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Production of Blastospore of Entomopathogenic *Beauveria bassiana* in a Submerged Batch Culture

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The principal objective of this study was to determine the optimal liquid culture conditions in shake flasks for maximal sporulation of *Beauveria bassiana*. The optimal initial pH for the spore production of *B. bassiana* using Potato Dextrose Broth was 5.2. The screening in shake flasks of carbon and nitrogen sources resulted in the identification of an optimal medium based on 3% sucrose and 1% casamino acid, with a C : N ratio of 22 : 4. Using this medium, a production level of 5.65×10^7 spores per *ml* was obtained after 5 days of culture. Using 3% corn meal, 2% corn steep powder, and 2% rice bran, the maximum spore concentration of 8.54×10^8/*ml* was achieved 8 days after inoculation at 25°C in a rotary shaking incubator operated at 200 rpm. This represents a yield gain of approximately 2.89 times that of pre-optimization.

KEYWORDS : *Beauveria bassiana*, Blastospore production, Liquid medium, Optimal culture conditions

In recent years, there has been a resurgence of interest in the use of fungi for the control of insect pests. This revival of interest has led to the large-scale production of several promising fungi candidates, and also to the marketing of the first commercial mycoinsecticides. *Beauveria bassiana* (Balsamo) Vuillemin is a fungus with a broad natural distribution; its potential to control more than 70 insect pests has been responsible for a substantial increase in interest in the large-scale production of the fungus for applications in the field (Thomas *et al.*, 1987). Moreover, this fungus also appears to be innocuous to most non-target organisms.

Efforts to improve potential control agents often center around an appropriate mass-production method for the suitably large-scale production of the infective propagules. The mass production of insect-pathogenic fungi is a necessary prerequisite for any large-scale field application employing these fungi. The most frequently utilized technique for the cultivation of fungal spores is either a surface culture with a solid substrate, such as moistened wheat bran, millet or rice, or a submerged culture with a liquid medium (Feng *et al.*, 2000). Submerged cultivation may have the advantage that fungi can be rapidly generated using conventional deep-tank fermentors, and the scale-up of this process is relatively easy. Submerged cultivation also has a number of highly desirable attributes: they are cheaper, particularly on a very large scale; environmental factors (pH, pO_2, pCO_2, nutrient levels) can be more readily controlled, and growth can be easily monitored (Bartlett and Jaronski, 1988). Harvesting from submerged fermentations is also made substantially easier, as the spores can be readily collected and concentrated via centrifugation, filtration, *etc.*, and then dried.

The entomopathogenic hyphomycete fungi are generally easy to grow on a large scale, and can be cultivated on cheap media in submerged cultures. Depending on the strain, medium, and culture parameters, the fungal biomass is increased via vegetative growth forming either hyphal filaments, often with copious branching, or various forms of flocs and pellets of mycelia (Brown *et al.*, 1988). The majority of fungal isolates are also capable of forming single cells via schizolytic separation at the septa or mechanical fragmentation of the hyphae, and can also be generated from the hyphae by yeast-like budding (Rombach, 1989; Jackson *et al.*, 1990). Hyphal cells, which are also referred to as hyphal bodies or blastospores, are usually the only type of propagules produced in liquid fermentation schemes (Thomas *et al.*, 1987; Jenkins *et al.*, 1993).

Previously, we isolated the entomopathogenic *B. bassiana* KK5, which exhibits particularly high infectivity to aphids. Despite its high levels of infectivity, the large-scale application of this fungus for the control of aphids in the field is possible only in cases in which the fungus can be grown and can produce high spore titers economically, on readily available substrates. The primary objective of the present study was to evaluate the influences of different carbon and nitrogen sources and main culture parameters on blastospore yields in submerged fermentation cultures of the *B. bassiana* KK5 isolate. First, the

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effects of differing pH values, temperatures, medium compositions, and C/N ratios were optimized in shake flasks. Sporulation was then optimized using different substrates. Finally, blastospore production was carried out using these optimized parameters.

Materials and Methods

Fungal cultures. *B. bassiana* KK5 was isolated from a soil sample and was selected from 11 strains of *B. bassiana* after screening for the strain with the highest mortality against *Myzus persicae*. Colonies of *B. bassiana* KK5 were white or slightly colored, with a white fluffy-to-powdery appearance. The conidiogenous cells were short and ovoid, and terminated in a rachis. The rachis elongated after the production of each conidium, thus resulting in a long zig-zag extension. The *B. bassiana* cells were stored at −80°C in sterile cryovials containing sterile 0.05% Tween 80 with 10% glycerol.

