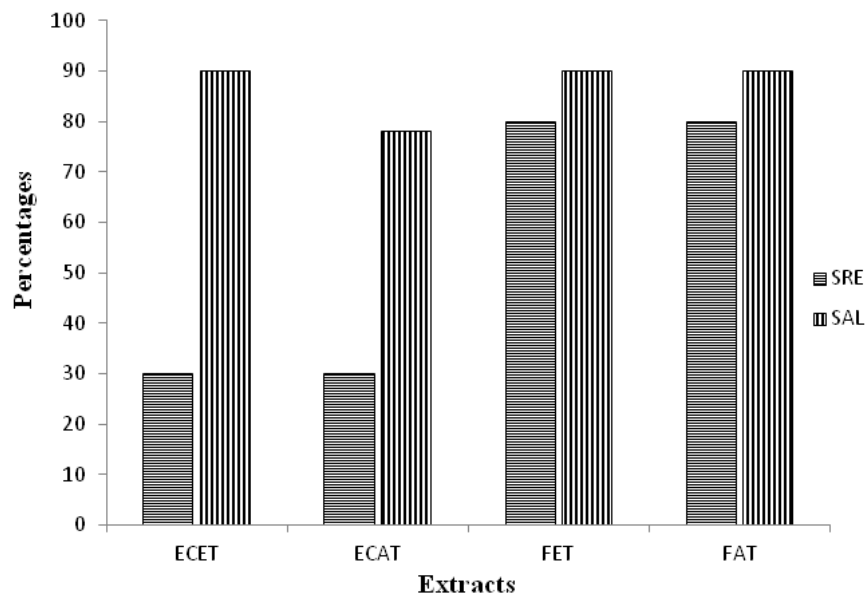
Figure 2. Antibacterial activity of different extracts from the leaves and bark of the cashew tree on reference and food strains. ECET: ethanol bark extract, ECAT: acetyl acetate bark extract, FET ethanol leaves extract, FAT: acetyl acetate leaves extract; SRE: reference strains and SAL: isolated food strains.
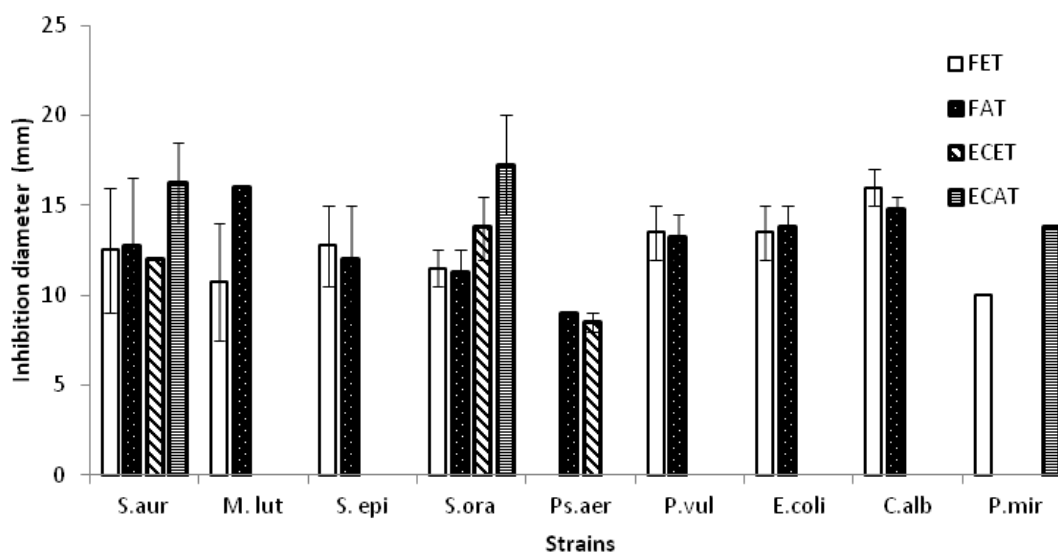

Figure 3. Mean inhibition zone diameter of some extracts in the reference strains. S. aur: *Staphylococcus aureus*, M.lut: *Miccrococcus luteus*, S. epi: *Staphylococcus epidermidis*, S. ora: *Streptococcus oralis*, Ps.aer: *Pseudomanas aeruginosa*, P.vul: *Proteus vulgaris*, E.coli: *Escherichia coli*, C.alb: *Candida albicans*, P.mir: *Proteus mirabilis*; ECT: ethanol bark extract, ECAT: acetyl acetate bark extract, FET ethanol leaves extract, FAT: acetyl acetate leaves extract.

(p<0.05). Therefore, the result showed that *S. aureus* and *S. oralis* were sensitive to the four extracts and the largest diameters (16.25 and 17.5 mm) were obtained with the acetyl acetate bark extract. With *S. aureus*, the three other extracts displayed approximately the same diameter (12.5 mm) whereas with *S. oralis*, a difference

was observed on the diameters of those extracts. Besides *P. aeruginosa*, sensitive only to acetyl acetate leaf extract and the ethanol bark extract, has the lowest inhibition diameter (8.5 mm) whereas *M. luteus* (only sensitive to extracts from leaves) has the largest inhibition diameter with acetyl acetate extract (16 mm).
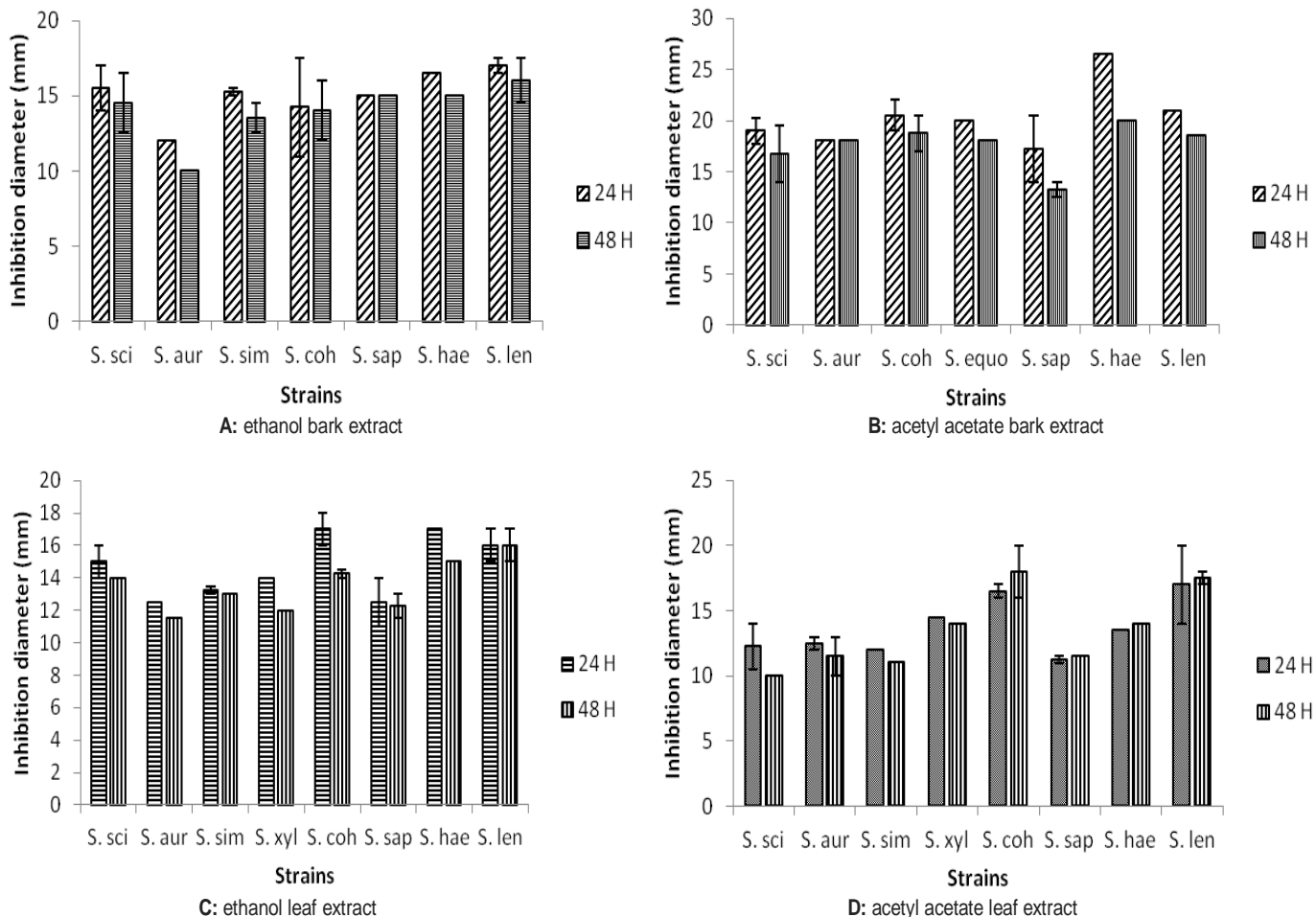
Figure 4. Mean inhibition zone diameter of four extracts on isolated food strains during 24 and 48 h of incubation. *S. sci: Staphylococcus sciuri; S. aur: Staphylococcus aureus; S. sim: Staphylococcus simulans ; S. coh: Staphylococcus cohnii ; S. equo : Staphylococcus equorum ; S. sap: Staphylococcus saprophyticus ; S. hae: Staphylococcus haemolyticus; S. len: Staphylococcus lentus; S. xyl: Staphylococcus xylosus.*

Susceptibility of isolated food strains in the presence of cashew's ethanol and acetyl acetate extracts

Figure 4 presents the mean inhibition diameters of different cashew extracts on food isolated strains during 24 and 48 h of incubation. The results revealed no significant difference ($p > 0.05$) between the inhibition diameters of the ethanol and acetyl acetate bark extracts whatever the incubation time (24 and 48 h) on isolated food strains. Regarding the leaf extract, the inhibition diameters of the ethanol extract of the isolated food strains were significantly different between the strains ($p < 0.05$) at 24 h and highly different ($p < 0.001$) after 48 h. The inhibition diameters of acetyl acetate extract on food isolated strains were very different after 48 h but no statistical difference was observed at 24 h. More other, the results show that these diameters were not statistically different ($p < 0.05$) over time (24 and 48 h) regardless of the type of extract and the organ used.

