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SHORT COMMUNICATION

Suitability of agricultural by-products as production medium for spore production by *Beauveria bassiana* HQ917687

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Abstract

Purpose The seemingly harmful effect of chemical insecticides to the environment, plants and human health, has led to prominent role of entomopathogenic fungi (ENPF) as biopesticide. However, the success of ENPF as biocontrol agent depends on their amenability to easy and cheap mass multiplication. Solid state fermentation (SSF) has emerged as an appropriate technology for management of agro-industrial residues and for their value addition. In this study, the potentiality of agricultural by-products for mass multiplication of *Beauveria bassiana* was determined.

Methods Two sets of SSF experiments with agricultural by-products [tea leaf waste, wheat bran, husk of rice, pigeon pea and urad, and seed cake of jatropha and pongamia] as substrates; one with fortification of nutrients, while other with no nutrient addition were performed. The substrates (200 g) were inoculated with *B. bassiana* spore suspension (1×10^8 spores/ml) and incubated at 28 °C for 14 days. After the incubation period, *B. bassiana* spore concentrations were enumerated.

Results Maximum spore production was observed in rice husk, while jatropha seed cake showed least spore production. Addition of nutrient supplements in the substrates showed slight (pongamia seed cake) to significant (rice husk, tea leaf waste) increase in spore production. The results showed constant increase in spore production with increase in C/N ratio of the substrates.

Conclusions Mass production processes directly influences the cost, shelf life, virulence, and field efficacy of fungal pathogens. The results from this investigation are expected to pave the way towards commercialization of *B. bassiana* by augmenting the research in fungal mass production.

Keywords *Beauveria bassiana* · Solid state fermentation · Spore production · C/N ratio · Bioefficacy

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Introduction

The use of chemical insecticides/pesticides has become a common practice around the world. The peril of their use has affected environment by contaminating soil, air and water, while also negatively impacting health of non-target plants and animals (Sharma et al. 2014). Potential volatility and pesticide drift of chemical pesticides can lead to widespread environmental contamination to several hundred miles. In addition to killing insects or weeds, pesticides could be toxic to a host of other organisms including birds, fish, beneficial insects, and non-target plants. In humans, low-dose exposure of pesticides is being linked to immune suppression, hormone disruption, diminished intelligence, and reproductive and neural abnormalities (Aktar et al. 2009). In this regard, biological insecticides based on entomopathogenic fungi (ENPF) are achieving prominence in pest management. ENPF species, such as *Metarhizium anisopliae* (Metsch.), *Verticillium lecanii* (Zimmerman) Viegas, *Nomuraea rileyi* (Farlow) Samson, *Paecilomyces farinosus* (Holm ex S. F. Gray) Brown and Smith, and *Beauveria bassiana* (Balsamo) Vuillemin have been reported for the control of various insect/pests (Shah and Pell 2003). Amongst these, *Beauveria bassiana* with an extremely broad host range is already being marketed as commercial biopesticide for the control of several agricultural insects (Feng et al. 1994; Zimmermann 2007). However, the market share of mycoinsecticides is very low compared to chemical insecticides, mainly due to lack of suitable mass multiplication techniques (Bhanu Prakash et al. 2008). Moreover, growth and sporulation in ENPF have been documented to be limited by the requirement of appropriate nutritional and the pH conditions (Mishra and Malik 2012). Thus, for a successful biocontrol programme using ENPF requires the production of adequate quantities of a good quality inoculum capable to support their optimum growth and sporulation. In this regard, the agro-industrial wastes, with rich nutrient source and a solid support to promote the growth of filamentous fungi, could be a good choice for mass multiplication of ENPF. The presence of carbon, other nutrients sources and moisture in the agricultural wastes provides suitable conditions for the development of microorganisms, and thus open up great possibilities for their reuse in the process of SSF (Torkashvand et al. 2015). Large amount of the agro-industrial wastes are mainly composed of cellulose, hemicelluloses and lignin, commonly categorized as lignocellulosic materials (Kundu et al. 2012). Lignocelluloses residues are the most useful substrates for the growth of filamentous fungi, which produce cellulolytic, hemicellulolytic and ligninolytic enzymes during SSF (Mussatto et al. 2012). Although, the percentage of cellulose, hemicellulose and lignin is different to each waste since it varies from one plant species to another, and also according to the process that the agricultural material was submitted (Mussatto et al. 2012; Kundu et al. 2012). The presence of sugars, proteins, minerals and water make the agro-industrial wastes a suitable environment for the development of microorganisms, mainly fungal strains due to their ability to grow on complex solid substrates and to produce a wide range of extra cellular enzymes (Bhanu Prakash et al. 2008).