Media and culture conditions. Conidial inocula for liquid culture studies were obtained from 2 week-old sporulated culture on Potato Dextrose Agar (PDA; Difco) plates at 25 ± 1°C, which had been previously inoculated with stock cultures. After 2 weeks of incubation at 25°C, sterile 0.02% (v/v) Tween 80 was poured onto the culture on the surface of the PDA and the spores were collected by scraping the culture with a glass hockey stick. The spores harvested in sterile 0.02% (v/v) Tween 80 were thoroughly vortexed and diluted to achieve a suspension of 5×10^7 conidia/*ml*. One milliliter of the 5×10^7 conidia/*ml* spore suspension was poured into 250 *ml* Erlenmeyer flaks containing 50 *ml* of sterilized medium, and then incubated at 25°C on a rotary shaker at 200 rpm. The spores were counted with a Hemacytometer at 24 h-intervals after 2 days of growth. Unless otherwise specified, these conditions were maintained throughout all experiments in this study.

Growth temperature profile. Ten microliter inocula of a 10^6/*ml* spore suspension grown in PDA were inoculated onto the centers of PDA plates, and the plates were incubated at temperatures of 25, 27, and 30°C. After 10 and 14 days of incubation at the specified temperatures, the diameters of the mycelia were measured to assess the effects of temperature on fungal growth. This experiment was conducted in triplicate.

Effect of shaking speed on spore production. The inoculated Erlenmeyer flasks were incubated at 25°C in a rotary shaking incubator at shaking speeds of 150, 200 and 250 rpm. The number of blastospores was determined for each sample at 24-hour intervals for 6 days. This experiment was conducted in triplicate.

Effect of initial medium-pH on sporulation. The initial pH of the liquid medium were adjusted to 3.0, 4.0, 4.5, 6.0 and 6.5 using 2 N HCl, after which the media were sterilized at 121°C for 15 min. The original PDB without pH adjustment was also utilized in the experiment - the pH of the original sample was 5.2. The inoculated samples at various initial pH were incubated at 25°C in a shaking incubator at 200 rpm. Sporulation was assessed at 24-hour intervals for 3 days.

Effect of medium component on sporulation. Different sterile liquids were utilized used to determine the optimal medium compositions for the sporulation of *B. bassiana* by many factors, including the carbon to nitrogen ratio, carbon and nitrogen sources, and additives. Rice meal and corn meal were made from rice and corn grain by grinding. Sweet potatoes were cut into 1 mm-thick slices and then dried for 24 h at 55°C and ground into powder. The ground cereals or sweet potato were screened with a 180 μm sieve. The various media were then incubated at 25°C on a rotary shaking incubator at 200 rpm.

Counting spore number. The spore suspension was throughly vortexed and diluted with sterile 0.05% (v/v) Tween 80. The spore number of the desired dilution was counted with a Hemacytometer (Superior, Marienfeeld, Germany) and a light microscope (Olympus BH2, Japan) at a magnification of × 400.

Results

Effect of temperature on fungal growth. The mycelial growth of *B. bassiana* KK5 10 and 14 days after incubation at different temperatures were examined, and the results are provided in Table 1. The optimal temperature for radial fungal mycelial growth was 25~27°C. After 14 days, the fungal growth at 25°C was slightly higher than that at 27°C. At 30°C, the growth declined sharply. In this study, the optimal temperature (25~27°C) for high spore production matched the optimal growth temperature of *B. bassiana*.

Microscopic observation of blastospore. The blastospore of *B. bassiana* KK5 formed in liquid culture is

Table 1. The radial growth of mycelia at different temperatures

Temperature (°C)	The radial growth (mm) after	
	10 days	14
25	44.8	58.8
27	44.1	56.4
30	6.4	6.7

Ten microliter inocula of 10^6/*ml* spore suspensions grown in PDA were inoculated onto the centers of the PDA plates, and the plates were incubated at different temperatures.