From these results, it appears that inhibition diameters obtained after 24 h were higher than those measured after 48 h. Unless the level of the ethanol extract (Figure 4A), *S. saprophyticus* strains had the same diameter at 24 and 48 h whereas with acetyl acetate extract the same diameters (18 mm) were obtained with *S. aureus*. The larger diameters were obtained on *S. lentus* (17 and 16 mm) at 24 and 48 h with the ethanol extract while with acetyl acetate extract the larger diameter (26.5 and 20 mm) were obtained on *S. haemolyticus.*

Regarding leaf extracts (Figure 4C and D); it is observed that the inhibition diameters after 24 h were higher than those obtains after 48 h except for *S. lentus* that displayed the same inhibition diameters (16 mm) both at 24 and 48 h (Figure 4C). As shown in Figure 4D, it is observed that the mean of inhibition diameters at 24 h are higher than those obtained after 48 h with *S. sciuri*, *S. aureus*, *S. simulans* and *S. xylosus* whereas with *S. cohnii*, *S.saprophyticus*, *S. haemolyticus* and *S. lentus*,

Table 1. Minimum inhibitory concentrations of ethanol and acetyl acetate cashew' (bark; leaves) extracts on the reference strains.

Strains	MIC (mg/ml)			
	ECET	ECAT	FET	FAT
S. aureus	0.313	0.313	0.313	0.313
M. luteus	-	-	0.078	0.625
S. epidermidis	-	-	0.039	0.313
S. oralis	0.625	0.313	0.625	0.625
E. faecalis	-	-	-	-
Ps. aeruginosa	0.156	-	-	0.625
P. mirabilis	-	0.313	0.625	-
P. vulgaris	-	-	0.313	0.625
E. coli	-	-	0.625	1.25
C. albicans	-	-	0.156	0.313

ECET: ethanol extract of the bark, ECAT: acetyl acetate bark extract, FET: ethanol extract of the leaves, FAT: acetyl acetate leaf extract.

Table 2. Minimum inhibitory concentrations of cashew bark and leave extracts on food strains.

Strains	MIC (mg/ml)			
	ECET	ECAT	FET	FAT
S. sciuri	0.313	1.25	1.25	1.25
S. aureus	0.313	0.313	0.625	1.25
S. simulans	2.5	-	2.5	2.5
S. xylosus	0.078	-	0.313	0.625
S. cohnii	0.156	0.313	1.25	1.25
S. equorum	-	0.078	-	-
S. saprophyticus	0.156	0.078	0.313	0.313
S. haemolyticus	0.313	0.313	0.625	0.625
S. lentus	0.313	0.078	1.25	1.25

ECET: ethanol bark extract, ECAT: acetyl acetate bark extract, FET : ethanol leaves extract, FAT: acetyl acetate leaves extract.

the mean of inhibition diameters obtained after 48 h were higher than those measured at 24 h.

Besides, it also appears from the results that *S. sciuri*, *S. aureus*, *S. cohnii*, *S. saprophyticus* and *S. lentus* are sensitive to the four extracts (Figure 4). For these 5 strains, the larger diameter was obtained with of acetyl acetate bark extract excepted for *S. saprophyticus* recording their largest inhibition diameter (15 mm) in the presence of ethanol bark extract. From these results, it appeared that *S. aureus* and *S. haemolyticus* displayed the same sensibility towards ethanol extracts of the two organs while for the acetyl acetate leaf extract, only *S. haemolyticus* have shown the highest diameters.

Minimum inhibitory concentration (MIC) of cashew extracts on the reference strains

Table 1 shows the minimum inhibitory concentrations (MIC) of cashew extracts (bark and leaf) on the reference strains. The computed MIC values ranged from 0.039 and 0.625 mg/ml for both cashew's leaf and bark depending on the strains. For acetyl acetate bark extract, the MIC was 0.313 mg/ml with three strains (*S. aureus*, *S. oralis* and *P. mirabilis*). Considering ethanol bark extract, the largest CMI was observed on *S. oralis* (0.625 mg/ml), while the most sensitive strain to this extract was *Ps. aeruginosa* with 0.156 mg/ml as MIC.

For ethanol leaves extract, the highest MIC (0.625 mg/ml) was observed with *S. oralis*, *P. mirabilis* and *E. coli* while the lowest MIC (0.039 mg/ml) was observed on *S. epidermidis*. By testing the acetyl acetate leaf extract, the highest MIC obtained (0.625 mg/ml) was obtained on *M. luteus, S. oralis*, *P. aeruginosa* and *P. vulgaris* while the lowest MIC (0.313 mg/ml) was obtained with *S.

aureus*, *S. epidermidis* and *C. albicans*. The analysis of these results shows that *S. aureus* has the same MIC (0.313 mg/ml) for all the tested extracts.

MIC of cashew extracts on food isolated strains

Table 2 presents the MIC of cashew barks and leaves extracts on the isolated food strains. The results showed that the MIC varied according to strains and type of extracts (leaf and bark). MICs range from 0.078 to 2.5 mg/ml. Among tested strain, *S. xylosus* was more sensitive to the ethanol bark extract with a MIC of 0.078 mg/ml while the least sensitive strain to this extract was *S. simulans* with a MIC of 2.5 mg/ml. For acetyl acetate bark extract, the highest MIC (1.25 mg/ml) was obtained on *S. sciuri* while the lowest MIC (0.078 mg/ml) was obtained on *S. equorum*, *S. saprophyticus* and *S. lentus*.

With the ethanol leaf extract, the highest MIC (2.5 mg/ml) was obtained on *S. simulans* while the lowest MIC (0.313 mg/ml) was obtained, with the same extract, on *S. xylosus* and *S. saprophyticus*. Using the acetyl acetate leaf extract, the highest MIC (2.5 mg/ml) was obtained on *S. simulans* while the lowest one (0.313 mg/ml) was obtained from *S. saprophyticus*. Finally, the results showed that *S. haemolyticus* has recorded the same MIC (0.313 mg/ml) with bark extracts. The same tendency was observed on the leaves extracts (0.625 mg/ml).

Minimum bactericidal concentration (MBC) of cashew bark and leaf extracts on reference strains

Table 3 presents the minimum bactericidal concentration (MBC) of cashew bark and leaf extract on reference strains. The results show that the MBCs varied according to the bacterial strains and organs used (bark and

Table 3. Minimum bactericidal concentrations of cashew bark and leave extracts on reference strains.

Strains	MBC (mg/ml)			
	ECET	ECAT	FET	FAT
S. aureus	1.25	0.313	1.25	0.625
M. luteus	-	-	0.625	1.25
S. epidermidis	-	-	20	5
S. oralis	0.625	0.625	5	5
E. faecalis	-	-	-	-
Ps. aeruginosa	20	-	-	10
P. mirabilis	-	0.625	5	-
P. vulgaris	-	-	1.25	5
E. coli	-	-	10	10
C. albicans	-	-	0.313	0.625

ECET: ethanol bark extract, ECAT: acetylacetate bark extract, FET: ethanol leave extract, FAT: acetylacetate leave extract.

Table 4. Minimum bactericidal concentrations of cashew bark and leaf extracts on food isolated strains.

Strains	MBC (mg/ml)			
	ECET	ECAT	FET	FAT
S. sciuri	10	10	10	20
S. aureus	>20	20	10	10
S. simulans	>20	-	5	10
S. xylosus	>20	-	5	5
S. cohnii	1.25	0.625	5	2.5
S. equorum	-	0.625	-	-
S. saprophyticus	1.25	0.078	1.25	1.25
S. haemolyticus	>20	20	5	5
S. lentus	>20	20	20	10

ECET: ethanol bark extract, ECAT: acetylacetate bark extract, FET: ethanol leave extract, FAT: acetylacetate leave extract.

leaves). Thus, the MBC varied from 0.313 to 20 mg/ml. With the barks, *S. oralis* displayed the lowest MBC (0.625 mg/ml) in the presence of the ethanol extract and the largest MBC (20 mg/ml) was obtained with the same extract on *P. aeruginosa*. With acetyl acetate extract, *S. aureus* was the strain that displayed the lowest MBC (0.313 mg/ml) whereas the largest MBC (0.625 mg/ml) was obtained on *S. oralis* and *P. mirabilis*.

With leaf extract, the highest sensitivity was observed on *Candida albicans* using the ethanol extract displaying a MBC of 0.313 mg/ml while the highest MBC obtained with this extract was 20 mg/ml on *S. epidermidis*. For the acetyl acetate leaf extract, *S. aureus* and *C. albicans* showed the lowest MBC (0.625 mg/ml) while the highest

MBC (10 mg/ml) was obtained on *P. aeruginosa* and *E. coli*. Considering the CMB results, it appeared that *S. oralis* was more sensitive to both bark (0.625 mg/ml) and leaf (5 mg/ml) extracts.

MBC of cashew bark and leaves extracts on food isolated strains

Table 4 presents the minimum bactericidal concentrations of cashew' bark and leaf extracts on food isolated *Staphylococcus* strains. These results show that the MBC of the bark and leaf extracts varied from 0.078 to 20 mg/ml depending to the *Staphylococcus* species tested.

Using barks, the highest observed MBC (10 mg/ml) was obtained with the ethanol extract on *S. sciuri* while the lowest MBC (1.25 mg/ml) was obtained with this extract on *S. cohnii* and *S. saprophyticus*. Indeed, with 20 mg/ml; the ethanol extract had no bactericidal effect on *S. aureus, S. simulans*, *S. xylosus*, *S. haemolyticus* and *S. lentus*. The acetyl acetate bark extract gives the largest MBC (20 mg/ml) with *S. aureus*, *S. haemolyticus* and *S. lentus* while the lowest MBC (0.078 mg/ml) was obtained on *S. saprophyticus*.

With the leaves, the largest MBC (20 mg/ml) was obtained on *S. lentus* (with ethanol extract) and *S. sciuri* (with acetyl acetate extract). The lowest MBC (1.25 mg/ml) was obtained on *S. saprophyticus* independently to the extract. From these results, it appears that *S. cohnii* was more sensitive to the acetyl acetate extract than the ethanol extracts independently to the organ used.

Evaluation of bactericidal and bacteriostatical effects of cashew barkand leaves extracts

To evaluate the effect of two types of the cashew bark and leaves extract on the reference and food isolated strains, the ratio of MIC and MBC parameters were calculated.The observation of these results allows concluding that these extracts have both bactericidal and bacteria-statical effects on reference and food-isolated strains. However, it was observed that the ethanol bark extract had no bactericidal effect on food-isolated strains (Tables 5 and 6).