All over the world, a large amount of wastes is generated every year from the industrial processing of agricultural raw materials. It is estimated that in 1990s, the global output of wheat straw residues was 709.2 million metric tons, while that for rice straws was 673.3 million metric tons (Belewu and Babalola 2009). Similarly, a large amount of rice husks (one-fifth by weight of rice grain production), and wheat husks is also produced every year in the process of rice/wheat threshing (Sharma et al. 2014). The most of these wastes are used as animal feed or burned as alternative for elimination, which generates uncontrolled emissions, while also wasting a potential energy resource. Husks are usually disposed off by combustion, thus producing ash, fumes, and toxic organic gasses. Incineration of rice husks produces rice husk ash (RHA), considered to be a world-wide environmental problem due to its role in air and water pollution (Sharma et al. 2014). Likewise, the plants of jatropha (*Jatropha curcas*) and karanja (*Pongamia pinnata*), which have received a great deal of attention recently due to their great potential for use in the production of biodiesel, are expected to generate huge quantity of residual deoiled seed cake (Achten et al. 2008). In India alone, jatropha production is forecasted to generate approximately 1000 kg seed cake per hectare crop, while karanja plantations is estimated to produce around 0.145 million metric tons of defatted oilseed cake per annum (Doshi et al. 2014). The disposal of defatted cake leftover after expelling oil from these oilseeds will be one of the major problem in coming years, since jatropha and karanja being non-edible crops and their seeds being toxic, these cakes cannot be used as cattle feed (Joshi and Khare 2011). However, the use of these seed cakes as fungal growth medium in SSF not only reduces their inherent toxicity, but also provides low cost substrate medium for biopesticides production (Ameen et al. 2011; Joshi and Khare 2011).

The success of microbial control of insects/pests depends not only on their pathogenicity, but also on the successful mass production of the microbial control agents. For a successful integrated pest management programme, the agents like the ENPF should be amenable to easy and cheap mass multiplication. The use of agroindustrial wastes in SSF for production of ENPF is of particular interest due to their availability and low cost, besides being an environment friendly alternative for their disposal. In the present study, SSF was used for the determination of suitability of different agricultural by-products for mass multiplication of ENPF, *Beauveria bassiana*.

Materials and methods

Microorganism

Beauveria bassiana HQ917687 used in this study for SSF was isolated from soil samples collected from Northern Uttar Pradesh, India (Mishra et al. 2015). The fungal isolate was maintained on Potato Dextrose Agar slants at 4 °C. Spore suspension was prepared from spores harvested from five days cultured slants by adding 10 ml of 0.1 % sterile Tween 80, vortexed for five min, and filtering the resulting spore suspension through a sterilized 8 μm membrane filter disk.

Agricultural waste substrate

The agricultural waste substrates tested for SSF were: tea leaf waste, wheat bran, husk of rice, pigeon pea and urad, and seed cake of jatropha and pongamia. The substrates were dried at room temperature and grinded to make a powder and filtered using a sieve (mesh size, 0.8 mm). The initial carbon and nitrogen content of the substrates, as well as their carbon to nitrogen ratio were determined by CHN analyzer. The pH of the substrates was measured by utilizing 5 g of the sample diluted in 50 ml of distilled water. The obtained suspension was well homogenized and pH was determined using a meter (HI 2212 pH meter, Hannah instruments, USA). The initial moisture content of the powdered substrate was adjusted between 40 and 67 %, depending upon the substrate type.