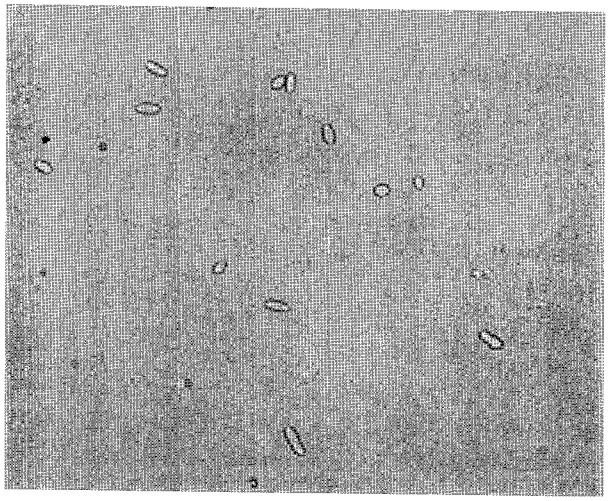

Fig. 1. Microscopic observation of *B. bassiana* blastospore. × 400 magnification.

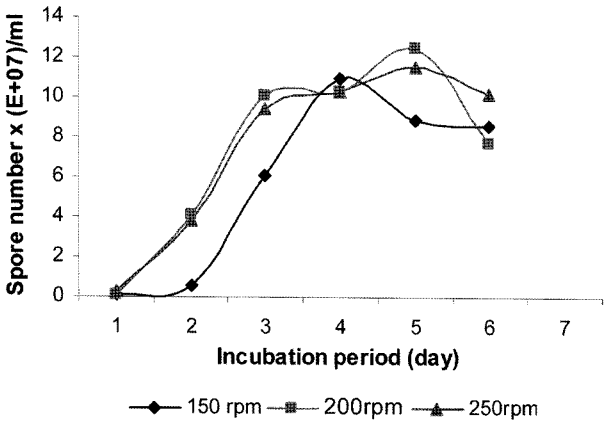

Fig. 2. Effect of shaking speed on blastospore production.

depicted in Fig. 1. The blastospores were measured to be less than 5 μm. The spores evidenced ellipsoidal morphology.

Effect of shaking speed on spore production. The spore production at various shaking speeds was evaluated and the results are shown in Fig. 2. Shaking speeds of 200 rpm and 250 rpm evidenced similar spore production patterns for the first 4 days, but after 5 days, spore production was higher at 200 rpm (12.5×10^7 spores/*ml*) than at 250 rpm (11.53×10^7 spores/*ml*). A shaking speed of 150 rpm produced the lowest number of spores (10.97×10^7 spores/*ml*). After 6 days, many mycelia were observed at all three shaking speeds, and this resulted in sharp declines in the spore numbers.

Effect of initial medium-pH on sporulation. The effect of initial medium-pH on sporulation was assessed using PDB as the culture medium, and the results are provided in Fig. 3. The highest spore number (12.08×10^7 spores/*ml*) was achieved after 5 days at a pH of 5.2, which was the natural pH of the PDB medium. The lowest levels of spore production were observed at pH 6.5.

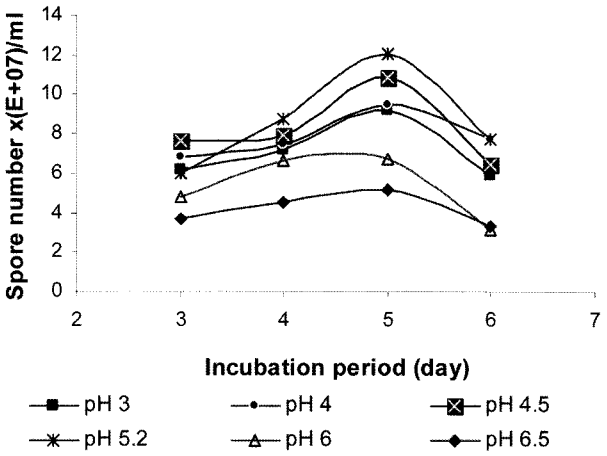

Fig. 3. Effect of initial medium-pH on spore production.

Table 2. Carbon to nitrogen ratio in liquid cultures[a] used to assess yields of *B. bassiana*

C : N	Carbon	Nitrogen
11.88	2% Sucrose	2% Casamino acid
14.51	2.4% Sucrose	1.6% Casamino acid
22.4	3% Sucrose	1% Casamino acid
32.3	3.32% Sucrose	0.68% Casamino acid
53.95	3.6% Sucrose	0.4% Casamino acid

[a]All the culture broth contained same carbon concentration [C] of 16 g/*l*.