DISCUSSION

In this study, four extract (aqueous, ethanol, acetyl acetate and dichloromethane) of leaf and back were tested on 19 strains. The ethanol and acetyl acetate extracts of the two organs have inhibited the growth of several microorganisms while dichloromethane and aqueous extracts have no effect on the strains tested at the dose of 20 mg/ml. The ethanol and acetyl acetate

Table 5. Bactericidal and bacteria-static effects of cashews bark and leaf extracts on reference strains.

Strains	MBC/MIC			
	ECET	ECAT	FET	FAT
S. aureus	4	1*	4	2*
M. luteus	-	-	8	2*
S. epidermidis	-	-	512	16
S. oralis	1*	2*	8	8
E. faecalis	-	-	-	-
Ps. aeruginosa	128	-	-	16
P. mirabilis	-	2*	8	-
P. vulgaris		-	4	8
E. coli	-	-	16	8
C. albicans	-	-	2*	2*

ECET: ethanol bark extract, ECAT: acetylacetate bark extract, FET: ethanol leave extract, FAT: acetylacetate leave extract ; * = with bactericidal effect.

Table 6. Bactericidal and bacterio-static effects of cashews bark and leave extracts on food isolated strains.

Strains	MBC/MIC			
	ECET	ECAT	FET	FAT
S. sciuri	32	8	8	16
S. aureus	-	64	16	8
S. simulans	-	-	2*	4
S. xylosus	-	-	16	8
S. cohnii	8	2*	4	2*
S. equorum	-	8	-	-
S. saprophyticus	8	1*	4	4
S. haemolyticus	-	64	8	8
S. lentus	-	256	16	8

ECET: ethanol bark extract, ECAT: acetylacetate bark extract, FET: ethanol leave extract, FAT: acetylacetate leave extract ; * = with bactericidal effect.

extracts have inhibited the growth of yeast, Gram+ and Gram- bacteria. The same observations were made on the ethanol extract by Aiswarya et al. (2011). Indeed, these authors have shown that the cashews apple's ethanol extracts (1 mg/ml) inhibited the growth of Gram+ (*Bacillus cereus*) and Gram- (*K. pneumoniae*) bacteria. However, our results with aqueous extract contradict those reported by other authors in Nigeria during their work on the same kind of extract revealing that cashew leaf aqueous extract are able to inhibits, at 0.1 mg/ml, the growth of *B. cereus*, *Salmonella typhi* and *Shigella dysenteriae* (Ifesan et al., 2013). The difference observed with ours work can be explained by the fact that the

strains used are not the same and, additionally, strains are not from the same origin. Moreover, the *A. occidentale* leaves aqueous extract were known to make, at 200 mg/ml, an inhibitory effect on the growth of many bacteria such as *S. aureus*, *E. coli*, *P. aeruginosa*, *S. dysenteriae* and *Salmonella typhimurium* (Omojasola and Awe, 2004). Comparing our results to those obtained by Omojasola and Awe (2004), the observed difference can be linked to the concentration (20 mg/ml) we used in our study that was tenfold lower than that reported by the latter authors.

Regarding the dichloromethane extract that has no effect on the strains, we can say that this solvent does not concentrate enough the compounds that can inhibit the microorganism's growth. Nevertheless during one of their studies, Ngari et al. (2013) showed that the *Euclea divinorum* root dichloromethane extracts can inhibits, at 200 mg/ml, the growth of many bacteria among which *E. coli*, *S. aureus* and *B. subtilis*. Comparing the inactivity observed with dichloromethane extracts in the present study to those reported by Ngari et al. (2013), we can say that the difference may be not only be due to the fact that we did not use the same plant but also because the concentration they used (200 mg/ml) in their study was higher than ours (20 mg/ml).

Our study reveals that all the extracts were more active on food strain (in majority Gram+) than on reference strains (Figure 2). It was also observed that the extracts were more effective against Gram positive bacteria than Gram negative one. The results obtained in this study corroborate those of Agedah et al. (2010) when they showed that Gram positive bacteria are more sensitive to the cashew ethanol leaf extract than Gram- ones at the same concentration. These results can be explained by the fact that Gram positive bacteria are devoid of outer membrane in their cell walls. Thus, the outer membrane may be responsible of the difference observed in the sensitivity level between the Gram + and - in presence of the extract. Among all the tested extracts, we denote that leaves extracts (ethanol and acetyl acetate) are more effective than bark's. From this fact, we can speculate that the active antimicrobial agents may be more concentrated in the leaves than in barks. These results are in agreement with the assertion made by the traditional elders that, for the treatment of bacterial infections, the backs are mostly used than leaves (Chabi Sika et al., 2013). The antimicrobial activities observed may be explained by the presence of large chemical groups such as tannins, flavonoids, alkaloids, saponines, steroids or triterpenes (Amvam et al., 1998; Cowan, 1999; Kolodziej et al., 1999; Omojasola and Awe, 2004; Sujatha et al., 2011).

Considering the inhibition zone diameters, it was noticed that no significant difference was observed between the diameters of ethanol and acetyl acetate bark extracts regardless of duration (24 and 48 h) on both reference and food isolated strains. These results

corroborate those of Bolou et al. (2011) when they found no difference between the inhibition diameters of ethanol and acetyl acetate *Terminalia glaucescens* extracts on the growth of *S. typhi*. But, Arekemase et al. (2011) in their study denoted a significant difference between the inhibitions diameters at a concentration of 200 mg/ml. The high concentration they use in their study and the difference of strains might be the reasons of this difference observed between our results and those of Arekemase et al. (2011).

Regarding the leaf extracts, the inhibition diameters of ethanol extract on the food-isolated strains were different ($p < 0.05$) at 24 h and highly different ($p < 0.001$) at 48 h. The same observations of efficiency were made by Arekemase et al. (2011) by showing that the inhibition diameters of cashew leaf ethanol extract were significantly different among the studied strains. Besides, our results also show that the sensitivity of ethanol leaf extract was more efficient than the ethanol bark extract while acetyl acetate leaf extract was more efficient than the acetyl acetate bark extract (Figure 4). These results corroborate those of Hamid and Aiyelaagbe (2011) when they demonstrated, *in vitro*, that the alcohol extract of *Alafiabarteri* had a better inhibition effect on the growth of many bacterial (*S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*) than the acetyl acetate extract. In the same way, Bolou et al. (2011) also demonstrated that the *T. glaucescens* acetyl acetate extract was more efficient than the ethanol extract on *S. typhi*. Taking into account the previous results from various studies, we can speculate that ethanol, as solvent, allow a better solubility of antimicrobial agent of the leaves than the barks; contrary to acetyl acetate in which the antimicrobial agent of barks are more soluble than that of leaves.

The MIC was variable depending on the strains and extracts (Tables 2 and 3). With the barks, the greater sensitivity was observed on *S. xylosus* (ethanol extract) and on *S. equorum*, *S. saprophyticus* and *S. lentus* (acetyl acetate extract) at the concentration of 0.078 mg/ml. With the leaves, at the concentration of 0.039 mg/ml, ethanol extracts inhibit the growth of *S. epidermidis*. These concentrations were higher than those reported by Akash et al. (2009) when they proved that *S. aureus* and *B. subtilis* were more sensitive to the ethanol leaf extract of *Anacardium occidentale* with MIC = 15.62 µg/ml. Nevertheless, our MIC values were lower than the 50 mg/ml reported by Arekemase et al. (2011). However, the greatest MIC was 0.625 mg/ml on all references strains and 2.5 mg/ml on *S. simulans* (among food-isolated strains) independently of the type of extracts. Akash et al. (2009), in one of their study, reported lower values (31.25 µg/ml) than ours; the difference observed may be explained by the divergent extraction methods and the different origins of strains used. Indeed, depending on the extraction methods, the antimicrobial agents extracted may have different concentrations. Thus, it appears that the extraction methods probably may have a major impact on the quantity of extracted antimicrobial agents.

The MBC were variable with regard not only to the strains but also to the type of extracts (Tables 3 and 4). With the bark extracts, the lower MBC was 0.078 mg/ml on *S. saprophyticus* with acetyl acetate extract and the greatest MBC was 20 mg/ml on *P. aeruginosa* with ethanol extract and on food-isolated *S. aureus*, *S. haemolyticus* and *S. lentus* with acetyl acetate extract. With the leaf extracts, the lowest MBC was 0.313 mg/ml on *C. albicans* with ethanol extract while the greatest MBC was 20 mg/ml on *S. epidermidis*, *S. lentus* with ethanol extract and *S. sciuri* with acetyl acetate extract. These results contrasted those of Quelemes et al. (2013) reporting a range of 6.75 to 27 µg/ml as MBC during an *in vitro* test of cashew gum on *S. aureus*, *E. faecalis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*.

The ratio parameters of the MIC and MBC shows that, according to the stains, leaf and bark extracts have both bactericidal and bacterio-statistical effect on reference and food-isolated strains (Tables 5 and 6). Thus, the acetyl acetate extracts have more bactericidal activity than ethanol extracts. Thus acetyl acetate leaf extract has a bactericidal effect on *S. aureus*, *M. lentus* and *C. albicans* regarding reference strains and on *S. cohniu* for food isolated strain. For the bark extracts, the bactericidal effect was observed on *S. aureus, S. oralis* and *P. mirabilis* regarding reference strains and on *S. cohniu* and *S. saprophyticus* for the food isolated strain. These results are similar to those reported by Dramane et al. (2010) which showed that the dichloromethane extract of *Erythrina senegalensis* has a bactericidal effect on many microorganisms (*C. albicans*, *E. faecalis*, *S. epidermidis*, *S. aureus*, *P. mirabilis*, *E. coli* and *P. aeruginosa*). The difference in bactericidal effect can be explained by the difference in the solvents used during the extraction and the different plants species used.

