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Evaluation of locally available substrates for conidial biomass production of *Beauveria bassiana* MCC0044 employing Solid Substrate Fermentation

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Abstract: The *Beauveria bassiana* (MCC0044) is a unique strain with a wide range of activity against the insect pest. Various agricultural byproducts were attempted to get high Yield and also maximize the production at low cost. Corn, Wheat, Rice, Pearl millet and Sugarcane bagasse were evaluated for mass production without adding extra nutrients. Among all substrates, Rice supported maximum spore production (8.13×10^9 conidia/g of substrate) for 10 days at 28±2°C, under natural day light.

Key words: *B. bassiana*, *Helicoverpa armigera*, Solid substrate Fermentation , Agricultural by-products and conidia production.

I. Introduction

The growing demand for reducing chemical inputs in agriculture and increased resistance to insecticides have provided great impetus to the development of alternative forms of insect-pest control. Entomopathogenic fungi are potentially the most versatile biological control agents due to their wide host range. These fungi comprise a diverse group of over 90 genera with approximately 750 species, reported from different insects (**Rai *et al*., 2014**).

B. bassiana, as most entomopathogenic fungi in the Hypocreales, can disperse from sporulating cadavers (**Gottwald and Tedders, 1982; Long *et al*., 2000; Shah and Pell, 2003**), as infections in migrating insect hosts (**Feng *et al*., 2004**) or by infectious conidia on wind currents (**Hajek, 1997**). *B. bassiana* has potential control on the growth of insects like White fly, Thrips, Aphids and Mealy bugs. Growth of *B. bassiana* requires conditions of prolonged high moisture for the airborne and water borne spores to germinate. When ready to produce the spores, the fungi grow out of the body.

The spores appear Chalky white, i.e white powdery conidial masses. *B. bassiana* can be produced *in vitro* by Solid-state fermentation (SSF) and Submerged fermentation (Smf). SmF is usually faster and easier to control system parameters however volumetric spore productivity by SmF is relatively slow compared to SSF. SSF enables aerial conidia to be produced which are similar to those produce naturally on the surface of insect cadavers and are superior to mycelia and blastospores produced under SmF conditions (**Feng *et al*., 1994; Wraight *et al*., 2001; Roberts and St. Leger, 2004**). The conidia produced by *Beauveria sp.* can be used directly as natural granules or extracted through sieving and formulated as powder, granules or oil concentrate or any other suitable formulation depending on the target insect pest (**Yadav *et al*., 2013**)

Solid-state fermentation (SSF) has shown tremendous potential in effective utilization and value addition of agro industrial byproducts (**Pandey *et al*., 2000a**). Development of simple and reliable production system follows the basic multiplication procedures of submerged liquid fermentation for the production of blastospores, which are short lived, and hydrophilic (**Romback, 1989**) or solid state fermentation (**Rousson *et al*., 1983**) for the production of aerial conidia. However, the most viable mass production technologies include making use of a biphasic strategy in which the fungal inoculum is produced in liquid culture, which is further utilized for inoculating the solid substrate(s) for conidia production (**Burges and Hussey, 1981**).

The use of agricultural residues or by-products such as Cassava bagasse, Coffee husk and Pulp, Sugar cane bagasse make SSF even more interesting as it can supply good efficiency and stability of the final product (**Roussos *et al*., 2000**). Solid state fermentation (SSF) is a suitable technological process for the mass production of biological control agents (BCAs). Conidiospores produced by SSF are cost-effective and present good stability and viability.

II. Materials and Methods

Source of cultures and its maintenance

The *B. bassiana* strains were isolated from the dead cadavers of *H. armigera* collected from Maize crop at Bowrampet village, Medak district. The cultures were labeled them as RSBB6278 and RSBB0005. Fungal mycelia on dead cadavers were touched by a sterile needle and streaked onto Chloramphenicol emended potato dextrose agar plates and grown at 28^0C for 7 days. Then, conidia from these plates were harvested and stored in

20% glycerol at -40^{0}C as frozen stock culture. For further studies the *B. bassiana* isolates were maintained on PDA.

Preparation of seed inoculum

The *B. bassiana* culture was grown on PDA for 7 days at 28^{0}C. After complete sporulation, the conidia were harvested by adding 10 ml of sterilized water with 0.02% Tween -80 to each plate. Then, the fungal conidia were scraped from each plate and collected into 10 ml centrifuge tubes and vortexed for 5-10min to separate conidia from mycelia and produce a homogenous suspension. Conidial suspension was filtered through double layered cheese cloth, to get a clear conidial suspension and diluted to get a required concentration of 1X 10^8 conidia/ml.

