

Growth, Sporulation and Biomass production of native entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin on a suitable medium

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Abstract

Potato Dextrose Agar, Saboured Dextrose Agar, Saboured Dextrose Yeast Agar, Malt Extract Agar, Czepeck Dox Agar Media were evaluated for mass production of the *Beauveria bassiana* (Balsamo) Vuillemin strains. Results show Potato Dextrose Agar is the most suitable substrate for examined fungi as it yielded highest conidial count (6.5×10^6 conidia/g) and colony forming unit (8×10^8 cfu/g) where as Saboured Dextrose Agar recorded minimum conidial count (12.2×10^{12} cfu/g) and colony forming unit (9.5×10^9 conidia/g). This study also shows the preparation of formulations of *B. bassiana* on best substrate and its viability at different storage temperature. Results show Talc based formulation at refrigeration temperature as the best and the cells were viable up to three months of storage.

Keywords: *Beauveria bassiana*, formulations, temperature

Introduction

Beauveria bassiana Bals. Vuill. (Ascomycota: Sordariomycetes) is one of the important entomo-pathogenic fungus used as bio control agent. Among fungal bio pesticides, it is the potential microbial alternative to chemical insecticides as this strain grows on variety of substrates, has high virulence, transcuticular penetration, broad host range and is safe to human beings, animals and the environment (McCoy, 1990) [10]. *B. Bassiana* is an entomopathogenic fungus that attacks a wide range of agricultural pests by contact and penetration (Feng *et al.* 1994; Nadeau *et al.* 1996) [5, 13]. The success of microbial control of insect pests depends not only on the isolation, characterization and Pathogenicity, but also on the successful mass production (Bhadauria *et al.* 2012) [1]. Species of genus *Beauveria* (*B. bassiana* and *Beauveria brongniartii*) are produced by different companies to control various insect pests including termites, whiteflies, aphids, maize borer and other insects (Strasser *et al.*, 2000; Wraight *et al.* 2001) [23, 24]. Both solid and liquid state fermentation are used for the mass production of *B. bassiana* (Pham *et al.*, 2010) [15]. The liquid phase provides active growing mycelia and blastopores, while the solid phase provides support for development of the dry aerial conidia (Lomer *et al.* 1997) [8]. The conidia produced by *Beauveria* 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) [25].

The Mycoinsecticide used as biological control agents for insect species has increased the global attention during last few decades. The development of a suitable formulation is

essential for successful utilization of Mycoinsecticide. Biological and physical properties of the formulation must remain viable for at least one year for commercialization to take place (Couch and Ignoffo, 1981) [3]. According to Moore *et al.* (2000) [11], fungal spores are living organisms and their viability diminishes with time depending on environmental conditions. For the commercial production of fungal spores, there is need to obtain an ideal, cheap and highly productive culture medium. The shelf life of microbial pesticide is an important factor for effective insect control (Consolo *et al.* 2003) [2]. So, the present study was conducted to identify best agricultural substrate and carrier for production of *B. bassiana* formulations and evaluation of the storage temperature on its viability.

Materials and Methods

The present research was conducted in the Bio control Laboratory of Department of Crop Science and Entomology, College of Agricultural Biotechnology and College of Agriculture, Loni.

Microorganisms

The isolation of *B. bassiana* was used in this study. These isolates, *B. bassiana* (Accession No 3319), were obtained from National Fungal Cultural Collection of India (NFCCI), Pune. These cultures were maintained at 4°C in potato dextrose agar (PDA) slants and sub cultured after every three months.

Substrates

Five solid substrates such as Potato Dextrose Agar, Saboured

Dextrose Agar, Saboured Dextrose Yeast Agar, Malt Extract Agar, and Czepeck Dox Agar Media were selected to study the suitability of different substrates for mass production of *B. bassiana*. Each Media Were prepared separately by observing the contents in it and autoclaved at 121°C for 30 min. Each media were prepared separately. After autoclaving, the substrates were cooled at room temperature in laminar Air Flow with U.V light ON and use. These substrates were inoculated individually with all the three isolates of *B. bassiana*.

Inoculum

B. bassiana isolates were produced in Erlenmeyer flasks (250 ml) containing 100 ml of PDA, incubated at 25°C for 15 days under static conditions. The conidia suspension was prepared by adding 40 ml of sterilized distilled water containing Tween 80 (0.1%) and stirred for 30 min on a magnetic stirrer. The spores were counted in Neubauer's chamber.