Conclusion

This study highlights the antibacterial activities of the cashew leaf and bark extracts. The activity of these extracts was remarkable on *Staphylococcus* food-isolated and reference strains in the study. The results show that the ethanol and acetyl acetate leaf extract were more efficient than the ethanol bark extract. Moreover good activity in the leaves than the bark was detected. These results partly justify the use of this plant by traditional healers in the treatment of certain diseases such as dysentery, diarrhea, skin diseases and urinary tract infections.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Microwave mutagenesis of high-producing rennet strain from *Bacillus subtilis*

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In order to acquire a high-producing rennet strain from *Bacillus*, different microwave irradiation intensities and durations were tested. The effect of microwave irradiation power and time were studied and the microwave mutagenesis parameters were optimized. The results indicated that the best conditions for microwave mutagenesis that produced better performed mutant strains were 450 W, 4 min. Under these conditions a viable strain with high-producing rennet (Strain No.: BsB1.1) was achieved whose genetic stability is good and rennet activity reaches 516.12 U/g which has increased by 29.0% compared with the original strain in the experiment.

Key words: *Bacillus subtilis*, high-producing rennet, microwave mutagenesis.

INTRODUCTION

Rennet is the zymin used for milk or goat milk solidification in the cheese making and it is the crucial enzyme for milk solidification in cheese production. As the main additive, rennet activity has a greater influence on the quality of cheese products and will determine the texture and tastes of the products.

The current method used to obtain chymosin mainly involves three types: the first is extraction of enzyme from the stomachs of unweaned calves. This process is expensive and the supply is subject to the fluctuations in the veal market. The second is from plants, but its production is restricted by time, geographical conditions and so on, therefore it is also not suitable for mass production. The third is from microorganism. Rennet produced by microorganism is not only in low costs but its

productivity can be expanded unlimitedly, so microorganism milk-clotting enzyme is one of the most promising development directions. However, the rennet-producing capability was relatively weak and therefore lacked of positive utilization value.

Mutagenesis could overcome the disadvantage of weak rennet-producing capability of wild-type strains. Microwave irradiation induces polar molecules around the cell walls to produce intense electric polarity oscillation (Kirsschvink, 1996; Chen et al., 2006), damages hydrogen bonds and base stacking forces, causes changes to DNA structure as well as the damages on the surface of microbial cells (Woo et al., 2000), and therefore results in chromosome changes and the production of mutants with desirable variation of genetic

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characters (Hong et al. 2004; Li et al. 2009a,b; Pan et al. 2008; Selvakumar et al. 2008; Lamsa et al. 1990). Compared with other mutational approaches (for instance: chemical mutagenesis and UV light irradiation), the advantages of microwave irradiation include the avoidance of toxic substance production, the convenience of operation and the potential obtaining of numerous mutants in short time (Xu et al., 2009). For instance, Song et al. (2008) observed higher mutation rate and obtained anaerobic hydrogen producing strain H-8, hydrogen production of the obtained variant HW195 was 50.7% higher than that of the original strain. Xu et al. (2009) also obtained high yields of ideal strains L-lactic acid bacteria by microwave mutagenesis. Nonetheless, no practical application of microwave mutagenesis for *Bacillus subtilis* has been reported. This study has the following aims: to obtain mutant strains of *B. subtilis* BsB1.1 with high rennet - producing capability by microwave mutagenesis, to establish optimum mutagenesis conditions, and to produce reference materials for the study of rennet with efficient rennet -producing capability.

MATERIALS AND METHODS

Bacterial strain

The wild-type rennet -producing *B. subtilis* BsB1.1 isolated from the cheese of Co. YILI of the People's Republic of China. The strain BsB1.1 had been identified as *B. subtilis* based on biochemical tests, and a 1.5-kbp sequence of the 16S rRNA gene region which was fully determined and analyzed by the Clustal W program (Gen Bank accession: KC492497). The strain BsB1.1 was able to grow in Casein media and was found possessing rennet -producing capability. Pure cultures were maintained in casein agar (CA) slants at 4°C until needed.

Medium

Casein medium

Preparation of crude enzyme: Inoculate bacteria fluid at 2% proportion on casein fluid nutrient medium at 30°C, shake cultivation for 2 days at 180 r/min, and then put all the fluid into centrifugal tube, centrifuge for 30 min at 3,500 rpm. And the supernatant in the tube is crude enzyme.

Determination of rennet activity

Rennet activity was determined according to Arima method. Take 5 mL 100 g/L skim milk, preserve heat for 5 min at 35°C in water bath, and then add 0.5 mL fermentation broth, mix them in the vortex mixer and record accurately the time from enzyme adding to milk solidification.

Sohxlet Unit (SU): The enzyme number in 40 min solidifying 1 mL 100 g/L skim milk.

$$SU = (2400 \times 5 \times D) / (T \times 0.5)$$

Where, T represents the coagulating time, and D represents the dilution multiple of the enzyme.

Determination of proteolysis test

Five milliliters 1.2% casein solution was added to 1 mL enzyme that has been diluted tenfold, and heat preserved for 10 min at 35°C. Enzyme reaction was terminated with 5 mL 0.44 mol/L TCA, and heat continually preserved for 20 min. Reaction mixture was filtered with filter paper. Two milliliters filtrate was taken and 5 mL 0.55 moL/lNa$_2$CO$_3$ solution and 1 mL 0.7 mol/L Folin reagent added. Heat was preserved for 20 min at 35°C, and absorbance measured at 660 nm wavelength.

Proteolysis activity = （A × K × V）/ （N × t）

Where: A represents the absorbance at 660 nm wavelength; K represents the tyrosine micrograms/μg when light absorption is "1" in the standard curve; V represents the total volume/mL of enzymatic reaction; t represents the time/min of enzymatic reaction; N represents the dilution multiple of the enzyme.

Screening of original strain

Preserved strain from the plate in the laboratory was selected and coagulating effect observed. Rennet activity and proteolysis activity of the strain was determined. The strain that have favorable coagulating effect, high rennet activity and low proteolysis activity was original one.

Screening of the best mutagenic time

Ten milliliters *B. subtilis* suspension was taken into 50 mL triangular flasks. The triangular flasks were sealed with sealing film, and the triangular flasks placed into a container filled with ice, and then the container put in a microwave for treatment. The mutagenic power was 450 W, and the mutagenic time 2, 3, 4, 5 and 6 min, respectively. After irradiation, the triangular flasks were moved on a clean bench; and 100 ul suspension taken from each triangular flask, respectively for dilution to 10^{-4}, 10^{-5}, and 10^{-6} times. They were coated on the casein culture plate after refrigeration and protection from light for 3 h. The *B. subtilis* suspension was coated before treatment under the same conditions, and protected from light for cultivation for 2 days. And then the clump counts were added up. The death rate, the positive and negative mutation rate was calculated and the best mutagenic time determined. Each experiment was done for 3 times.

Screening of the best mutagenic power

Ten milliliters of *B. subtilis* suspension was taken into 50 mL triangular flasks. The triangular flasks were sealed with sealing film, and the triangular flasks placed into a container filled with ice, and then the container put into a microwave for treatment. The mutagenic time was 4 min, and the mutagenic power 350, 400, 450 and 500 W, respectively. After irradiation, the triangular flasks were moved on a clean bench; and 100 ul suspension taken from each triangular flask respectively for dilution to 10^{-4}, 10^{-5} and 10^{-6} times. They were coated on the casein culture plate after refrigeration and protection from light for 3 h. The *B. subtilis* suspension was coat before treatment under the same conditions, and protected from light for cultivation for 2 days. And then the clump counts were added up. The death rate, the positive and negative mutation rate was calculated and the best mutagenic time determined. Each experiment was done for 3 times.

Table 1. Screening results of three *Bacillus Subtilis* preserved in the laboratory

Strain No.	curd effect	Chymosin activity (U/g)	Proteolysis activity (U/g)
BsB1	Better flavor, tight and smooth texture, moderate hardness with the color of cream yellow and even gloss	399.96	1.280
BsB2	Favorable flavor, basically even but slightly soft texture, relatively tight tissue with a slight change of color and luster	363.60	2.633
BsB3	Acceptable flavor, loose tissue with a slight change of color and luster and fragility	355.52	2.898

Screening of the mutagenic strain

Mutagenic strains were selected with bigger curds circle and little bacteriostasis circle, coagulating effect of the mutagenic strains were observed, respectively and rennet activity, proteolysis activity and stability of the strains determined. The screened strain that has favorable coagulating effect, high rennet activity, low proteolysis activity and genetic stability were target strains.

Texture analysis of the cheese by rennet from original strain and objective strain

Crude rennet solution was prepared by the same way. Then the rennet form original and objective strain was used to make cheese separately to contrast the texture by texture analyzer.

RESULTS AND ANALYSIS

Screening of original strains

The results of screening original strains are shown in Table 1. BsB1 strain has a better flavor, tight and smooth texture, moderate hardness with the color of cream yellow and even gloss. Rennet activity of BsB1 strain is 399.96 U/g, and its proteolysis activity is 1.280 U/g. BsB2 strain has a favorable milk-clotting enzyme flavor, basically even but slightly soft texture, relatively tight tissue with a slight change of color and luster. Rennet activity of BsB2 strain is 363.60 U/g, and its proteolysis activity is 2.633 U/g. BsB3 strain has the characteristics of an acceptable flavor, loose tissue with a slight change of color and luster, and fragility. Rennet activity of BsB3 strain is 355.52 U/g, and its proteolysis activity is 2.898 U/g. BsB1 strain has the best coagulating effect, maximum rennet activity and minimum proteolysis activity. Therefore, BsB1 strain is chosen as the original strain of this experiment.

Effects of mutagenic time on death rate and positive mutation rate

With the extension of the mutagenic time, the death rate of the strain has increased to some extent (Figure 1). When the mutagenic time is 4 min, the death rate of the suspension is 73%. With the extension of the mutagenic time, the positive mutation rate increases. When the death rate of the suspension is 73%, the positive mutation rate reaches the maximum. After that, with the increase of mutagenic power, the positive mutation rate has continuously decreased. Therefore, 4 min as the mutagenic time of microwave mutagens is chosen.

Effect of mutagenic power on death rate and positive mutation rate

The experimental result is shown in Figure 2. With the increase of the mutagenic power, the death rate of the strain has increased to some extent. When the mutagenic power is 450 W, the death rate of the suspension is 76%. With the increase of the mutagenic power, the positive mutation rate increases. When the death rate of the suspension is 76%, the positive mutation rate reaches the maximum. After that, with the increase of mutagenic power, the positive mutation rate has a tendency to decrease. Therefore, choose 450 W as the mutagenic power of microwave mutagenesis.

