Micología Aplicada International
ISSN: 1534-2581
dcarrera@colpos.mx
Colegio de Postgraduados
México

Brand, D.; Soccol, C. R.; Sabu, A.; Roussos, S.
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Micología Aplicada International, vol. 22, núm. 1, enero, 2010, pp. 31-48
Colegio de Postgraduados
Puebla, México

MICOLOGIA APLICADA INTERNATIONAL, 22(1), 2010, pp. 31-48

www.micaplint.com

Review

PRODUCTION OF FUNGAL BIOLOGICAL CONTROL AGENTS THROUGH SOLID STATE FERMENTATION: A CASE STUDY ON *PAECILOMYCES LILACINUS* AGAINST ROOT-KNOT NEMATODES

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Accepted for publication May 18, 2009

ABSTRACT

Root-knot nematodes cause annual losses of about USD $100 billion worldwide. Development of natural resistance to nematicides by nematodes and the tendency to withdraw chemical pesticides/nematicides from the market led to the search for new methods of control. Biological control of root-knot nematodes with *Paecilomyces lilacinus* is being investigated thoroughly, but there is a lack of information on the production systems. Solid state fermentation is a suitable ecofriendly biological process for the mass production of biological control agents. Conidiospores produced are cost-effective and show good stability and viability for field applications on a commercial scale. Studies on bioreactor design are essential for scaling up solid-state fermentation processes, but they are scarce yet. We did an in-depth analysis on the production of fungal spores by solid state fermentation for commercial scale application against root-knot nematodes.

Key words: Biological control, *Meloidogyne incognita*, nematodes, *Paecilomyces lilacinus*, solid state fermentation.

INTRODUCTION

Environmentally sound and economically feasible alternatives for pest control are now a subject of numerous studies due to the development of resistance to pesticides in targeted pathogens, as well as the withdrawal of commercial pesticides from the market due to environmental and public health concerns[33]. Plant-parasitic nematodes, especially root-knot nematodes (RKN), are major pests of several economically important crop plants, causing severe yield loss. Methyl bromide, the most widely used soil fumigant against nematodes, has been banned in most developed and developing countries, because of the serious threats it poses to the environment. So there is an increasing demand all over the world for ecofriendly nematicides.

Biological control agents (BCAs), such as fungi, offer great scope for field application, but the development of a viable bioprocess for its commercial production is not an easy task. *Paecilomyces lilacinus* (Thom) Samson is a known soil hyphomycete, and it parasitizes RKN eggs and females showing great nematicidal activity. Growth physiology of filamentous fungi is an important factor considering their production as BCAs. The German manufacturer Prophyta produces a commercially patented strain of *P. lilacinus*, which is under continuous research worldwide[51,53,56]. Field applications of BCAs are mainly accomplished by means of fungal conidiospores, which must be virulent and viable for long periods of storage. Solid state fermentation (SSF) offers many advantages for large scale and cost effective production of conidiospores.

The present review deals with the relevance of controlling RKN in agriculture, and the application of the BCA *P. lilacinus* produced under SSF. Existing methods for controlling nematodes are discussed with emphasis on biological control research and practices using the spores of *P. lilacinus*.

PLANT-PARASITIC NEMATODES

Nematodes are roundworms that belong to the phylum Nematoda. They are the most abundant creatures on earth, occupying different ecological niches and living as parasites of humans, animals and plants. Parasitic nematodes can cause a large-scale multiplication and invasion of their hosts[70].

Phytoparasitic nematodes can devastate several economically important crops, causing significant losses in yield. These nematodes are obligate parasites, and they have developed different parasitic strategies and relationships with their hosts to attain enough nutrients for development and reproduction. The products of nematode parasitic genes can be expressed as morphological structures (*e.g.*, stylet), which allow researchers to assess the level of parasitism in a particular host plant, where nematodes can develop critical physiological functions in the interaction with their host[24].

The groups of phytoparasitic nematodes that have great economical importance are the sedentary endoparasites, which include the genera *Heterodera* and *Globodera* (cyst nematodes) and *Meloidogyne,* or RKN. Others include several migrant nematodes, such as species of *Pratylenchus* and *Radopholus*. Root knot and cyst nematodes have complex interactions with their hosts and they have extremely different characteristics for their parasitic cycle[112].