Solid state fermentation

Growth of *B. bassiana* on agricultural by-products was performed to select best substrate for spore production. The study of SSF was carried out in Erlenmeyer flasks (500 ml) according to the method described by Bhanu Prakash et al. (2008). With each of the substrate used for solid state fermentation, two sets of experiments were performed. In first set of experiment, substrates were fortified with a fixed concentration of several essential nutrients (Dextrose 1.5 %, $NaNO_3$ 0.25 %, KH_2PO_4 0.1 %, $MgSO_4$ 0.05 %), while for second set of experiments, no nutrient addition was made. The substrates (200 g) were inoculated with 1 ml of spore suspension at 1×10^8 spores/ml, and incubated at 28 °C for 14 days. After the incubation period, 1 g of the fermented substrate was mixed with of 30 ml of distilled water and 0.01 % of Tween 80. After 30 min of agitation, the mixture was filtered through a nylon sieve of 200 µm and the spore concentration was evaluated using an automatic cell counter (Mishra et al. 2013).

Evaluation of insecticidal activity under simulated field condition

The fungal growth on rice husk (with nutrient supplementation), showing maximum spore production was evaluated for its biocontrol potentiality against *Musca domestica* larvae. The assay was performed under simulated filed condition during the period of April, 2010. The assay was done on decaying waste matrix according to the method modified from Mishra et al. (2013). Decaying waste matrix was inoculated with substrate (200 g) [after 14 day of fungal growth] and mixed thoroughly. After the inoculation, 200 *M. domestica* larvae (2nd instar) were added to each tray. Experiment was done in triplicate. Trays were kept at room temperature within a netted cage (80 × 65 × 60 cm), and checked daily for the formation of pupae and emergence of flies for next seven days. Number of pupae formed or flies emerged were counted together, and rest of the larvae, which could not be accounted for, were considered as dead. The experiment was performed for 8 weeks, during which test larvae were introduced in the system at the beginning of each week.

Results and discussion

Nutrient characterization of agricultural waste residue

Table 1 shows the nutrient content of different agricultural substrate used for SSF. Carbon content of different agricultural waste substrate varied between 25 and 57 %, while C/N ratio showed variation between 2.4 and 22.6 (Table 1).

Spore production on agricultural waste residue

Rice husk with maximum *B. bassiana* spore production (4.3×10^8 spore/g), followed by wheat bran (2.1×10^8 spore/g) was observed to be the best substrate (Table 2). Substrates supplemented with nutrients showed comparatively higher spore production, with maximum spore production in rice husk (1.8×10^9 spore/g). Least spore production was observed with jatropha seed cake, which showed spore production of 7.6×10^3 and 4.8×10^4 spore/g, without and with nutrient supplement, respectively.

The result from this study showed a constant increase in spore production with increase in C/N ratio of the substrates (Fig. 1). The jatropha and pongamia seed cake showing minimum spore production had lowest value of C/N ratio, due to their high nitrogen content. However, the low spore count in these medium may also be due to their inherent toxicity (as discussed below). The rice husk with highest C/N ratio of 22.7, showed maximum spore production in both types of medium. Wheat bran with a C/N ratio of 19.5, proved to be another medium supporting better spore production (5.4×10^8 and 2.1×10^8 spore/g, with and without nutrient supplement, respectively). The

Table 1 Nutrient characteristics of different agricultural-by products used as substrate for solid state fermentation

Substrate	Carbon (%)	Nitrogen (%)	C/N
Wheat bran	53.4	2.74	19.49
Rice husk	57.35	2.53	22.67
Pigeon pea husk	26.24	6.53	4.0
Urad husk	25.42	6.05	4.2
Pongamia seed cake	41.5	15.75	2.63
Jatropha seed cake	46.08	18.73	2.46
Tea leaf waste	37.35	3.23	11.56

Table 2 The *B. bassiana* spore production on different agricultural-by products with or without nutrients supplementation during solid state fermentation