Screening of mutagenic strain

From Table 2, coagulating texture of BsB1.1 strain is the tightest and the coagulating time is much more shortened; rennet enzyme activity of BsB1.1 strain is 516.12 U/g, and its proteolysis activity is 1.296 U/g. Coagulating texture of BsB1.2 strain is slightly tight and the coagulating time is more shortened; rennet activity of BsB1.2 strain is 457.20 U/g, and its proteolysis activity is 2.530 U/g. Coagulating texture of BsB1.3 strain is slightly tight and the coagulating time is slightly shortened; rennet activity of BsB1.3 strain is 417.36 U/g, and its proteolysis activity is 2.784 U/g. BsB1.1 strain has the best coagulating effect and its milk-clotting enzyme activity has increased most. So BsB1.1 strain is the target strain
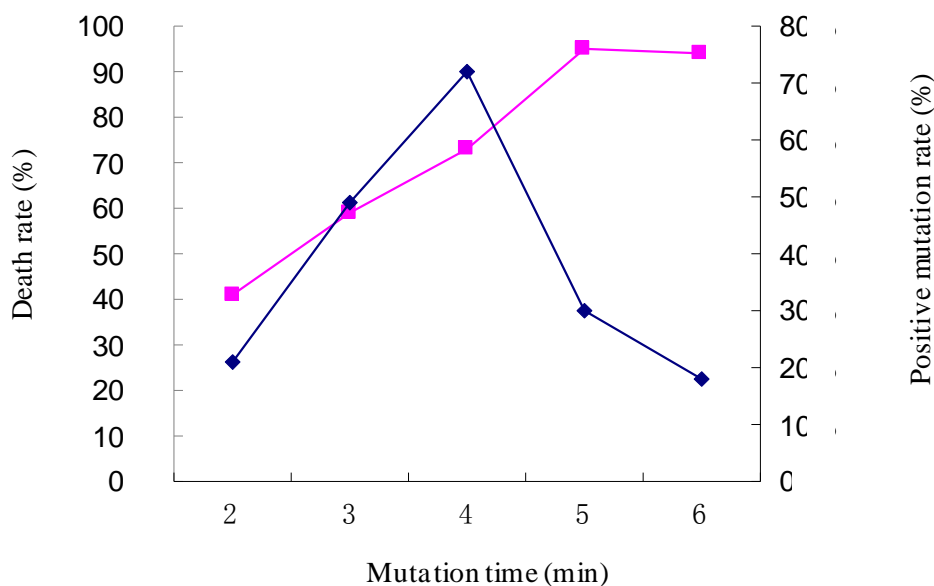
Figure 1. Effects of mutagenic time on death rate and positive mutation rate. ■ Death rate (%), ▲ Positive mutation rate (%).
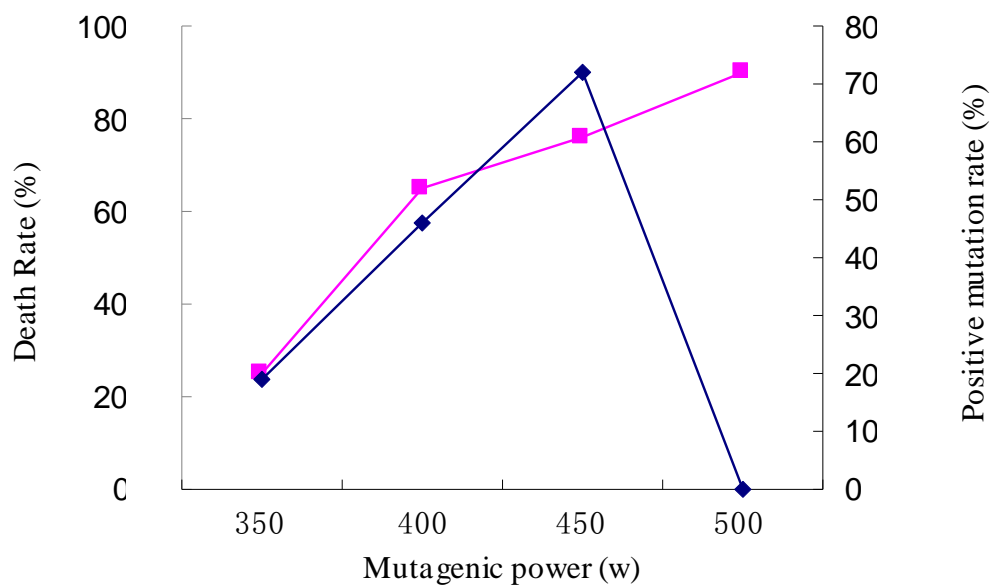

Figure 2. Effect of mutagenic power on death rate and positive mutation rate. ■ Death rate (%); ▲ Positive mutation rate (%).

**Table 2.** Screening results of strains after microwave mutagenesis of *BsB1*

Strain No.	Coagulating effect	chymosin activity (U/g)	Proteolysis activity (U/g)
BsB1.1	Tightest texture and coagulating time much more shortened	516.12	1.296
BsB1.2	Slightly tight texture and coagulating time more shortened	457.20	2.530
BsB1.3	Slightly tight texture and coagulating time slightly shortened	417.36	2.784

Table 3. Inspection results of stability.

Generation Number	Chymosin activity (U/g)	Proteolysis activity (U/g)
1	516.12	1.2964
2	510.06	1.1433
3	511.36	1.1495
4	509.27	1.1478
5	510.52	1.1497
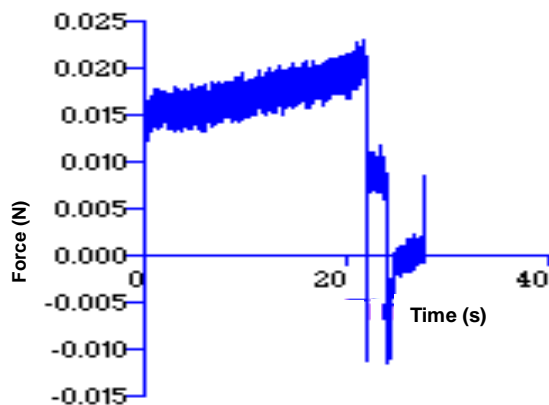
(a)
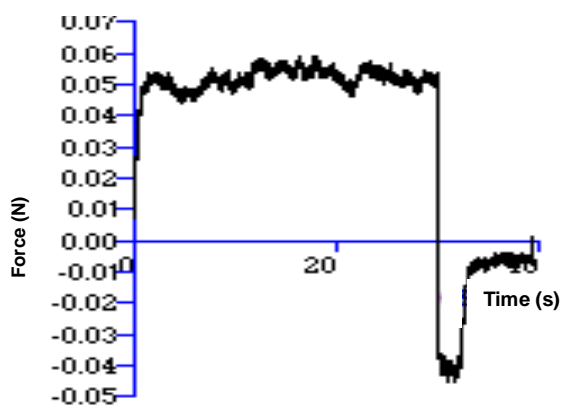
(b)

Figure 3. Texture analysis of the cheese by (a) rennet from original strain and (b) objective strain.

of this experiment. Its rennet activity has significantly improved, which has increased by 29.0% compared with the original strain.

Genetic stability of the strain

Some genetic types of mutant strain by artificial mutation were not stable, which prone to mutation or production fell back. So it was needed to verify its genetic stability. The data in Table 3 indicates that when BsB1.1 strain screened after mutagenesis inherits to the fifth generation, rennet activity is relatively stable, which shows that BsB1.1 strain has a favorable genetic stability and its characters can be stably inherited.

Texture analysis of the cheese by rennet from original strain and objective strain

From Figure 3, the texture analysis of the cheese by rennet from original strain and objective strain were obtained. The area blew the horizontal axis indicated the viscosity. It was found that the viscosity was had risen significantly through the microwave mutagenic, which enhanced the stability of the cheese effectively. So, it could be confirmed that the microwave mutagenic was an effective way to gain high-producing rennet strain from *B. Subtilis.*

DISCUSSION

Microwave mutagenesis studies have been widely carried out. Sasaki (1976-1978) had studied the mutant effect of the microwave on the mile bacteria, viruses, spores and lower eukaryotes yeast. It has been found that the production of Nisin from lactobacillus increased 42.13% than the original strain through microwave mutagenesis.

It is also shown from the experimental results that the best microwave mutagenic time of BsB1 strain is 4 min and the best mutagenic power is 450 W. After mutagenesis, the rennet activity of BsB1.1 strain has increased by 29.0% and has a favorable genetic stability. Therefore, microwave mutagenesis is an effective way to cultivate viable *B. subtilis* strains with high-producing rennet. The mechanism of microwave mutagenic may be due to the following aspects: (1) Microwave is an electromagnetic wave, which can cause the rotation of the polar molecules such as water, proteins, nucleotides, fat, and carbohydrate. Especially when the water molecules were under microwave irradiation of 2450 MHZ, it can rotate back and forth 24.5×10^8 times in 1 s,

which causing intense friction between the molecules. So it makes the hydrogen bonding and base stacking of intracellular DNA molecular chemical impaired, eventually cause the molecular structure of DNA change, and Lead to genetic variation. (2) The microwave has a strong penetrating effect, causing water molecules produce vigorous rotation, to change the cell wall permeability. It is easier to make intracellular enzyme secreted. (3) Strong thermal motion of molecules caused by the microwave was easy to cause inactivation of the enzyme, and make it easier for the intracellular enzyme secreted. (4) Microwave mutagenic effects of micro-organisms, in addition to the thermal effects, there are still non-thermal effects. But the mechanism of microwave mutagenic on the high-producing rennet strain from *B. subtilis* is still by further experiments to prove it.

From the experimental results it is indicated that the microwave treatment is a new technology for strain to improve the rennet. The technique is easy and safe to manipulate and the equipment requirement is low. It may be possible to apply this technology industrial microbial breeding engineering.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Low cost medium for recombinant endoglucanase II production by *Pichia pastoris*

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The suitability of crude glycerol (75% (v/v) glycerol content) from a biodiesel production plant, and soybean meal and rice bran hydrolysates as replacements in the culture medium for commercially available glycerol, peptone and yeast extract, respectively, to form a low cost culture medium for the growth of, and recombinant endoglucanase II production by, *Pichia pastoris* was evaluated. The use of 1% (v/v) crude glycerol as a carbon source, and a mixture of 0.175% (v/v) soybean meal and 0.15% (v/v) rice bran hydrolysate in the presence of 0.5% (w/v) ammonium sulfate was found to be a suitable medium in the presence of 3% (v/v) methanol as the inducer. Under these conditions, the highest level of extracellular endoglucanase II activity (61.5 U/mL) was found after 120 h culture at 30°C and pH 6.0.