Phytoparasitic nematodes and agricultural losses

Nematodes invade all vascular parts of the

plants, but those species that infect the root are economically very important. Due to the presence of other root pathogens, it is very difficult to estimate the actual loss caused by nematode infection. It is estimated that overall yield losses are more than 10%, reaching 20% in some crops. In monetary terms, worldwide losses certainly exceed USD $100 billions annually. The impact of plant parasitic nematodes in agriculture can also be estimated by the strategies employed in their control. However, in recent decades the utilization of chemical pesticides is being discouraged due to severe environmental problems, including ground water contamination, avian and mammalian toxicity, and accumulation of pesticides in food materials[8].

The root-knot nematodes (RKN)

Nematodes of the genus *Meloidogyne* are also known as RKN, because they develop knots in the roots of infected plants during their parasitic life-cycle. Root knots are giant cells of plants and, once developed, nematodes use them as a source for their nutrition. *Meloidogyne* species have great economic importance, as they can cause severe damage to several important crop plants. Important species of the genera include *M. arenaria* (Neal) Chitwood, *M. fallax* Karssen, *M. hapla* Chitwood, *M. incognita* (Kofoid and White) Chitwood, *M. javanica* (Treub) Chitwood, *M. naasi* Franklin, *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos and Almeida, *M. trifoliophila* Bernard and Eisenback, among others. *M. incognita* and *M. javanica* are widely distributed around the world. *Meloidogyne* species attack several crops, which belong to Solanaceae (tomato, potato), Cucurbitaceae (water melon, cucumber), Leguminosae (beans), and other families. They also cause severe damages to some other staple crops, such as cereals (rice, maize, soybean, banana, plantains, sweet potato, yam), as well as to industrial crops, such as tobacco, coffee, sugar cane, sugar beet, cotton, and black pepper Economic losses have also been reported in fruit crops, such as guava, pineapple, papaya, and grapes[57]. Infections by RKN cause leaf chlorosis, gall development on roots and subsequent drastic reduction of the root system, stunted plant growth and wilting occur during severe infestations[70].

Life-cycle of RKN. Nematodes are soilborne pathogens and they feed themselves on the roots. The life-cycle include the stages of eggs, juveniles and adults. Nematodes hatch in the soil as second stage juveniles (J2). The vermiform juveniles are motile, they infect the roots preferentially in the zone of elongation or at the site of a lateral root emergence, and perforate the root walls with the stylet. After penetration, the nematode migrate intercellularly into the vascular cylinder, where it establishes a feeding site that constitutes the giant cells. These cells contain a granular cytoplasm and a great number of knots that are necessary for their development. This site becomes differentiated by cell division and swelling, causing the formation of galls or root-knots where the female is sheltered. The galls thus formed contain a gelatinous matrix, where the female lays hundreds of eggs[23,24,48].

METHODS FOR NEMATODE CONTROL

Since 1950, the control of phytoparasitic nematodes has been based on chemical pesticides, although several of them are being withdrawn from the market due to issues related to the environment and pub-

lic health. Methyl bromide was widely used against nematodes, but now it has been withdrawn from the market because of its adverse effects on the ozone layer. Nematodes also developed resistance against most of the known pesticides, and this triggered worldwide research for new alternative agents and methods for nematode control[32,107]. Possible control measures change with climate conditions, socio-economical situation of the country, crop economy, availability of chemical pesticides, resistant cultivars, and the suitability of agricultural practices.

Resistant plants

Plants are resistant to nematodes when they have a reduced level of reproduction. Nematode resistance genes are present in several crops, and are an important component of various multiplication programs in tomatoes, potatoes, cotton, soybean, and cereals. Resistance to nematodes can be either broad with action against several species of nematodes or narrow against only selected specific biotypes[113].

Several resistance genes, dominant or semidominant, were identified, cloned, and subjected to various studies[114,115]. The *Mi* gene of tomato is the most widely studied gene, which confers resistance against *Meloidogyne incognita, M. javanica*, and *M. arenaria*, but not to *M. hapla*. Resistance to nematodes is accompanied by a hypersensitive response in the host plant, with a region of necrotic cells of plants being visible around the head of the invading nematode within 12-24 h of inoculation on tomato roots[63]. The coded protein contains a nucleotide binding site and a leucine rich repeat protein motifs that are found in numerous plant resistance genes against a variety of pathogens[8,112].