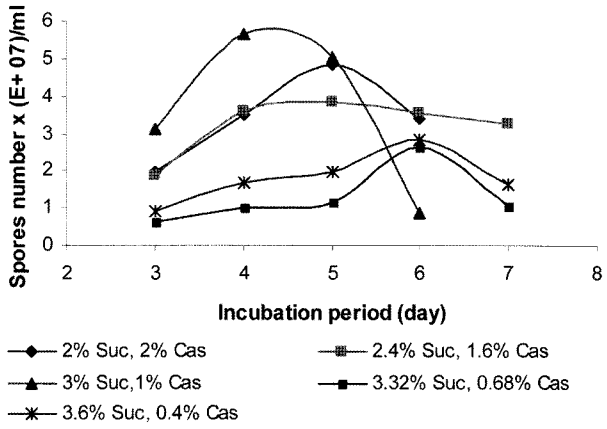

Fig. 4. Effect of carbon:nitrogen ratio on spore production. Suc - Sucrose. Cas - Casamino acid.

Effect of C : N ratio on spore production. Blastospore production was carried out in media containing sucrose and casamino acid with different carbon-to-nitrogen ratios (Table 2). The carbon concentration, which was constant in all media used in this experiment, was 16 g/*l* (Fig. 4). A carbon concentration of 8 g/*l* was also tested, but this

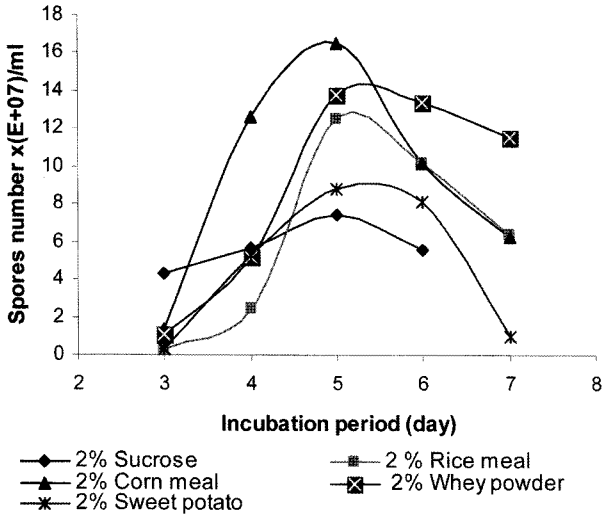

Fig. 5. Effect of carbon source on spore production. The media containing 2% peptone and 2% of different carbon sources were examined to determine the optimal carbon source for spore production.

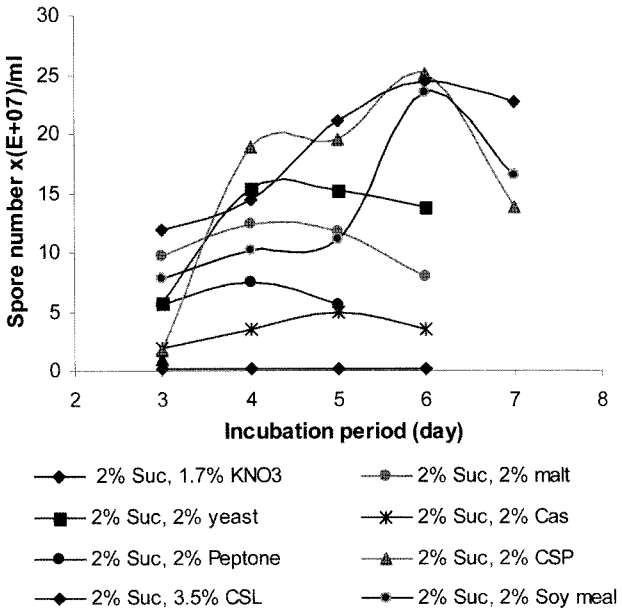

Fig. 6. Effect of nitrogen source on spore production. Suc - Sucrose. Malt - Malt extract. CSP - Corn steep powder. CSL - Corn steep liquor.