Insect Bioassay

Two isolates of *B. bassiana* conidial suspension was sprayed on Maize leaves at 1.0 x 10^8 conidia/ml and taken into the petridishes. Then the second instar larvae of *H. armigera* were introduced on the surface of the treated Maize leaves. A total of 30 larvae were used for each isolate @10 larvae/replication. Three replications were maintained for each treatment. Control Maize leaves was sprayed with only sterilized water. The plates were incubated at 27±1°C. Percent mortality of *H. armigera* was recorded from forth day after treatment until 11th day. Mycelial growth and sporulation of *B. bassiana* were observed on dead cadavers.

Studies on morphological characteristics of *B. bassiana*

For studying colony characteristics of *B. bassiana*, culture was inoculated on PDA medium. The plates were then incubated at 28^{0}C for 10 days. Both Macroscopical and Microscopical characters were assessed when they were 10 days old. Colony colour was observed by naked eye and the culture on the slide mounts was examined under Phase contrast microscope (Nikon Eclipse E-200) under 400 X magnification.

Sample preparation for scanning electron microscope

The *B. bassiana* culture was grown on PDA plates for 5 days at 28^{0}C and the culture was placed on specimen stubs with double sided adhesive tape and sputter coated with gold - palladium in a JFC- 1600 autofine coater. Samples were then scanned by the JSM5600JEOL SEM operated at 5-10 KV. Pictures were taken at appropriate magnification and printed in the required size. At least 20 measurements were made on the morphological characters studied under light and SEM microscopy.

Molecular characterisation of *B. bassiana*

The *B. bassiana* isolate was confirmed at species level through molecular characterization.

DNA extraction and gene sequencing

The fungus was derived from single conidial spore grown on SDAY plates. To do this, conidia from culture on SDAY plate were suspended in 0.1% tween 80 up to 10^5 conidia ml^{-1} and were plated on Potato Dextrose Agar (PDA) (Merck, Germany) and incubated for 2 days at 25°C under 12:12 photoperiod. At the end of the incubation period, single colony was used to inoculate into 250 ml flask containing 100 ml Potato Dextrose Broth (PDB) (Merck, Germany). Liquid culture was shaken at 250 RPM at 28ºC for 1 - 2 weeks. After the incubation, mycelia were collected by filtering, frozen into liquid nitrogen, crushed with mortar and 50 mg fungal mycelia were used for DNA extraction. Total genomic DNA was extracted using Nucleospin Plant kit (50 preps). Isolated DNAs were stored at -20ºC until use.

The ITS1-5.8S-ITS2 region of the nuclear rRNA-complex was examined for the isolate to confirm strain identification. Oligonucleotide primers ITS4 (5'– CTCCGCTTATTGATATGC–3') as reverse primer and ITS5 (5'– GGAAGTAAAAGTCGTAACAAGG–3') as forward primer were used for ITS PCR-amplification (White *et al*., 1990). PCR amplifications were performed in a total volume of 50 µl, which included 5 µl 10X *Taq* DNA polymerase reaction buffer, 200 µM of each dNTPs, 50 pmol each of the opposing amplification primers, 2,5 unit *Taq*-DNA polymerase (Fermentase), and 50 ng genomic DNA. Thirty-five cycles were conducted in thermocycler (Eppendorf, master cycler gradient, Hamburg, Germany): After the denaturation at 95ºC for 5 min, 95º C for 1 min, 55ºC for 55 s, 72ºC for 2 min, with a final extension at 72ºC for 10 min.

Approximately 1200 bp fragment of EF1-α was also amplified by using primers (EF1T (5'-ATGGGTAAGGARGACAAGAC-3') and 1567R (5'-ACHGTRCCRATACCACCSATCTT-3') (**Rehner & Buckley, 2005**). PCR conditions were adapted essentially as described by Rehner & Buckley (2005). PCR products were separated on 1% Agarose gel and visualized under UV light. Amplification products were extracted from Agarose gels with the QIAquick Gel Extraction Kit (50) and sent to Oscimum Biosolutions (Hyderabad, India)) for sequencing. Obtained sequences were used to carry out BLAST searches by using the

NCBI GeneBank database to confirm isolate identification. The strain has been deposited as *Beauveria bassiana* MCC0044, Microbial culture collection, Pune, Maharashtra, India -411 021.