Crop rotation

Important method for maintenance and improvement of soil fertility, and for enhancing yield. In crop rotation, various crops are followed in a certain order in the same soil. With the same succession of crops reproducing in a regular time cycle, rotations can be biennial, triennial, and so on. Crop rotation is a very good strategy that can always be adopted against nematode species with narrow ranges of plant-host, which is not the case of *Meloidogyne* sp. However, the order of plants and the time intervals between susceptible crops depend on the nematode species. In the case of species of *Meloidogyne*, which lay eggs in a gelatinous matrix, at least three years between harvests must have passed. Cultivation and harvests of high economic value crops are often done intensively, and it is difficult to find an order of suitable plants that does not affect the economic equilibrium[66] in a particular farm.

Chemical control

Plant-parasitic nematodes are more vulnerable as juveniles (J2) in soil, when searching for the roots of host plants. Once an endoparasitic nematode species penetrates a root, chemical control is more difficult as compounds have to be non-phytotoxic. There are several nematicides that can be used effectively against nematode pests of many annual crops, but there appears to be little progress for management of nematodes in many susceptible perennial crops without repeated application of nematicides[36].

There are two kinds of chemical products that can be utilized against plant parasitic nematodes: soil fumigants and nematicides. Their application to soil depends on the form of the formulation, it can be by injection, spraying, mechanical means, or through irrigation pipes. Fumigation prod-

ucts are usually applied before planting and, in the case of pesticides, they are applied at the time of planting. Fumigants are highly effective against nematodes, their efficacy is related to their high volatility at ambient temperatures. All fumigants have low molecular weights, and are available as gases or liquids. As they volatilize, the gas diffuses through the spaces between soil particles where the nematodes are killed. The most widely used fumigant is methyl bromide, which is mainly applied for high valued crops, such as strawberries and tomatoes, and in lesser amount to grains and commodities. However, methyl bromide has been banned in developed countries since 2005. In developing countries, substances with methyl bromide will be withdrawn from field application by the end of 2015.Other fumigants, such as chloropicrin, dazomet and meta sodium showed good activity against nematodes when applied. Nematicides are available as liquids, granules or solids, and they inhibit the nematode development through contact or by systemic action. They can control a wide range of nematodes. Some nematicides are species specific, consisting mainly of organophosphates and organocarbamates, and they differ in their action depending on the nematode species[107].

Biological control

An eco-friendly pest management strategy that utilizes deliberate introduction of living natural enemies to lower the population level of a target pest[27]. These enemies are commonly referred to as BCAs, which must demonstrate some characteristics for success in the field, including ability for rapid colonization of the soil, persistence, virulence, predictable control below economic threshold, easy production and application, good viability under storage, low cost of production, compatibility with agrochemicals, and safety[50].

In nature, it is observed that many natural enemies, such as viruses, bacteria, rickettsias, fungi, and others, can attack plant parasitic nematodes, but in the search for suitable BCAs more attention has been given to fungi and bacteria. Biological control can be either natural (*i.e.*, when a natural population of a particular organism inhibits the growth and development of nematodes), or induced (*i.e.*, when BCAs have been introduced artificially). There are two approaches for introduction: microbial pesticide application for rapid control of a pest, and the introduction or mass release of a biocontrol agent to provide long lasting control. The suppression can be specific or non specific, when only one or two organisms are involved[2,22,44,49].

Researchers have made several attempts to utilize bacteria for nematode control. Nematicidal bacteria are of two types: nematode parasites and rhizobacteria. The most studied bacteria are *Pasteuria penetrans,* an obligate endoparasite of *Meloidogyne*, followed by strains of *Pseudomonas*[3,20,65,74,96,97,98,106].

Nematophagous fungi are organisms that control the development of plant-parasitic nematodes by way of attacking nematodes or their eggs, and they utilize them as a source of nutrients. They are classified or named on the basis of different action mechanisms that they develop against the nematodes, in a particular phase of the life-cycle. The nematode-trapping fungi develop special mycelial structures in the form of traps in response to the presence of nematodes in the soil. These structures may adhere to the nematode cuticle[43]. The endoparasitic fungus develops inside the nematode, and starts its nematicidal action once their spores are ingested or adhered

to nematodes. There are fungal species that are parasites of root-knot and cyst nematodes, including their eggs or females[28,64,69]. There are several important points that need consideration while developing commercially viable BCAs. These points include: 1) While selecting BCAs from the environment, care must be taken for the proper maintenance of the strain; 2) Thorough studies must be conducted on ecology, physiology and taxonomy of potential BCAs; 3) Laboratory and/or field tests are needed to identify the most virulent strains; 4) Economic feasibility for mass production of selected strains; 5) Studies on formulation strategies and compatibility with application techniques; and 6) Risk assessment trials, BCAs should be safe to humans and other non-target species.