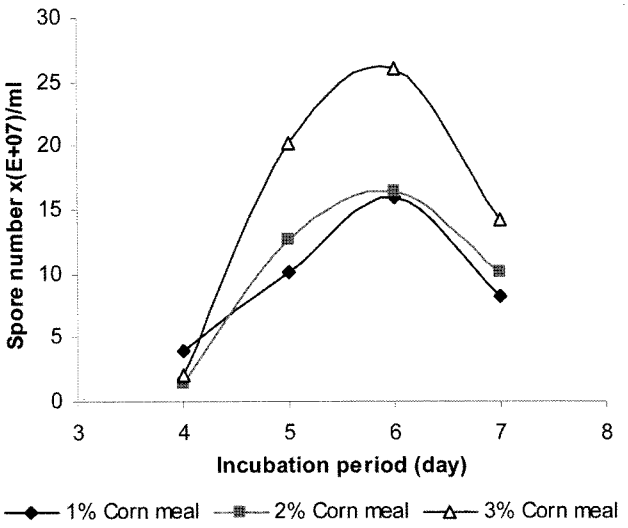

Fig. 7. Effect of corn meal concentration in medium on spore production.

resulted in a very low spore yield ($< 1 \times 10^7$ spore/*ml*). A medium containing 3% sucrose and 1% casamino acid with a C : N ratio of 22.4 was identified as the optimal C : N ratio for sporulation.

Effect of carbon source on spore production. Five different carbon sources were tested: sucrose, rice meal, corn meal, whey powder, and sweet potato. 2% peptone was used as the nitrogen source. The results of these tests showed that the highest spore production (16.5×10^7 spores/*ml*) was achieved using corn meal as a carbon source (Fig. 5). Sucrose produced the lowest number of spores, probably because sucrose did not contain the nutrients contained in the other substrates (rice meal, corn meal, whey powder, and sweet potato). Therefore, corn meal was utilized as the carbon source in all subsequent experiments in this study.

Effect of nitrogen source on spore production. Using 2% sucrose as a carbon source, different nitrogen sources were tested to determine the optimal nitrogen source for blastospore production in a shaking flask culture. Fig. 6 demonstrated that the media containing corn steep liquor or corn steep powder (CSP) generated the most blastospores (25×10^7 spores/*ml*). The fungal strain could not utilize potassium nitrate as a single nitrogen source, and no spores were produced in the medium containing potassium nitrate.

Effect of corn meal concentration on spore production. The media containing 2% CSP and different concentrations of corn meal were tested in order to determine the optimal corn meal concentration for spore production. Fig. 7 demonstrated that a medium containing a corn meal concentration of 3% produced the highest number of blastospores (26×10^7 spores/*ml*) after 6 days.

Effect of CSP concentration on spore production. Sporulation was assessed using media containing 2% corn meal and different concentrations of CSP. Fig. 8 shows that the highest blastospore yield was observed with a CSP concentration of 2%. The 3% CSP sample produced less spores than the 2% CSP, probably because the C : N

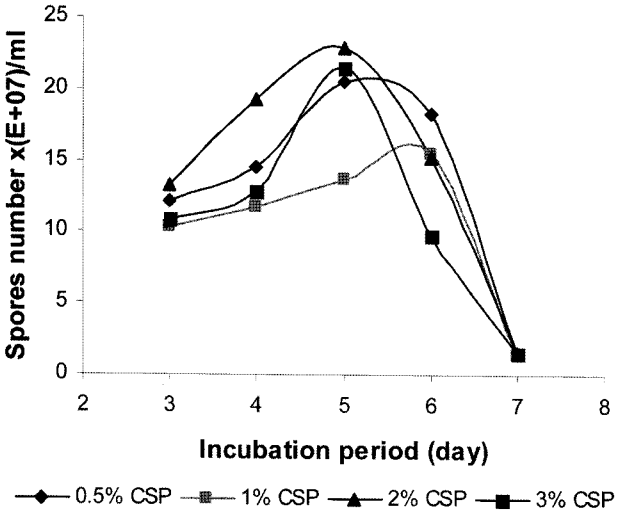

Fig. 8. Effect of CSP concentration in medium on spore production. CSP - Corn steep powder.