Growth of *B. bassiana* on different solid substrates

The solid substrate mass production provides a physical support for the fungus to produce aerial conidia the infective propagules which were best suited for storage and formulations. Different solid substrates like Potato Dextrose Agar, Saboured Dextrose Agar, Saboured Dextrose Yeast Agar, Malt Extract Agar, and Czepeck Dox Agar Media were used for estimating the sporulation of different isolates of *B. bassiana* and its viability at 25±2°C. 1 ml of the spore suspension of fungi was aseptically inoculated into each Conical Flask which was incubated at 25±2°C at relative humidity ≥50% for 15 days (Sahayaraj and Namasivayam, 2008 [20]; Rajanikanth *et al.* 2010) [18]. To avoid clumping, after a week of inoculation. After incubation of 20 days, 1 g of homogenous samples were taken from each Conical Flask and transferred to 9 ml of sterilized distilled water containing Tween 80 (0.01%) solution. The suspension was filtered through sterilized double layered muslin cloth. Counting of spores were made after the serial dilution of the suspension using double ruled Neubauer haemocytometer for determining the number of conidia in 1 g of the sample by using the following formula:

Conidia/g = Average number of cells per square × dilution factor × 10⁹

To determine colony forming unit per gram (cfu/g), prepared serial dilutions were placed on PDA medium (1 ml/plate). The plates were gently rotated for uniform spreading of spore suspension and incubated at 25±2°C. Each treatment had three replications. The cfu counts were recorded on 4th day after plating and calculated by using the following formula:

$$\text{Cfu/g} = \frac{\text{Average number of colonies} \times \text{dilution factor}}{\text{Volume Plated}}$$

Formulation of *B. bassiana* conidia with talc and charcoal

To prepare dry formulations, talc powder and charcoal were used as carriers. Polyethylene bags showing full sporulation of *Beauveria* growth were cut open and grains were crushed to fine powders which were passed through sieve of 80 µm mesh size. This powder was dried at room temperature (28±2°C) under aseptic conditions. 100 g of dried conidial powder of *B. bassiana* isolates were mixed with carrier material. (Talc and charcoal) in proportion of 1:2 and dried under aseptic conditions for 24 h. Carboxyl methyl cellulose (1%) was added. After thorough mixing these formulations were packed into sterilized polyethylene bags and stored at 4 and 25°C for three months. The viability of *B. bassiana* was calculated by determining the number of colony forming units by standard pour plate method, 1 g portions was homogenized in 9 ml of sterilized water, serial dilutions were prepared and aliquots of the dilutions were placed on PDA after every fortnight (Prasad and Rangeshwaran, 1999) [16].

Formulation of *B. bassiana* conidia with oil

Conidia of *B. bassiana* isolates were also formulated in sunflower oil. Oil was initially blended with Tween 80 in 9:1 ratio and 45 ml of mixture was blended with 15 g of conidia to get the Suspension concentrates (SC) formulation and stored (Devi and Hari, 2009) [4]. They were stored at 4 and 25°C for three months to determine the number of colony forming units of the formulation, 1 ml portions were homogenized in 9 ml sterile water, serial dilutions were prepared and aliquots of the dilutions were placed on PDA within 15 days intervals.

The statistical analysis was done through analysis of variance (two-way ANOVA) at 5% level of significance.

Results and Discussion

Effect of substrates

Solid substrates Potato Dextrose Agar, Saboured Dextrose Agar, Saboured Dextrose Yeast Agar, Malt Extract Agar, and Czepeck Dox Agar Media exhibited significant differences in their utility to yield conidia and colony forming unit. Among these substrates, maximum mean conidial count (34.0×10⁹conidia/g) (Table 1a) and colony forming units (30.5×10⁹cfu/g) (Table 2b) was recorded in Potato Dextrose Agar. Czepeck Dox Agar Media gave minimum conidial count (11.5×10⁹conidia/g) and cfu count (9.5×10⁹cfu/g) (Figure 1). Among *B. bassiana* strains, the mean conidial count varied. The maximum mean conidial count (25.8×10⁵ conidia/g) and cfu count (22.8×10⁹cfu/g) was recorded for *B. bassiana* NFCCI 3319. The substrates which were rich in nutritional composition produced not only high spore count but also higher colony count (Rajnikanth *et al.* 2010). Hence, Potato Dextrose Agar was the best substrate followed for mass multiplication of *B. bassiana* which may be due to the presence of rich source of carbon and adequate source of nitrogen.

Table 1: conidial count of *B. bassiana* isolates cultivated on different substrates.