Ecological and economical consequences should be carefully considered when releasing BCAs into the environment. Programs must only be considered and approved when benefits are greater than costs[46]. BCAs are generally highly species specific, but they may have indirect impact on non-target species, causing environmental imbalance. However, if the agent introduced demonstrates high efficacy, this problem can be easily overcome once a BCA reduces their own population by feedback[80].

Infection process. The infection process of nematophagous fungi has been elucidated through the application of biochemical and molecular biology techniques. This includes the characterization of the enzymes involved in the penetration of eggshell or the nematode body wall, as well as the identification of nematicidal toxins. Nematodes are provided with two distinct barriers against infection: the eggshell and the cuticle. Eggshells of RKN are composed of three layers: the outer vitelline layer composed mainly of proteins; the middle layer composed of chitin; and an inner layer of lipoprotein. The thickness of these layers varies considerably among different nematode genera. The cuticle plays an important role in motility, maintenance of morphology, integrity, and it also provides protection from the environment and pathogens[68]. Fungi as nematicides are usually applied to soil as spores, which must be active and virulent when they colonise the rhizosphere of plants where sedentary females and eggs are found. Spores will germinate and form appressoria, which is a hyphal structure that secretes extracellular enzymes depending on the recognition of the host surface hydrophobicity[62]. The fungus is also capable of adjusting the pH to regulate its optimum enzyme activity, as described for *Metarhizium anisopliae*[103,104]. This is required as there is a relation in serology and functions of enzymes secreted by nematophagous fungi[94,95]. Serine proteases and chitinases from *Paecilomyces lilacinus* strains were purified and characterized from culture media containing egg yolk and chitin[9,40,53]. Enzymes produced by the commercial strain 251 of *P. lilacinus* were applied to *M. javanica* eggs, and it resulted in significant differences in the eggshell and reduction in egg hatching[51]. Similar studies were conducted with eggs of the nematode *Globodera palida* applying an endochitinase and a protease from *Verticillium chlamydosporium* resulting in surface damage when compared to the untreated control[105]. Park *et al.*[79] studied the influence of leucinostatins, a secondary metabolite produced by *P. lilacinus*, in the colonization of *M. javanica* eggs, revealing positive results. They further proved that chitinase activity can be related to parasitism, and it does not have a direct role in the degradation of pathogens cell wall.

Paecilomyces lilacinus. Fungal species that is found in the majority of agricultural soils, and it can be frequently isolated from eggs and females of the nematode *Meloidogyne*. *Paecilomyces* belongs to the division *Eumycota*, class *Deuteromycetes*, order *Moniliales* and family *Moniliaceae*. *P. lilacinus* shows fast hyphal growth. The conidiophores are up to 600 µm in height, and develop groups of lateral branches, from which 2-4 bottle-shaped phialides develop. Conidiophores of the genus *Paecilomyces* ramify in grouped branches or irregularly. The conidia are separated from the phialides in the form of chains. Conidia are ellipsoid, 2.5-3.0 µm long and 2.0-2.2 µm broad, lilac in colour. The facultative egg parasite *P. lilacinus* is sometimes capable of infecting mobile nematode stages or sedentary females, but it is most aggressive against eggs[42,71].

The use of *P. lilacinus* as a BCA depends on several factors, such as age, virulence, viability, inoculum concentration, method of application, and environmental conditions (soil type, fertility, organic matter, fertilizers, temperature, pH, host susceptibility)[50]. Several studies were completed for utilizing this fungus in the control of plant parasitic nematodes, and generated important information useful for the development of a BCA. However, there is a lack of detailed information on the production system to be utilized. The information dealing with fermentation parameters, such as water content, aeration, type of bioreactor, inoculum, and pH is also limited.

Temperature has a significant effect on the culture of *P. lilacinus*. Best growth and biomass development was obtained in potato dextrose broth (PDB) at temperatures between 24-30 C. Also the soil temperature demonstrated a great influence in the control of *M. incognita* with *P. lilacinus*[12,13]. Cabanillas *et al.*[14] studied the survival of *P. lilacinus* spores produced in potato dextrose agar (PDA), using carriers such as alginate pellets, diatomaceous earth, wheat grain, soil and soil plus chitin. They also studied its nematicidal activity against *M. incognita* in microplot experiments.

The fungus grown in wheat was also tested against *M. javanica* on tobacco, with or without the addition of nematicides like phenamiphos and ethoprop in microplot experiments for two years having vetch as winter culture. The fungus survived in the soil, although it was not capable of controlling nematode development, showing that the type of root system has an important role in control by *P. lilacinus*[37].