Key words: Biodiesel glycerol, soybean meal, rice bran, endoglucanase II, *Pichia pastoris.*

INTRODUCTION

The high cost of nutrient medium for the cultivation of recombinant *Pichia pastoris* in the production of recombinant proteins is a major factor that needs to be considered and optimized for large scale production (Yadav et al., 2011). Therefore, the development of a suitable culture medium using low cost raw materials is of interest. The growth and recombinant protein production level in recombinant *P. pastoris* depends on various conditions and on the composition of the culture medium (Batista et al., 2013).

P. pastoris is a methylotrophic yeast that is widely used as a host for the production of recombinant proteins.

Recently, the improvement of individual cellulase enzymes from *Trichoderma reesei* has been attempted by genetic engineering in *P. pastoris* in order to enhance their cellulose degradation ability (Boonvitthya et al., 2013).

Cellulases, which degrade cellulose, consist of endo-glucanases (EGs), cellobiohydrolases and beta-glucosidases. The EGs act on the amorphous regions of cellulose, and EGII is one of the most abundant EGs produced by *T. reesei* and has the highest catalytic efficiency (Qin et al., 2008; Zhang et al., 2012).

The estimated cost of enzyme production at an industrial scale depends to a large extent upon the growth medium used in the process (Hajii et al., 2008; Batista et al., 2013). Previous studies have reported on the fermentation using pure glycerol as the carbon substrate because the salts in raw glycerol exert important inhibitory effects on many microorganisms (Petitdemange et al., 1995). However, yeast strains that can grow on raw glycerol have been reported (Papanikolaou et al., 2002). In order to reduce the medium costs, the crude glycerol produced during biodiesel manufacturing, which contains macro elements, such as calcium, potassium, magnesium, sulfur and sodium (Kitcha and Cheirsilp, 2011), has been used as a carbon source in *P. pastoris* culture medium to increase the cell concentration in the logarithmic growth phase (Celik et al., 2008). In addition, the waste agricultural products of soybean meal and rice bran have been used as organic nitrogen sources since they provide a rich but inexpensive source of nutrients (Xiao et al., 2007; Sereewatthanawut et al., 2008).

The aim of this study was to determine the suitability of crude glycerol, obtained as the by-product of biodiesel production, and soybean meal and rice bran as a substitute for high purity commercial glycerol, peptone and yeast extract, respectively, in the formation of a low cost culture medium for the growth of, and recombinant (r)EGII production by, *P. pastoris.*

MATERIALS AND METHODS

Microbial strain

The genetically engineered *P. pastoris* X-33 yeast strain, which is transfected with the pPICZαA plasmid encoding for the *T. reesei* EGII gene under the methanol inducible *AOX1* promoter (Boonvitthya et al., 2013) was provided by the Biocatalysts Research Unit, Chulalongkorn University. The transformed line was maintained on YPD (2% (w/v) peptone, 1% (w/v) yeast extract and 2% (w/v) dextrose) agar. Expression of the rEGII as a secretory product was controlled under the methanol inducible AOX1 promoter and repressed by glycerol.

Preparation of crude glycerol, the byproduct of biodiesel production

The crude glycerol was obtained from the Energy Absolute Public Co. Ltd., biodiesel production plant at Kabinburi Industrial Zone in Prachinburi, Thailand, which uses refined bleached deodorized palm oil and methanol in the transesterification process with an alkaline catalyst. This crude glycerol preparation contained 75% (v/v) glycerol and so the volume of this crude glycerol added to the medium was set accordingly to give the desired final glycerol concentration (v/v) ignoring the impurities. The crude glycerol was adjusted to pH 7.0 prior to addition to the culture medium.

Preparation of soybean meal and rice bran hydrolysates

Soybean meal was obtained from Lactasoy Co. Ltd., Prachinburi, Thailand, while rice bran was obtained from a rice mill in Pathumthani, Thailand, and separately treated in 1 N sulfuric acid (H_2SO_4) at 33% (w/v). Each mixture was autoclaved at 121°C for 40 min, cooled down and distilled water added to the required volume as well as 10 N NaOH to set the pH to 7.0. The suspension was then filtered and the filtrate stored at 4°C until use. The total nitrogen content was analyzed by the Kjeldahl method (AOAC, 1990).

Medium optimization by a sequential univariate approach

The effect of the nutrient composition, in terms of the concentration of crude glycerol, methanol, soybean meal and rice bran hydrolysates and ammonium sulfate, as well as the medium pH and culture temperature and time, was investigated upon the growth of, and the production of rEGII by, *P. pastoris* in that order as follows.

For the initial growth of *P. pastoris* to form the starter inoculum, the crude glycerol concentration was varied in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer pH 6.0, 0.4% (v/v) *Pichia* trace minerals (PTM) and 1% (v/v) glycerol) by substitution of the pure glycerol with the crude glycerol preparation at a final glycerol concentration of 0, 0.5, 1, 1.5, 2 and 2.5% (v/v). *P. pastoris* was grown in each of these media (50 mL) in 250-mL baffled flasks at 30°C with shaking at 250 rpm for 21 h. The growth of the yeast was then monitored in terms of the yeast dry cell weight (DCW) and optical density, as detailed below.

For the induction of rEGII production by the recombinant *P. pastoris*, the yeast was first grown in the optimum crude glycerol concentration supplemented BMGY medium (BMcGY), harvested by centrifugation (10,000 x*g*, 3 min), washed in sterile water and then resuspended and transferred into 100 mL BMMY medium (as per BMGY except the glycerol was replaced with methanol) except that the methanol concentration was varied at 0, 0.5, 1, 2, 3 and 4% (v/v) for induction of the AOX-1 promoter and as a carbon source for the yeast. The cell suspension was then grown in 250-mL baffled flasks at 30°C with shaking at 250 rpm for 144 h. For each selected methanol concentration, the same amount (final concentration) of fresh methanol was added to the culture each day (0, 24, 48, 72, 96 and 120 h). At 12, 24 h and then every 24 h thereafter a 5-mL aliquot of the cell suspension was removed to ascertain the yeast DCW and extracellular rEGII activity.

The effect of substitution of peptone with soybean meal hydrolysate (at 0, 0.025, 0.05, 0.1, 0.125, 0.15, 0.175 and 0.2 (v/v)) upon *P. pastoris* growth in the BMcGY medium (optimal crude glycerol level determined as above), and upon the subsequent growth and rEGII production level in BMMY medium was then evaluated as above, except using the optimal methanol concentration in the BMMY medium, respectively.

The replacement of both the peptone and yeast extract in the BMGY and BMMY media with a combined organic nitrogen source of soybean meal and rice bran hydrolysates was then evaluated as above, except using the optimal soybean hydrolysate level and varying the rice bran hydrolysate at 0, 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0% (v/v) in place of the yeast extract.

Table 1. Effect of the crude glycerol concentration as a replacement for pure glycerol in BMGY medium on the growth of recombinant *P. pastoris*.

Crude glycerol concentration (% (v/v))	Dry cell weight (g/L)[a]
0	2.08 ± 0.07
0.5	4.02 ± 0.10
1.0	8.17 ± 0.06
1.5	7.61 ± 0.13
2.0	6.22 ± 0.03
2.5	5.58 ± 0.17

[a]Data are shown as the mean ± 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

Next, the optimal modified BMcGY and BMMY media were supplemented with ammonium sulfate at 0, 0.1, 0.5, 1, 1.5 and 2% (w/v) and the growth or growth and rEGII production levels, respectively, were evaluated as above. Finally, the effect of the pH (5.5 to 7.0) of the optimal media, followed by that of the cultivation temperature (25 to 35°C), upon the recombinant *P. pastoris* growth and rEGII production levels was evaluated in the same manner.

Analytical methods

Enzyme assay

The evaluation of the extracellular rEGII activity in the culture supernatant assay was performed by monitoring the hydrolysis of carboxymethyl cellulose (CMC) as the total reducing sugar, evaluated using the dinitrosalicylic acid DNS method (Miller, 1959). Each reaction contained 0.5 mL of 2% (w/v) CMC solution in 50 mM acetate buffer (pH 4.8) and 0.5 mL of the diluted test enzyme solution. The reaction was incubated at 50°C for 30 min and then 3 mL of DNS reagent was added, incubated in a boiling water bath for 5 min, and then cooled. Thereafter, the absorbance was measured at 540 nm to estimate the quantity of reducing sugars produced in the assay (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar/mL/min under the above assay conditions. Glucose (0-2 μg/mL) was used to form the standard curve.

Measurement of protein concentration

The total protein content was determined following the method of Bradford (1976) using bovine serum albumin (0-25 μg/mL) as a standard and measuring the absorbance at 595 nm with a microplate reader (ANTHOS Zenyth 200, USA).

Determination of yeast cell concentration

The yeast DCW was determined by centrifugation (5000 xg for 5 min) of 5 mL of the culture broth, washing the cells with distilled water, and then drying at 105°C for 24 h. From the obtained mass and the starting culture volume analyzed the DCW was then calculated (g/L). In addition, the yeast cell concentration was estimated in terms of the optical density (OD) of the suspension at 600 nm (OD_{600}) AS compared to the medium alone (Charoenrat et al., 2013).

Data analysis

Data WEre presented as the mean ± one standard deviation (SD). The statistical significance of the difference between means was tested by analysis of variance (ANOVA) and Duncan's multiple range tests (DMRT), with significance accepted at the $P < 0.05$ level.

RESULTS AND DISCUSSION

In this study, different cultivation media compositions and cultivation conditions were evaluated in order to attempt to reduce the cost of efficient rEGII production by *P. pastoris*. The culture medium composition is known to be a major influence on the growth and enzyme production, especially the carbon and nitrogen sources (Broach, 2012; Charoenrat et al., 2013).