Substrate	Moisture content (%)	Spore production (conidia/g)	
		w/o nutrient supplement	With nutrient supplement (dextrose 1.5 %, $NaNO_3$ 0.25 %, KH_2PO_4 0.1 %, $MgSO_4$ 0.05 %)
Wheat bran	48–56	2.1×10^8	5.4×10^8
Rice husk	51–54	4.3×10^8	1.8×10^9
Pigeon pea husk	50–62	3.4×10^6	6.7×10^7
Urad husk	47–53	8.1×10^6	3.2×10^8
Pongamia seed cake	40–57	1.4×10^5	5.6×10^5
Jatropha seed cake	44–64	7.6×10^3	4.8×10^4
Tea leaf waste	55–67	4.2×10^7	3.7×10^8

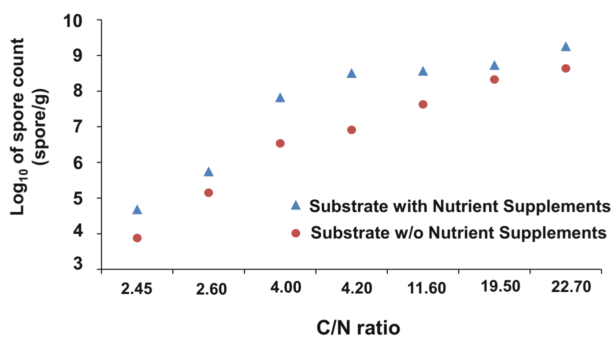

Fig. 1 Spore count of *B. bassiana* obtained after 14 days of growth on SSF with respect to C:N ratio of agricultural substrates

growth of *Paecilomyces lilacinus* performed with different C:N ratio (10:1, 20:1, 40:1 and 50:1) by adding sucrose in pongamia seed cake medium showed that by increasing C:N ratio, increase in fungal growth was observed, however, not much difference in growth was seen between C:N ratio 40:1 and 50:1 (Sharma et al. 2002). According to Jackson and Schisler (1992), the C:N ratio not only affects the mycelial and spore production, but also affects the biocontrol efficacy as it also impacts production of various secondary metabolites.

The results obtained in this study were in agreement with other literature reports, where rice grain was found to be the most suitable media for mass culture of *B. bassiana* (Sharma et al. 2002; Sahayaraj and Namasivayam 2008). Sahayaraj and Namasivayam (2008) reported rice (11.24×10^6 spore/g) and wheat grain (11.76×10^6 spore/g) as the most suitable substrate for *B. bassiana* spore production. However, in comparison to above studies, the present study which utilizes agricultural waste (unsuitable for human consumption) as substrate holds much significance. Moreover, utilization of food grains for biopesticide production would not be economical. Investigations concerning fungal enzyme production on seed oil cake have been reported (Joshi and Khare 2011). The low spore production obtained with seed cakes (jatropha and pongamia) in this study could be attributed to their organic content (C/N ratio) or presence of antinutritional factors such as phorbol esters, curcin and trypsin inhibitors (Ameen et al. 2011). Makkar et al. (2008) reported *Jatropha curcas* seed cake meal to be toxic to rats, mice, ruminants and humans. However, recent reports on reductions of cyanide, tannin, oxalate, trypsin inhibitors and other toxins from the *J. curcas* seed cakes during SSF with fungi (Belewu and Sam 2010; Ameen et al. 2011), indicates that jatropha if used as substrate, would not only help in biopesticide production, but the detoxified seed cake would also serves as protein rich feed for livestock. Literature reports on *B. bassiana* spore production on some other seed cakes as; gingelly seed cake (5.35×10^{10} spores/100 g) and cotton seed cake (4.31×10^{10} spores/100 g) medium, observed comparatively higher spore yield (Abraham et al. 2003).