Another *P. lilacinus* isolate was cultured in rice grains and tested under pot experimental conditions with the addition of chitin (0-1%, w/w) in tomato plants infected with *M. arenaria*. Results indicated that the combination of *P. lilacinus* and chitin were effective in the control of the tested nematode[19].

Tomato plants infected with *M. incognita* were protected at different levels by the fungus *P. lilacinus*. The protection level against this nematode by *P. lilacinus* was positively correlated with the quantity of fungal spores applied and the period of application. The best protection against the nematode in tomato plants was achieved with 10 g and 20 g of fungus cultured in wheat, which resulted in a 3 and 4 times enhancement of tomato yield, respectively, when compared to the plants affected with the nematode. The best protection achieved against the nematode was when the fungus was applied in soil 10 days before planting and during planting[14].

Spores of *P. lilacinus* produced in PDA were tested in microplot experiments alone and in combination with chitin to control

the nematode *M. incognita* in eggplant, tomato and chickpea. Results showed that the best treatment for the suitable growth and development of the plants, affected with *M. incognita* with lower gall formation, was the addition of fungal spore suspension and chitin. When the fungus alone was applied, the treatment was less effective. However, when chitin was employed alone, root galling was higher in all plants tested. It seems that chitin can be used as a substrate or food base for selective development of the biocontrol agent in the soil. Two hypothesis may explain the action of chitin against nematodes: chitin decomposition releases ammonia, which acts as a nematicide on J2 of RKN; or chitin may increase population of chitinolyitic microbiota, which parasitize nematode eggs and egg sacs[67].

P. lilacinus was also applied alone and in combination with bone meal, horn meal and several oil cakes to control *M. javanica* in tomato plants. Results indicated that *P. lilacinus* was effective for inhibiting and parasitizing females, egg masses and eggs. Addition of organic fertilizers showed increased activity and persistence of the fungus in soil[54].

The effect of culture conditions on *P. lilacinus*, *e.g.* spore size, ultra structure, and UV tolerance, was determined using aerial spores produced in PDA and submerged spores produced in media containing glucose and mineral solution. Aerial spores were more uniform in size, but were smaller than submerged spores, and the rodlet layer was found only in aerial spores. Aerial spores were more tolerant to UV radiation, showing better viability after drying and storage. Aerial and submerged spores showed similar nematophagous activity[39].

The German manufacturer Prophyta produces a commercially patented strain of *P. lilacinus* (PL 251), which is registered for sale in several countries. The product consists of a water dispersible granules that can be used for a variety of crop plants[31] for protection against nematodes. This strain was studied for the production of paecilotoxin and other toxins with anti-microbial activities showing no detectable levels[52,53]. The evaluation of its potential to control *M. incognita* and *M. hapla* in tomato plants proved that a single pre-planting application is sufficient for efficient nematode control[55,56].

Different molecular approaches were followed to identify and to monitor the activity of the fungus in the soil. Species specific primers were developed for the identification of *P. lilacinus* based on sequence information from the ITS region. The primers generated a single fragment of 130 base pairs, specific to *P. lilacinus,* which permitted to detect the fungus in soil, roots and nematode eggs. Also, through real-time PCR primers and a *Taq* Man probe, it was possible to quantify the population of the fungus[6]. RAPD (randomly amplified polymorphic DNA) markers were also used to monitor and to differentiate *P. lilacinus* from a strain of *Pochonia chlamydosporia*. Two specific fragments from each strain were chosen and cloned, sequenced, and used to design specific sequence-characterized amplification region (SCAR) primers. These markers were used in classical PCR reaction to determine the detection limits[116]. Phylogenetic analysis using 5.8S rDNA and internal transcribed spacer (ITS1, ITS2) sequences were conducted for identification and taxonomy of different *Paecilomyces* species[41].