Effectiveness of crude glycerol to replace commercially available glycerol in high cell density cultivation of *P. pastoris* in BMGY medium

Glycerol is regularly used as the principal initial carbon source in *P. pastoris* cultivation in order to increase the cell concentration. When utilizing the cheaper crude glycerol, a surplus byproduct of the expanding biodiesel production industry, in the BMGY medium, the highest level of yeast biomass production (8.17 g/L, DCW basis) was obtained with 1% (v/v) of biodiesel glycerol (Table 1). Crude glycerol concentrations above or below 1% (v/v) resulted in a lower yeast yield, in accord with the previously reported repression of yeast growth at high crude glycerol levels (Tang et al., 2009). Accordingly, *P. pastoris* was initially grown in BMGY medium with 1% (v/v) crude glycerol in place of the commercial glycerol (hereafter called BMcGY). Although this was a similar glycerol concentration to the commercial glycerol containing BMcGY medium, a likely economic saving would still be gained since, as compared to the pure glycerol (US$1.11 per kg), the crude glycerol is some ten-fold cheaper (US$0.11 per kg). Indeed, as a cheaper carbon source for biotechnological applications that may make them economically feasible, crude glycerol has been used as carbon source in many industrial fermentations (Chatzifragkou et al., 2011). However, the crude glycerol derived from biodiesel production typically possesses a low glycerol concentration of low value because of the impurities. Further refining of the crude glycerol will depend on the economy of production scale and/or the availability of a glycerol purification facility (Naresh and Brian, 2006).

Effects of the methanol concentration on BMMY medium

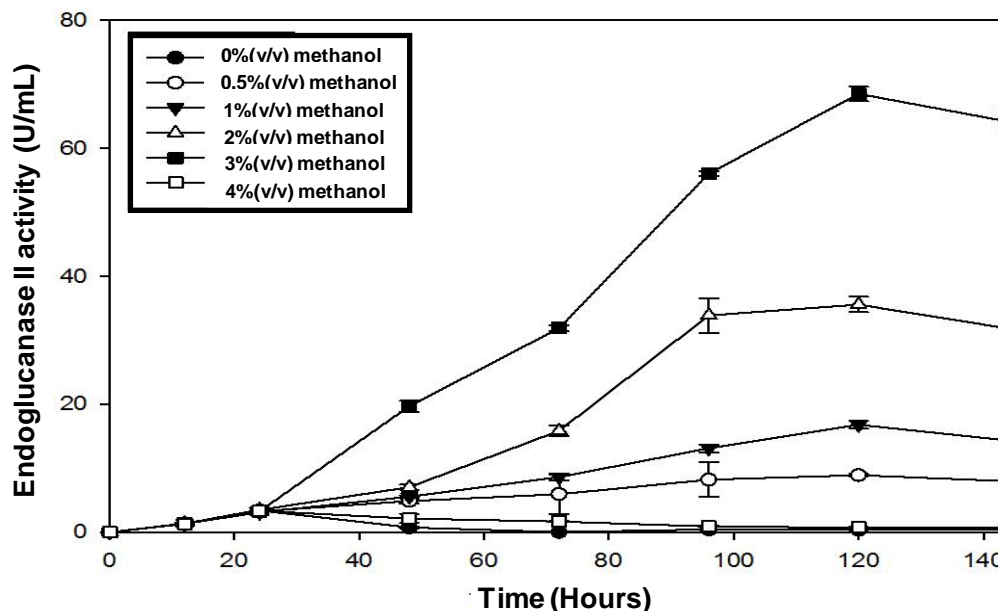Methanol is typically the widely accepted industrial choice

Figure 1. Effect of the methanol concentration on the growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* in BMMY medium. Data are shown as the mean ± 1 SD and are derived from triplicate experiments.

Table 2. The growth of recombinant *P. pastoris* in BMcGY[a] medium with different soybean meal hydrolysate concentrations as a replacement for the peptone.

Soybean meal hydrolysate (% (v/v))	Dry cell weight (g/L)[b]
0.0000	4.57 ± 0.17
0.0125	5.35 ± 0.05
0.0250	6.21 ± 0.10
0.0500	6.63 ± 0.13
0.0750	7.04 ± 0.09
0.1000	7.25 ± 0.10
0.1250	7.41 ± 0.11
0.1500	7.67 ± 0.04
0.1750	8.01 ± 0.13
0.2000	6.13 ± 0.12

[a]BMcGY = BMGY medium but with the 2% (v/v) pure glycerol replaced with 1% (v/v) crude glycerol (75% (v/v) glycerol) from a biodiesel production plant. [b]Data are shown as the mean ± 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

of carbon source for *P. pastoris* fermentations, and also serves as the inducer for recombinant protein expression under the control of the *AOX*1 promoter of *P. pastoris*. Following the initial growth of *P. pastoris* in BMcGY medium and transfer to BMMY medium without methanol no rEGII production was detected, as expected with the

AOX1 promoter. Increasing the methanol concentration up to 3% (v/v) correspondingly increased the rEGII level produced, especially at 2 and 3% (v/v) methanol, with the highest rEGII level obtained (68.5 U/mL) at 3% (v/v) methanol after 120 h (Figure 1). Increasing the methanol concentration further to 4% (v/v), however, resulted in almost no rEGII production, consistent with that previously reported in the production of human serum albumen in this yeast (Bushell et al., 2003). Accordingly, a methanol concentration of 3% (v/v) was used in the BMMY medium (hereafter called BMM$_3$Y).

Effect of organic nitrogen sources

As determined by Kjeldahl's method, the total nitrogen content of the soybean meal and rice bran hydrolysates were 0.52 and 0.31% (w/v), respectively, as compared to ~12% and ~9.5% (w/v) (Technical Data from HiMedia Laboratories Pvt. Ltd.) for the peptone and yeast extract, respectively.

Soybean meal hydrolysate replacement for peptone

The use of soybean meal hydrolysate in place of peptone in the BMcGY medium was found to support the growth of *P. pastoris*, with an increasing yeast yield being obtained with an increasing soybean meal hydrolysate concentration up to a maximum yeast yield (8.01 g/L, DCW basis) at 0.175% (v/v) soybean hydrolysate (Table 2). This compared reasonably well with the 8.17 g/L (DCW basis) obtained with the peptone containing
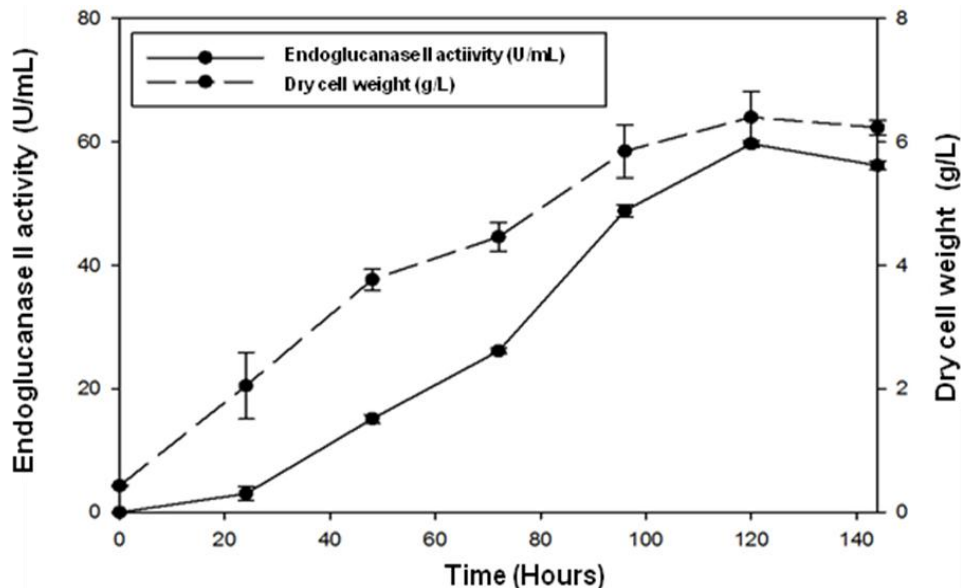
Figure 2. The growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* over time when grown in BMM$_3$YS media (soybean meal hydrolysate at 0.175% (v/v) as a replacement for peptone in the BMM$_3$Y media). Data are shown as the mean ± 1 SD and are derived from triplicate experiments.

Table 3. Effect of increasing rice bran hydrolysate concentration as a replacement for yeast extract in BMcGYS[a] medium on the growth of recombinant *P. pastoris*.

Rice bran hydrolysate (% (v/v))	Dry cell weight (g/L)[b]
0.0000	4.86 ± 0.12
0.0125	5.28 ± 0.15
0.0250	6.12 ± 0.19
0.0500	6.48 ± 0.05
0.0750	6.93 ± 0.09
0.1000	7.14 ± 0.23
0.1250	7.32 ± 0.09
0.1500	8.12 ± 0.11
0.1750	7.81 ± 0.22
0.2000	7.46 ± 0.10

[a] BMcGYS = BMGY medium but with the 2% (v/v) pure glycerol and 1% (w/v) peptone replaced with 1% (v/v) crude glycerol (75% (v/v) glycerol) from a biodiesel production plant and 0.175% (v/v) soy meal hydrolysate, respectively.
[b] Data are shown as the mean ± 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different (*P* < 0.05; ANOVA, DMRT).

BMcGY medium. Accordingly, the BMcGY and BMM$_3$Y media were modified to contain 0.175% (v/v) soybean meal hydrolysate in place of peptone (hereafter called BMcGYS and BMM$_3$YS, respectively).

When *P. pastoris* was cultured and induced in the BMM$_3$YS medium, the modified medium was found to support rEGII production, with a maximum level (59.7 U/mL) being obtained after 120 h (Figure 2). Although this was 1.15-fold lower than that with the peptone containing BMM$_3$Y medium, a likely economic saving is still gained (~99.4% decrease; http://www.quotenet.com/commodities/soybean-meal-price and HiMedia Laboratories Pvt. Ltd.). Note that the yeast yield obtained in the modified BMM$_3$YS medium essentially correlated with the obtained rEGII production level. Thus, soybean meal hydrolysate has the potential to be employed as a peptone replacement.

Rice bran hydrolysate replacement for yeast extract

The use of rice bran hydrolysate in place of peptone in the modified BMcGYS medium was found to support the growth of *P. pastoris*, with an increasing yeast yield being obtained with increasing soybean meal hydrolysate concentrations up to a maximum (8.12 g/L, DCW basis) at 0.15% (v/v) (Table 3). This compared reasonably well with the 8.17 g/L (DCW basis) obtained with the peptone containing BMcGY medium. Accordingly, the BMcGYS and BMM$_3$YS media were further modified to contain 0.15% (w/v) rice bran hydrolysate in place of yeast extract, hereafter called BMcGYSR and BMM$_3$YSR, respectively.