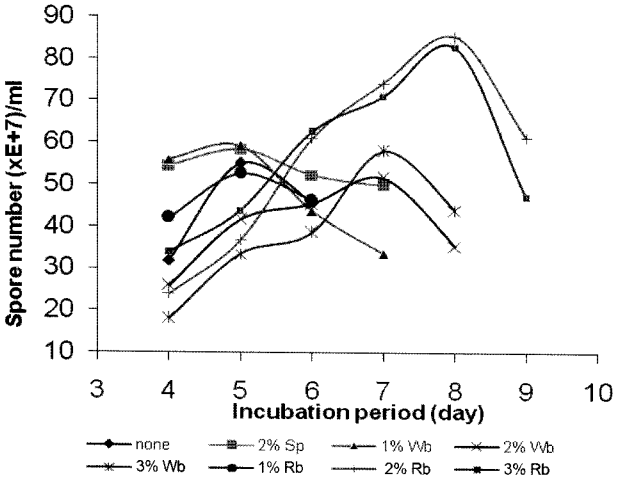

Fig. 9. Effect of medium supplement on spore production. The basal medium contained 3% corn meal and 2% CSP, and other supplements such as rice bran, wheat bran, or sweet potato were added to the basal medium. CSP - Corn steep powder. Sp - Sweet potato. Rb - Rice bran. Wb - Wheat bran.

ratio of the 3% CSP was suboptimal for spore production. The 1% CSP medium produced the fewest blastospores.

Effect of medium supplement on spore production. The effect of supplement to the medium composed of 3% corn meal and 2% CSP on spore production was evaluated, and the results are provided in Fig. 9. The supplement that produced the highest spore yield (8.54×10^8 spores/*ml*) was 2% rice bran after 8 days of incubation. After 5 days of incubation, the highest spore yield (5.59×10^8 spores/*ml*) was observed in the medium supplemented with 1% wheat bran.

Discussion

Beauveria bassiana (Balsamo) Vuillemin is considered a very important and promising fungal agent for use in the control of insect and mite pests (Feng *et al.*, 1994; Wright *et al.*, 2001; Shi and Feng, 2004). Previously, we have isolated the entomopathogenic *B. bassiana* KK5, which exhibits particularly high infectivity to green peach aphids. Despite its high infectivity, the large-scale application of this fungus for aphid control in the field is only possible if the fungus can be grown and produce a high titer of spores economically on readily available substrates. The entomopathogenic hyphomycete fungi are frequently easy to grow on cheap media in submerged cultures on a large-scale, and submerged blastospores can be economically produced in deep-tank fermentors.

The results demonstrated that the optimal temperature for spore production of *B. bassiana* KK5 was 25~27°C. Thomas (1987), Feng (1994), and Hallsworth (1999) have also demonstrated that 25°C was the optimal temperature for the liquid and solid production stages of *B. bassiana*. The optimal initial pH of 5.2 in this study was lower than that reported by Karthikeyan *et al.* (2008), who found an initial pH of 6~8 to be the most suitable for spore formation. The shaking speed also clearly affected spore production of the fungi due to the different aeration rates. Shaking speeds of 200 rpm and 250 rpm resulted in similar patterns of spore production, but after 5 days, a shaking speed of 200 rpm generated more spores than were detected at 250 rpm. Thus, we selected 200 rpm as the default speed for the other experiments.

The medium composed of 3% sucrose and 1% casamino acid (carbon concentration of 16 g/*l*) with a C : N ratio of 22.4 was determined to have the optimal C : N ratio for the sporulation of *B. bassiana* KK5. According to Vega *et al.* (2003), the highest spore yields of *B. bassiana* in liquid culture were obtained in media containing a carbon concentration of 36 g/*l* and a C : N ratio of 10 : 1, using sucrose and casamino acid. The blastospore production of *Metarhizium flavoviride* Mf189 was based on sucrose and brewer's yeast, with a C : N ratio of 1.6 (Issaly *et al.*, 2005). This indicates that the optimal C : N ratio differed with differing fungal strains.