GROWTH PHYSIOLOGY OF FILAMENTOUS FUNGI

Spore production of filamentous fungi

is an important stage in its reproduction. Spore production consists of the formation and liberation of conidiospores. Life-cycle of imperfect fungi comprises five steps, which are dormancy of the spore, germination, development of apical mycelium, and conidiogenesis. Normal development of the mycelium and suitable conidiogenesis are the main conditions required for a successful sporogenesis. The conidiospore production is directly related to the quantity and nature of carbon and nitrogen sources available in a culture media, and it depends on several other factors including method of inoculation, media salinity, carbon/nitrogen ratio, aeration, water content, among others[85]. Conidiospores are characterized by a low water activity, absence of cytoplasmic movements, and reduced metabolic activity. Under favourable conditions, spore germination takes place through the formation of a vegetative tube, which will be the base of a future mycelium. A spore is considered as germinated when the length of the longest germ tube is greater than the dimension of the swollen spore. Different techniques, other than microscopic examinations can be used to assess spore germination. Gompertz equation and logistic function can be used for analysing germination data[21]. Determination of optimal culture conditions for the large-scale production of conidiospores of filamentous fungi, which are used as BCAs, is highly significant for commercial applications. There are several studies carried out to enhance conidiospore production for BCAs. Chen *et al.*[17] studied the effect of addition of different carbon and nitrogen sources in the sporulation of *Coniothyrium minitans* under SSF using wheat bran as substrate. *P. fumosoroseus*, an entomopathogenic fungus, cultured under SSF and submerged fermentation (SmF) using the same media and culture conditions proved that in SSF the carbon source was mainly utilized for the production of biomass, while in SmF the fungus produced more insecticidal metabolites[5]. It is clear that when more biomass is synthesized by a fungus, more conidiospores are formed resulting in a higher spore yield by SSF than SmF.

SOLID STATE FERMENTATION

SSF can be defined as the growth of microorganisms in a moist solid substrate in the absence of liquid water. The water content in the moist solid substrate must be adequate to support growth and metabolism of microorganisms[15,77]. SSF can be carried out in two types of matrices, either in a natural substrate acting as solid substrate and a source of nutrients or a nutritionally inert support which must be impregnated with a liquid nutritive media[91]. The most widely used substrates are of amilaceous or lignocellulosic origin. Several materials are utilized as inert supports for SSF, such as sugar cane bagasse, amberlite, vermiculite, polyurethane foam, and polystyrene beads. SSF has several advantages over SmF, but the choice of the method should depend on the physiology of the microorganism and the end product. Comparative evaluations of SSF and SmF[38,61] indicated several advantages of SSF processes: simplicity of culture media; absence of liquid residues; reduction of contamination due to low water content; culture conditions mimic the natural environment; ease of aeration (humid or dry) because of porosity of the material; direct utilization of the fermented material; easy downstream processing because of high yields; and easy to dehydrate and dry the fermented product *in situ*.

There are certain disadvantages associ-

ated with SSF processes which include: excess of heat generation and subsequent difficulties in heat and mass transfer, problems with the control of fermentation parameters (*e.g.*, pH, water content), difficulty in biomass estimation and pre-treatment of the substrate, among others.

SSF processes simulate the living conditions of many higher filamentous fungi. Hence SSF is the cultivation method of choice for biotechnological processes, where it is required to consider morphological and metabolic differences in substrate-penetrating and aerial hyphae (*e.g.*, production of conidiospores)[38].

There is a lack of information about the influence of physico-chemical and nutritional parameters on the physiology and kinetics of growth and sporulation of *P. lilacinus* under SSF, and the methods for estimation of biomass. As filamentous fungi grow, hyphae penetrate into the solid matrix becoming impossible to separate substrate from the mycelium, and thus making difficult a direct measurement of biomass. Indirect methods for estimation of biomass are available through the analysis of biomass components, such as glucosamine, ergosterol[25,59,99], nucleic acids, and proteins[108]. Several aspects should be considered when selecting biomass components for assay: 1) They should be major components in the microorganism; 2) They should have little or no influence from the substrate; and 3) They must be consistently present throughout development. The most important method so far to assess biomass consists of measuring the production of CO_2 and the consumption of O_2 by the microorganism during fermentation. This permits the estimation of biomass and specific growth of the microorganism inside the reactor through correlations between biomass synthesis and oxygen consumption[81,93].

Important factors in SSF

Factors affecting SSF are purely based on the type of microorganism that is employed in the process. The microorganism in a SSF process can be either natural microbiota of the substrate or a pure culture. Ensiling and composting are two methods that utilize the natural microbiota. Pure cultures are mainly used for the production of fungal conidiospores, secondary metabolites, antibiotics, and other high value products[76]. There are several groups of microorganisms which can profusely grow in solid substrates; however, filamentous fungi are well known for their capacity to grow in substrates of relatively low moisture content due to their physiological, enzymological, and biochemical properties. The growth of filamentous fungi takes place combining the apical extension of hyphae and the generation of new hyphae by mycelial ramification. This allows fungal growth within the solid matrix to form a solid structure. The penetration of hyphae into the substrate enhances the access to available nutrients, promoting suitable metabolic activity[83].