Evaluation of solid substrates for mass production of *B. bassiana*

In the present work, different solid substrates were evaluated to select the best substrate for conidiospore production by *B. bassiana*. The experiments were carried out in Erlenmeyer flasks (1.0 L capacity) containing 100g of substrate with moisture content 55-60% was maintained. Fermentation was carried out without any external nutrient addition. Substrates were sterilized at 121^0C for 20min and cooled at room temperature before inoculation. The substrate was inoculated with 5 ml. conidial suspension of *B. bassiana* (1.0 x 10^6 cfu/ml.) separately and incubated at 28^0C for 12 days. After the end of incubation, 1.0 gm of fermented substrate was mixed with 9 ml of sterilized distilled water with 0.01% Tween -80 and serially diluted up to 10^-7 and TVC was done using standard plate count assay then incubated at 28 Deg.C for 4-5 days . Results were expressed as number of conidia per gram of substrate. Three replications were maintained for each substrate.

Lists of substrates used for SSF

1. Corn
2. Wheat
3. Rice
4. Pearl millet
5. Sugar cane bagasse

III. Results and Discussion:

Bioefficacy of *B. bassiana* against *H. armigera* under laboratory conditions

The strain RSBB6278 of *B. bassiana* caused 100% mortality on *H. armigera* larvae under laboratory conditions. The results (Table 1.0) shows that from the 4^{th} day to 9^{th} day, strain RSBB6278 was statistically more pathogenic than strain RSBB0005 ($F = 115.89$; $df = 2, 27$; $P = 0.001$). On 11^{th} day, 100% mortality was recorded by both strains. Mycelial growth and sporulation of *B. bassiana* was observed on dead cadavers of *H. armigera*, indicating and confirming the mortality was indeed caused by *B. bassiana*.

The LT_{50} for strain RSBB6278 is 5.1 day in the laboratory condition and day 6 for strain RSBB0005. Though LT_{50} value was slightly lower for strain RSBB0005, but it eventually killed 100% of *H. armigera* larvae within 11 days. Over all sluggishness was observed in the infected larvae. Blackening of the body parts were also observed after treated with fungus and sporulation was observed primarily on the mouth parts, thorax and eventually spreading to other parts of the insect cadaver (Plate 1.0).

Table 1.0 Mortality of *H. armigera* infested with *B .bassiana*

Strains	Days after treatment and percent mortality							
	4	5	6	7	8	9	10	11
RSBB6278	38±8.7a	56±8.8a	72±6.8a	88±7.2a	98±4.2a	100±0a	100±0a	100±0a
RSBB0005	27±5.2b	44±12.5b	54±5.1b	69±4.3b	84±6.8b	87±6.2b	97±4.8b	100±0a
Control	1±3.1c	1±3.1c	1±3.1c	1±3.1c	1±3.1c	1±3.1c	1±3.1c	1±3.1b

Values in the same column followed by same letter are not statistically different at 5 % level

Plate 1.0 Morphological abnormalities in 2rd instar larvae of *H. armigera* treated with *B. bassiana*

(Control)

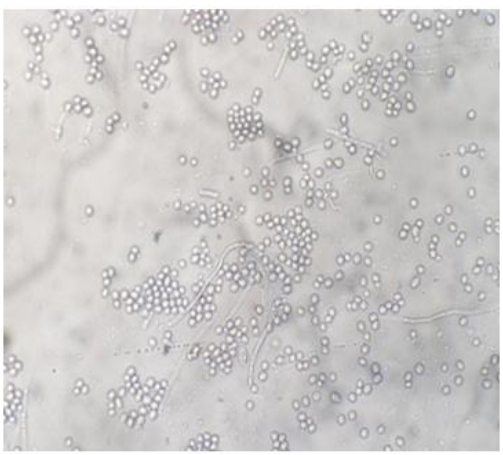
Brown deformed larvae after treatment with *B. bassiana*

Mycelial growth of *B. bassiana* on dead cadaver of *H. armigera*

Colony characteristics of *B. bassiana*

Colony white, hyphae cylindrical 3.5 µm wide hyaline, septate, conidiophores single or branched, abundant arising from vegetative cells globose to flask shaped (3-5 x 3-7 µm) with well developed conidiophore up to 20 µm long and 1-1.5 µm wide, conidia were borne at thread like apex of the phialide on a series of zig-zag branchlets, conidia globose (1-4 µm) to oval (1.5-5 x 1.0-3.0 µm), smooth and hyaline (Plate 1.1).