Among the five different carbon sources evaluated, the highest level of spore production was observed when corn meal was used as a carbon source in medium containing 2% peptone. Kumar *et al.* (2005) also demonstrated that corn grain medium supported the heavy sporulation of the fungus *Arthrobotrys dactyloides*. Corn meal has been generally considered a good substrate for fungal growth, has a high starch content (67.6~69.4%), and also contains other nutrients (Lee *et al.*, 2008). Corn meal supported the high growth rate of a variety of fungal strains, such as *Aspergillus niger*, *Fusarium moniliforme*, *Penicillium* sp.,

Cercospora sp., *Curvularia palesscens*, *Botryodiplopodia* sp., *Rhizopus* sp., and *Rhodotorula rubra* (Adesemoye and Adedire, 2005). The growth of the 8 fungal species were noted to be approximately the same or occasionally better in corn meal medium as compared to those grown on PDA, a widely used but expensive commercial fungal medium. Corn is also readily available across the globe, and is easy to grind finely, as a result of its low fiber content (Adesemoye and Adedire, 2005). The authors concluded that corn meal could be utilized in the formulation of an alternative to PDA.

Among the nine different nitrogen sources tested in this study, the media containing corn steep liquor or corn steep powder (CSP) generated the highest number of blastospores. Corn steep liquor (CSL), a byproduct of the corn wet-milling industry, is one of the cheapest nitrogen sources (White and Johnson, 2003). CSL contains water (~46%), proteins (~47%), amino acids, minerals, vitamins, reducing sugars, organic acids, enzymes, fat, and elemental nutrients (White and Johnson, 2003). These constituents can be readily assimilated into normal cell metabolism (Liggett and Koffler, 1948; White and Johnson, 2003). High cell density yeast cultures using a mixture of CSL and molasses as the culture medium have been previously reported (Kim *et al.*, 2007; Vu and Kim, 2009).

Three percent corn meal as a carbon source and 2% CSP as a nitrogen source were selected as the optimal basal medium for high-yield blastospore production. Further improvements were made to increase the spore yield via the addition of supplements to the basal medium. As a result, the medium that generated the highest spore yield (8.54×10^8 spores/*ml*) was 3% corn meal, 2% CSP, and 2% rice bran, with an incubation period of 8 days. After 5 days of incubation, the maximal blastospore yield (5.59×10^8 spores/*ml*) was achieved with a medium containing 3% corn meal, 2% CSP, and a supplement of 1% wheat bran. Brans of various crops are agricultural wastes and were utilized as the microbial culture medium (Jatinder *et al.*, 2006; Kim *et al.*, 1977; Kumar *et al.*, 2005). In a previous study, among a variety of tested brans, including the brans of pea, wheat, rice, gram, pigeon pea, and lentil, the maximum sporulation of various strains of the fungus *Arthrobotrys dactyloides* was achieved in rice bran medium (Kumar *et al.*, 2005). Rice bran has also been employed as a sole nutrition source for the culturing of *Streptomyces actuosus* (Wang, *et al.*, 2003) and *Lactobacillus brevis* (Ohtomo *et al.*, 2006). A mixture of 1% wheat bran, 2% starch, and 3% corn meal was also utilized as a submerged culture medium for the fungus *Monascus purbigerus*.

In the case of wheat bran supplemented into a basal medium of 3% corn meal and 2% CSP, the higher concentration of wheat bran generated less spores after 5 days of fungal cultivation, i.e.-the 1% wheat bran supplement generated the highest spore number, the 2% wheat bran produced a medium number of spores, and the 3% wheat bran produced the lowest spore number. Kumar *et al.* (2005) also mentioned that the poor growth of the fungus *Arthrobotrys dactyloides* on wheat bran might be attributed to its higher concentrations. Similar observations regarding the reduced growth of *Catenaria anguillulae* at higher concentrations of wheat bran in medium and mass culture were made by Vaish and Singh (2002).

In conclusion, this study assessed the effects of various initial culture medium pH values, carbon sources, nitrogen sources, C : N ratios, and medium supplements on the sporulation of *Beauveria bassiana* KK5 in liquid culture. The results demonstrated that the optimal temperature, shaking speed, initial pH, and C : N ratio were 25°C, 200 rpm, 5.2, and 22.4, respectively. Corn meal at a concentration of 3% was found to be the optimal carbon source, and 2% CSP was found to be the optimal nitrogen source. Further improvements in spore yield were accomplished via the addition of 2% rice bran to the 3% corn meal and 2% CSP. Using the optimal conditions and media, the maximal spore concentration of 8.54×10^8/*ml* was achieved, which was 2.89 times higher than the pre-optimization spore yield.

Acknowledgements

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