In SSF, the quantity of water present in the media is a function of the substrate water retention capacity. This quantity should be sufficient for the growth of microorganisms, without destroying the solid structure or reducing the porosity of substrate or support[34,35]. The water content in the substrate influences the morphology of the microorganisms, and serves as a carrier for enzymes, nutrients and metabolites, as well as in the solubilization of oxygen[73]. High moisture content of the substrate can lead to reduced porosity of the solid matrix, weak oxygen diffusion and a high risk for bacterial contamination. Low moisture levels result in limited growth of the microorganism, as the distribution of available nutrients in the substrate is not uniform[61].

The control and monitoring of the gaseous environment in aerobic SSF is a critical factor for the growth of microorganisms, which depends on the air flow rate through the substrate and the rate of O_2 consumption[84]. Aeration provides O_2 for aerobic growth and fungal metabolism, and it also helps to control moisture and temperature, and to eliminate CO_2 and some other volatile metabolites. Any adverse change in the gaseous environment may significantly affect the levels of production of biomass and enzymes.

The initial pH of the substrate in SSF is usually adjusted to support optimal growth of the microorganism. The pH value varies according to metabolic activity of the microorganisms. Acid production during fermentation or the formation of urea tend to decrease the pH. Sudden and drastic change of the substrate pH can be avoided using a solution of mineral salts with buffering capacity, as suggested by Raimbault and Alazard[84].

SSF and the production of Biological Control Agents (BCAs)

The most important aspect to be considered when selecting a BCA for commercial application is the availability of a cost-effective production and stabilization technology for manufacturing an effective formulation[102,110]. BCAs are mainly applied as spores and SSF offers several advantages: 1) The production of aerial fungal spores are more tolerant to UV radiation; 2) Higher spore stability; 3) Spore resistance to drying; and 4) Higher spore germination rates for longer storage periods. These better characteristics can be attributed to the presence of a hydrophobic rodlet layer formed during the production process[1,87]. Another advantage of SSF for production of BCAs is the utilization of agricultural by-products as substrate for fermentation. The generation of high amounts of agricultural residues causes serious environmental problems worldwide, so SSF allows efficient utilization and value addition. The use of agricultural by-products for SSF leads to a less expensive process for the production of BCAs on a large scale[100]. **Table 1** shows a list of microorganisms produced under SSF using a variety of substrates.

SSF processes are usually cost effective, and they require reduced labour. In many cases, the fermented substrate can be used for field application, and thus most technical difficulties associated with downstream processing and product formulation are ruled out. The products of SSF are usually air dried or rotavapor dried to be used directly[45]. Roussos *et al.*[88] studied different methods for the conservation of fungal spores produced under SSF, and found that temperature has a significant effect on spore viability in long term storage.

The mass production of fungal spores must be achieved for any commercial application of BCAs. Therefore, further in-depth studies for scaling up SSF processes are needed, such as the design and development of automated SSF bioreactors.

SSF Bioreactors

Bioreactors employed in SSF processes should provide adequate environmental conditions for the maximal growth and activity of microorganisms[75]. SSF bioreactors have been studied for the commercial production of biopesticides, metabolites, fermented foods, and other products[47,58,78,92,109]. Several problems in scaling up SSF processes have been found, such as variations in biomass production, high inoculum level, substrate sterilization, heat generation due to microbial metabolic activity, on-line

Table 1. Biological control agents produced through solid state fermentation in different substrates/ supports.

Microorganism	Substrate/support	Application
Coniothyrium minitans W.A. Campb. [= *Paraconiothyrium minitans* (W.A. Campb.) Verkley]	Oats	Fungal antagonist[72]
Epicoccum nigrum Link	Peat/vermiculite	Brown rot of fruits[58]
Verticillium chlamydosporium Goddard	Sand /barley bran mixture	Nematophagous[10]
Hirsutella rhossiliensis Minter & B.L. Brady	Corn grits	Nematophagous[16]
Trichoderma harzianum Rifai	Sugar cane bagasse	Fungal antagonist[86]
Paecilomyces lilacinus (Thom) Samson	Coffee husk	Nematophagous[11]
Metarhizium anisopliae (Metschn.) Sorokīn	Rice/sugar cane bagasse	Entomopathogenic[4]
Bacillus thuringiensis Berliner	Wheat	Insecticidal[109]
Beauveria bassiana (Bals.-Criv.) Vuill.	Potato waste	Entomopathogenic[101]

monitoring of aeration, or pH[60]. Bioreactor design for a particular SSF process should consider: the type of substrate or support to be used, particle size and mechanical resistance of the substrate, oxygen transfer, nature of the gaseous phase between the particles of substrate or support, morphology of microorganisms, and a suitable sterilization process[29,82].