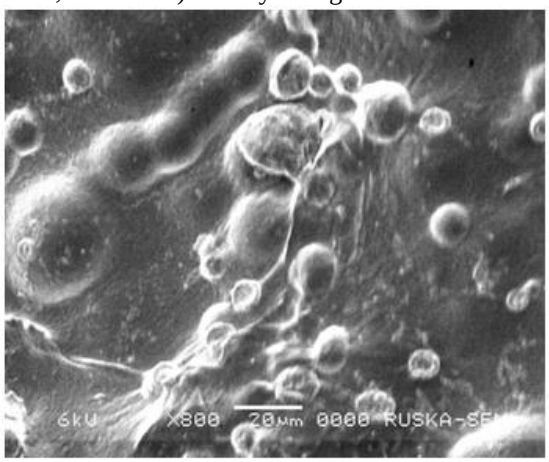
Plate 1.1 Colony characteristics of B. bassiana. A) White colour colony on PDA media B) Observation of Aerial conidia under 400X magnification

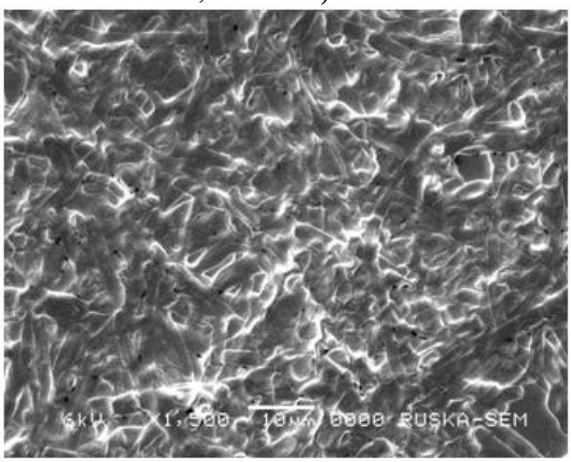


Morphological observations of *B. bassiana* by SEM

In this study observation of mycelium and conidia was performed on specimens fixed by double sided adhesive tape and sputter coated with gold - palladium in a JFC- 1600 autofine coater. The formation of fungal conidia and mycelia (amplification of X800 and X1500) were observed after 120 h of inoculation of *B. bassiana* on PDA slants. (Plate 1.2).

Plate 1.2 Morphological characteristics of *B. bassiana* A&B. The formation of fungal conidia (amplification of X800, after 120h) and mycelia growth of *B. bassiana* (amplification of X1500, after 120h)





Molecular characterization

Molecular characterization of *B. bassiana*

The fungal isolates were discriminated mainly by phylogenetic analysis based on PCR amplification and DNA sequencing studies (**Davolos D *et al.*, 2007**). Quick and simple methods for determining the species composition of fungal communities based on sequencing of particular regions of the fungal genome have proven a reliable alternative to traditional methods. Through targeted amplification of specific regions of the fungal genome via the polymerase chain reaction (PCR), researchers can now quickly and accurately identify all fungal species present within a community (**Horton & Bruns, 2001**).

The effective strains of *B. bassiana* was confirmed at species level through molecular characterization. Amplification with universal 18s primers gave an unambiguous sequence of 565bp for *B. bassiana* at the immediate 5' termnus of the 18s gene. Amplified *B. bassiana* sequences was initially blasted in Gene Bank (http://www.ncbi.nlm.nih.gov) to predict the family and/or order for each isolate. Both the strains showed 99% genetic similarity with available sequences of *B. bassiana* in gene bank (Table 1.1).

Sequence of *B. bassiana* strain

CCTTTTGGGGACAGCGGAGGGACATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAACCTACCTAT
CGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCAGCGGCCCGCCGGGGACCTC
AAACTCTTGTATTCCAGCATCTTCTGAAACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATC
CAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTCGACCTCCCCTTGGGGGGGTCGGCGTTGGGGACCGGCAGCACACCGCCG
GCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTAATACAGCTCGCACCGGAAC
CCCGACGCGGCCACGCCGTAAAACACCCAACTTCTGAACGTTGACCTCGAATCAGGTAGGACTACCC
GCTGAACTTAAGCATATCAATAAGCCGGGAGGAA