When *P. pastoris* was cultured and induced in the BMM$_3$YSR medium, the modified medium was found to support rEGII production reasonably well but not to as
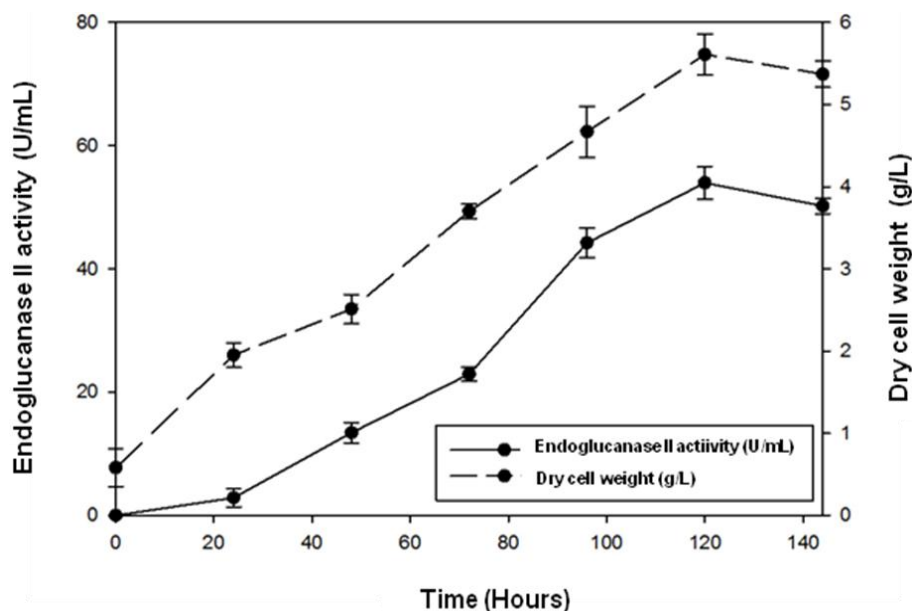
Figure 3. The growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* over time when grown in BMM3YSR medium (rice bran hydrolysate at 0.15% (w/v) as a replacement for yeast extract in BMM_3YS media). Data are shown as the mean ± 1 SD and are derived from triplicate experiments.

Table 4. Effect of ammonium sulfate as an inorganic nitrogen supplement in the BMcGYTRS[a] medium on the growth of recombinant *P. pastoris*.

Ammonium sulfate (% (w/v))	Dry cell weight (g/L)[b]
0.00	4.52 ± 0.07
0.10	8.26 ± 0.47
0.25	8.30 ± 0.12
0.50	8.34 ± 0.18
1.00	7.81 ± 0.26
1.50	7.66 ± 0.09
2.00	7.76 ± 0.13

[a]BMcGYRS = BMGY medium but with the 2% (v/v) pure glycerol, 1% (w/v) peptone and 2% (w/v) yeast extract replaced with 1% (v/v) crude glycerol (75% (v/v) glycerol) from a biodiesel production plant, 0.175% (v/v) soy meal hydrolysate and 0.15% (v/v) rice bran hydrolysate, respectively. [b]Data are shown as the mean ± 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

high an extent as in the other media. A maximum rEGII level (54.0 U/mL) was obtained after 120 h (Figure 3). Although the maximum level of obtained rEGII was 1.11- and 1.27-fold lower than the maximum amount obtained

in the BMM_3YS and BMM_3Y media, respectively, this loss of yield was still not as great as the likely financial savings gained (~98.4%; http://www.alibaba.com/Rice-Bran and HiMedia Laboratories Pvt. Ltd.). Therefore, the mixture of soybean meal and rice bran hydrolysates is a cheap nitrogen source that could be used for enzyme production.

Effect of the inorganic nitrogen source

The effect of supplementing the BMcGYSR medium with ammonium sulphate, as an inorganic nitrogen source, on the growth of *P. pastoris* was evaluated over the range of 0-2% (w/v). Increasing the ammonium sulphate concentration up to 0.5% (w/v) caused an increased yield of yeast, reaching a maximum of 8.34 g/L (DCW basis) (Table 4). Further increasing the ammonium sulphate concentration above 0.5% (w/v) reduced the yeast yield and so 0.5% (w/v) was taken as optimal for both media (hereafter called BMcGYSRA and BMM_3YSRA).

When the recombinant *P. pastoris* was cultured and induced in the BMM_3YSRA medium an increased yeast yield and rEGII production level, ~1.1-fold to 6.53 g/L and 61.7 U/mL, respectively was observed as compared to that in the BMM_3YSR medium without ammonium sulphate (Figure 4). This result is in agreement with previous reports that too high or too low ammonium ion concentration in the culture medium leads to a reduced cell growth (Cos et al., 2006; Yalcin and Ozbas, 2008).
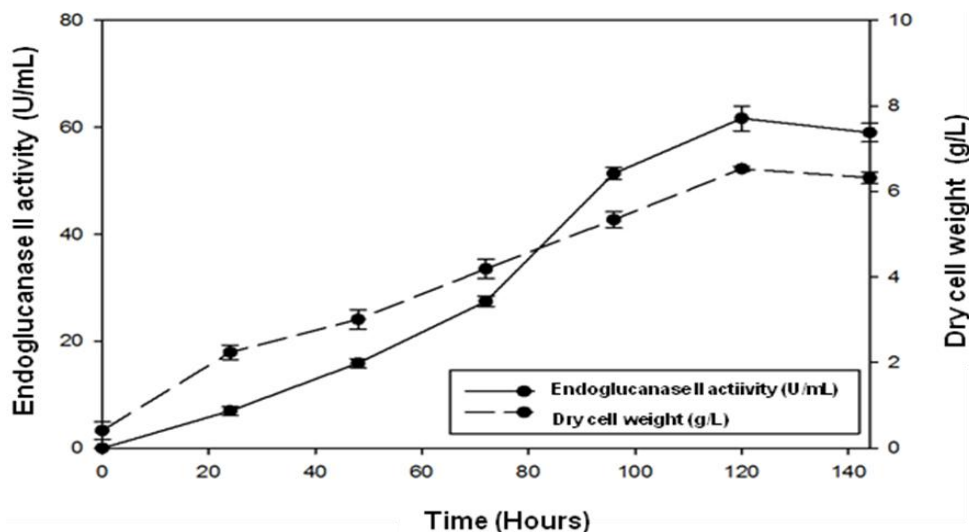
Figure 4. The growth of, and the extracellular recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* over time when grown in BMM_3YSRA media (BMM_3YSR with 0.5 (w/v) ammonium sulphate). Data are shown as the mean ± 1 SD and are derived from triplicate experiments.

Table 5. Effect of the medium pH on the growth of, and recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* in BMM_3 $YRSA^a$ medium.

pH	Dry cell weight (g/L)[b]	Endoglucanase II activity (U/mL)[b]
5.5	6.57 ± 0.18	60.30 ± 0.33
6.0	6.61 ± 0.04	61.42 ± 2.28
6.5	6.73 ± 0.22	24.07 ± 2.78
7.0	6.69 ± 0.22	19.78 ± 2.07

[a]BMM_3YRSA = BMMY medium but with the 0.5% (v/v) methanol, 1% (w/v) peptone and 2% (w/v) yeast extract and replaced with 3% (v/v) methanol, 0.175% (v/v) soy meal hydrolysate and 0.15% (v/v) rice bran hydrolysate, respectively, and supplemented with 0.5% (w/v) ammonium sulphate; [b]Data are shown as the mean ± 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

Table 6. Effect of the incubation temperature on the growth of, and recombinant endoglucanase II production (U/mL) level by, recombinant *P. pastoris* in BMM_3YRSA^a medium.

Temperature (°C)	Dry cell weight (g/L)[b]	Endoglucanase II activity (U/mL)[b]
25	6.21 ± 0.12	58.95 ± 1.45
30	6.67 ± 0.26	61.49 ± 0.25
35	1.17 ± 0.09	7.87 ± 0.14

[a]BMM_3YRSA = as per Table 5; [b]Data are shown as the mean ± 1 SD and are derived from triplicate experiments. Means followed by a different lowercase letter are significantly different ($P < 0.05$; ANOVA, DMRT).

Effect of the culture medium pH

With respect to the medium pH, no significant effect on the recombinant *P. pastoris* yield in the BMM_3YSRA medium was observed at pH values between 5.5 and 7.0 (Table 5). This is consistent with the reported ability of *P. pastoris* to grow within the pH range of 3.0-7.0 (Inan et al., 1999; Bayraktar, 2009). However, the level of obtained extracellular rEGII activity was markedly reduced at pH 6.5 (~2.5-fold) and pH 7.0 (~3.1-fold) as compared to that at pH 5.5-6.0 (Table 5), which may

reflect a higher protease activity in the culture medium or a decreased enzyme stability at the higher pH values (Cos et al., 2006).

Effect of incubation temperature

With respect to the cultivation temperature, the optimal yeast growth (as DCW) and rEGII production level was obtained at 30°C for the culturing and induction of the recombinant *P. pastoris* in the BMM_3YSRA medium (Table 6). Increasing the culture temperature from 30 to 35°C resulted in a marked (5.7- and 7.8-fold) decrease in the yeast and rEGII yields obtained, respectively. In agreement, it has previously been reported that 30°C was the most appropriate template for the growth of, and enzyme production by, *P. pastoris* in general (Shi et al., 2003; Macauley et al., 2005; Rahbarizadeh et al., 2006).

Conclusions

The culturing of recombinant *P. pastoris* for the production of rEGII was attained using BMcGYRS and BMM₃YRS media, which are the use of a 1% (v/v) crude glycerol (75% (v/v) glycerol content from a biodiesel production plant) plus 0.175% (v/v) soybean meal and 0.15% (v/v) rice bran hydrolysates as a replacement for the 2% (v/v) glycerol, 2% (w/v) peptone and 1% (w/v) yeast extract in standard BMGY and BMMY media, respectively. Although the optimal rEGII production required supplementation of this modified medium with 0.5% (w/v) ammonium sulphate, overall these media will provide a likely economic advantage for the industrial scale production of recombinant enzymes by *P. pastoris*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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