SSF bioreactors can be classified in accordance with the quantity of substrate utilized in the process. They are divided into two categories: laboratory scale (g-kg capacity) and pilot or industrial scale (kg-tons). Another type of classification is based on the design of fermenters, which may provide agitation or aeration devices. Laboratory scale bioreactor comprises simple devices, such as petri dishes, Erlenmeyer flasks, jars and Roux bottles, which are mainly utilized for screening of microorganisms and substrates. These small bioreactors cannot provide aeration and agitation controls. Autoclavable plastic bags are also useful and commonly used for the production of fungal inoculum. The utilization of plastic bags for the production of fungal spores from *Pochonia chlamydosporia* has been reported (substrate: rice and corn grains) for application as a BCA against nematodes[111].

Column type bioreactors are well studied as they provide on-line information of the microorganism's respiration. These reactors monitor respiration and other gaseous exchanges, and are mainly used for process optimization studies[84]. Column bioreactors can also monitor and control aeration, so they are used as a model for designing and manufacturing several other types of reactors. The substrate can be cooled by evaporation, and heat generation can be minimized through convection and heat exchange by glass walls with the help of a water bath. Barranco-Florido *et al.*[7] used this type of bioreactor for the selection of strains of *Verticillium lecanii*, an entomopathogenic fungus. They used sugar cane pith as a substrate, impregnated with mineral media, and cuticle of *Sphenarium purpurascens* for the production of proteases and chitinases. The production of conidia from

Beauveria sp. for the control of caterpillars was also investigated in column bioreactors[90], as well as the influence of aeration and moisture content on the sporulation of *Metarhizium anisopliae* var. *acridum*[4].

Zymotis is a large-scale fermenter, which is composed of heat exchange plates. It has a maximum capacity of 12 kg dry substrate and it can control temperature, moisture and aeration during fermentation. This reactor was used for the production of cellulases by *Trichoderma harzianum*[89], and for the production of fungal biopesticides[87].

The scaling-up from lab-scale bioreactors to pilot- and industrial-scale reactors is complex, so important factors should be taken into consideration, such as difficulties in heat removal, compaction of solid substrate media, effect of agitation on microbial growth, oxygen demand, substrate pretreatment, and material handling. Based on these factors, fermenters can be categorized as follows: unmixed, intermittently mixed, and continuously mixed reactors with or without air circulation. Tray fermenters are extensively used in industries, because they can be easily scaled-up, and built in wood, metal and plastic with or without perforations. Tray fermenters are usually placed in temperature regulated rooms and large incubation areas, although sterility is difficult to maintain.

Reactors designed on continuous agitation are called rotating drum reactors. These are perforated drums with a horizontal paddle mixer. Rotating drum reactors were designed to increase contact between the reactor wall and solid media, as well as to facilitate oxygen transfer. However, they have several disadvantages, such as agglomeration of substrate, difficulties in temperature regulation, low oxygen transfer, and alterations of substrate structure due to intense agitation. Using a similar device from the Zymotis bioreactor for reducing heat and providing high air flow, a novel bioreactor was designed and patented by the German company Prophyta. This new bioreactor is exclusively used for the production of the BCA *P. lilacinus*, strain PL-251. It has perforated plates where heat exchangers are located at the bottom, and the substrate on the top. The flow of sterile air is facilitated by perforated plates[29]. Another reactor patented by Durand *et al*.[30] was used for the production of fungal conidiospores in biological control. This reactor has a capacity of 50 L, and it is fitted with a planetary agitation device and controls for temperature, relative humidity, and sterilization[26].

Scaling-up is still a bottleneck for the widespread commercial application of SSF. However, the development of rational and computer-controlled processes during last decades brought about advances in SSF, namely: the modelling of microbial growth on solid substrates, and energy and mass transfer in different types of bioreactors. New methods are now available for measuring SSF parameters, such as water activity and biomass production, as well as statistical tools for process optimization. These breakthroughs in SSF will certainly promote the commercial production and application of BCAs[16,18,38,72].

ACKNOWLEDGEMENTS

D. Brand is grateful to IRD for her scholarship.

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