In this study, addition of nutrient supplements in the substrate medium showed significant increase in spore production. The increase in spore production for different substrates ranged from 2.5 to 39 times. The minimum effect of nutrient fortification was observed for wheat bran (increased by 2.5 times), followed by pongamia seed cake (increased by 4 times). The husk of urad (increased by 39 times) and pigeon pea (increased by 20 times) showed maximum increase in the spore production with nutrient supplementation compared to non-supplemented substrates. Balakrishnan et al. (2011) reported a 1.2 and 1.9 times increase in the biomass and spore production of *B. bassiana*, respectively, with 3 % supplementation of calcium chloride in molasses media. In the same study, fortification of molasses with lactic acid (3 %) showed an increase in the *B. bassiana* biomass by 1.3 times and spore production by 4.1 %. Compared to this, results from present investigation showed higher enhancement in *B. bassiana* spore productions. The comparison of present results with that from Balakrishnan et al. (2011) indicates the effect of substrate type on spore production inclination. Furthermore, spore production of a substrate media was also affected by the types and composition of nutrient supplements. Fortification of nutrient balances any nutrient deficiency in the substrates, thus providing better fungal growth and spore production. Increased spore production with the inclusion of sugars and nitrogen-containing compounds was also reported by Hallsworth and Magan (1994). Supplementation of nutrients was also reported to improve spore persistence and enhance insect infection under the conditions of low relative humidity.

Evaluation of insecticidal activity under simulated field condition

The substrate (fungal growth on rice husk with nutrient supplementation) when inoculated with experimental soil medium and test larvae of *M. domestica,* showed variable level of larval mortality. The larval mortality was 19.0 % (1st week), 21.3 % (2nd week), 26.1 % (3rd week), 32.2 % (4th week), 42.3 % (5th week), 43.6 % (6th week), 30.6 % (7th week), and 37.7 % (8th week). The initial increase in larval mortality could be due to increased spore count in the experimental medium. It is speculated that, residual substrate (rice husk) as well as soil in the experimental medium, act as growth medium for *B. bassiana*, supporting its spore production. A similar study based on simulated field condition involving livestock bedding was conducted by Watson et al. (1995). They evaluated the pathogenicity of *B. bassiana* strains (P89 and L90) against larvae of *M. domestica*, showing mortality of 56 (L90) and 48 % (P89) at the fungal dose of 1×10^{10} spore/cm^3. In their study, it was demonstrated that condition in bedding mixture supported sporulation and persistence of *B. bassiana* spore. Persistence of *B. bassiana* spore in bedding environment could provide inoculum for subsequent infection to insect larvae. However, with increase in time period, due to exhaustion of nutritive component, saturation in sporulation may occur. Moreover, an experimental scheme containing manure is accompanied by the release of CO_2 gas, thus leaving hydroxide ion in solution and subsequent elevation in pH of the system. With increase in pH in the bedding mix, fungistatic activity of soil increases, limiting growth of *B. bassiana* mycelia. The reduction in virulence may also be attributed to diminution in infectivity of aged spore compared to the newly formed one (Watson et al. 1995). The initial low mortality of insect larvae in this study may also stem from time required for acclimatization between *B. bassiana* growth system and experimental soil medium.

Mass production processes directly influence the cost, shelf life, virulence, and field efficacy of entomopathogenic fungi. Moreover, production medium has a significant impact on the attributes of resulting propagules, such as biocontrol efficacy, desiccation tolerance, and persistence of fungal pathogens. This study optimized various agricultural by-products as substrate medium in SSF study in expectation of their utilization in mass multiplication of ENPF, *B. bassiana*. Several of the agricultural wastes; such as rice husk, wheat bran and tea waste supported excellent sporulation in *B. bassiana*, showing potentiality to be used as substrate of choice for mass production of this fungi. Addition of nutrient supplements to the substrates showed slight (pongamia seed cake) to significant (rice husk, tea leaf waste) increase in spore production, revealing further possibility of improvement in SSF output. The results from this study are expected to pave the way towards the commercialization of isolated *B. bassiana* strain.

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Compliance with ethical standards

Conflict of interest Authors' declare that the present research work does not have any kind of financial, academic, commercial, political or personal conflicts with any institution or individual.

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