Table 1.1 Sequence producing significant alignments of *B. bassiana*

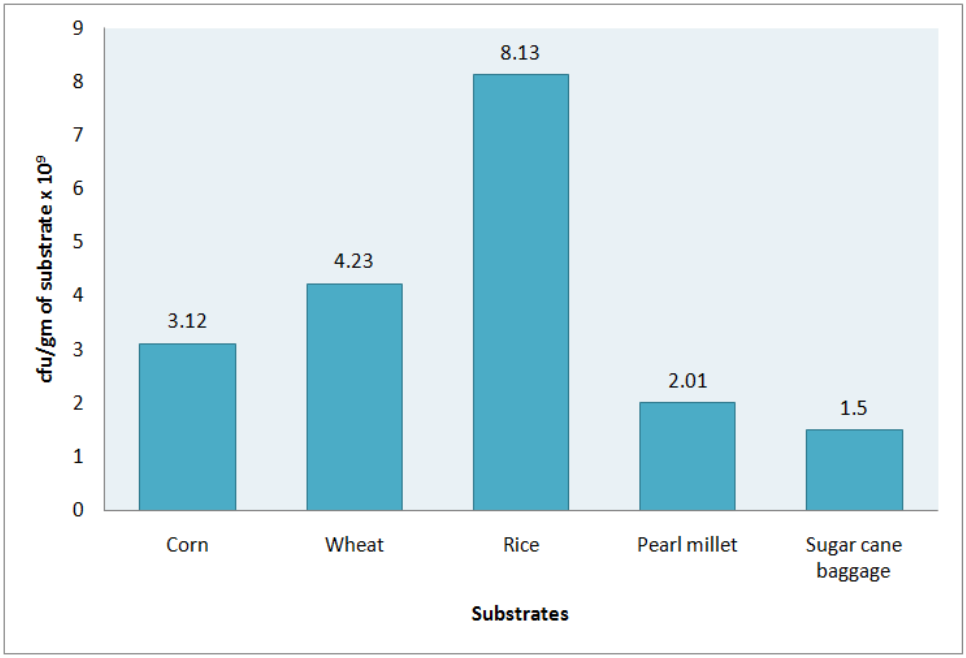
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU272502.1	Beauveria bassiana isolate 2698 18S ribosomal RNA gene, p	1014	1014	98%	0.0	99%
AB576868.1	Beauveria bassiana genes for 18S rRNA, ITS1, 5.8S rRNA, I	1013	1013	98%	0.0	99%
EU573330.1	Beauveria bassiana strain MRCIF3 internal transcribed space	1013	1013	99%	0.0	99%
EU821491.1	Beauveria bassiana isolate 2486 18S ribosomal RNA gene, p	1009	1009	98%	0.0	99%
EU821480.1	Beauveria bassiana isolate 2475 18S ribosomal RNA gene, p	1009	1009	98%	0.0	99%
EU821470.1	Beauveria bassiana isolate 2541 18S ribosomal RNA gene, p	1009	1009	97%	0.0	99%
EU272504.1	Beauveria bassiana isolate 2700 18S ribosomal RNA gene, p	1009	1009	98%	0.0	99%
EU821495.1	Beauveria bassiana isolate 2543 18S ribosomal RNA gene, p	1007	1007	96%	0.0	99%
EU821479.1	Beauveria bassiana isolate 2474 18S ribosomal RNA gene, p	1007	1007	96%	0.0	99%
EU272506.1	Beauveria bassiana isolate 2702 18S ribosomal RNA gene, p	1007	1007	96%	0.0	99%

Evaluation of solid substrates for mass production of *B. bassiana*

B. bassiana was tested for their optimum growth on various solid substrtaes. Among the substrates tested, significantly more conidia (8.13 x 10^9 cfu/g of substrate) recorded in Rice, followed by Wheat (4.23 x 10^9 cfu/g of substrate). Other substrates *viz.*, corn (3.12 x 10^8 cfu/of substrate), Pearl Millet (2.01 x 10^9 cfu/g of substrate), Sugarcane bagasse (1.5 x 10^9 cfu/g of substrate) was observed (Fig 1.0). Sharma *et al*., (2002) who found rice to be the suitable media for mass culturing of *B. bassiana* and the cereal was also suitable for the mass production of other deuteromycete fungi due to high starch content.

Rice was the best substrate for mass multiplication of *B.bassiana* which may be due to the presence of rich source of carbon and adequate source of nitrogen. It has been reported that the rice grain consists of 75-80% starch, 7% protein that enhanced the growth and sporulation (**Oko *et al.*, 2012**).

Fig 1.0 Comparison of spore production of *B. bassiana* on different solid substrates



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