Substrate	Conidial count (1×10^9 conidia/g) of different <i>Beauveria bassiana</i> isolates cultivated on different substrates (Mean* \pm S.D*)					Mean
	Beauveria bassiana NFCCI 3319					
	R1	R2	R3	R4	R5	
Potato Dextrose Agar	15.00	12.28	11.02	14.58	14.26	13.428
Saboured Dextrose Agar	16.24	13.56	12.04	09.08	12.84	12.752
Saboured Dextrose Yeast Agar	15.28	14.56	12.48	10.56	06.56	11.888
Malt Extract Agar	07.08	07.90	11.23	10.80	11.97	09.796
Czepeck Dox Agar Media	05.20	07.28	09.45	01.20	09.85	06.596
Mean	11.760	11.116	11.244	09.244	11.096	
CD (0.05)	CD (5%) substrates = 1.92 CD (5%) isolates = 0.97 CD (5%) substrates \times isolates = 2.58					

*Mean = Mean of three replications ** S.D = standard deviation

Table 2: Colony forming unit (cfu) of fungal isolates cultivated on different substrates

Substrate	Conidial count (1×10^5 conidia/g) of different <i>Beauveria bassiana</i> isolates cultivated on different substrates (Mean* \pm S.D*)					Mean
	Beauveria bassiana NFCCI 3319					
	R1	R2	R3	R4	R5	
Potato Dextrose Agar	10.74	08.24	07.52	10.49	10.02	09.402
Saboured Dextrose Agar	11.84	09.56	08.35	07.48	08.97	09.240
Saboured Dextrose Yeast Agar	11.36	11.24	07.98	05.98	04.65	08.242
Malt Extract Agar	05.24	02.34	05.64	06.87	05.38	05.094
Czepeck Dox Agar Media	01.25	06.87	04.35	02.69	04.89	04.010
Mean	08.086	07.650	06.768	06.702	06.782	
CD (0.05)	CD (5%) substrates = 1.92 CD (5%) isolates = 0.97 CD (5%) substrates \times isolates = 2.58					

*Mean = Mean of three replications ** S.D = standard deviation.

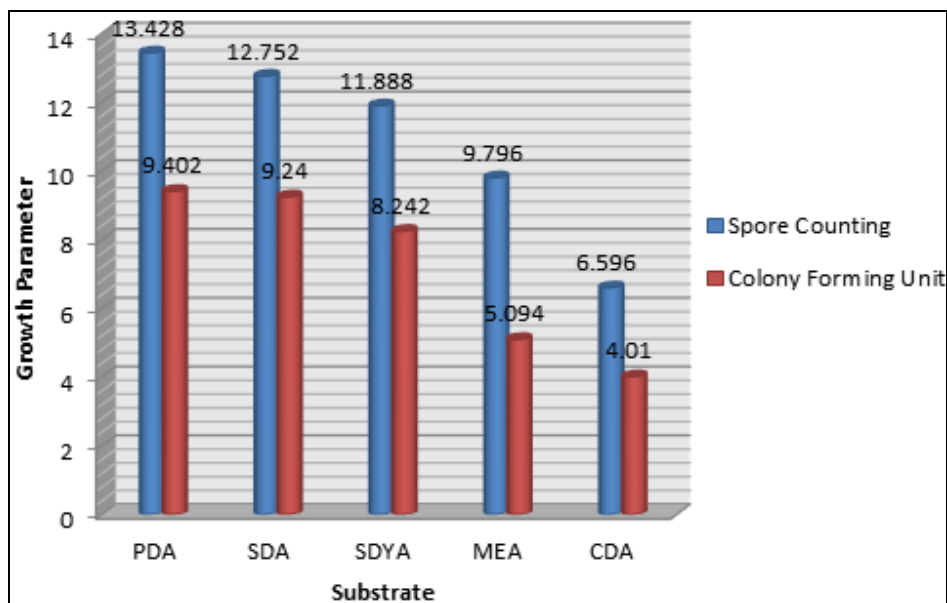


Fig 1: Conidia Count and Colony Forming Unit of *Beauveria bassiana* cultivated on different solid substrate.

It has been reported that the Potato Dextrose Agar consists of 75-80% starch, 7% protein and Saboured Dextrose Agar consists 75% starch, 25% amylase, which are rich sources of carbon and nitrogen that enhanced the growth and sporulation (Oko *et al.*, 2012) [14]. Mar and Lumyong (2012) [9] also reported that *B. bassiana* showed maximum growth on Potato Dextrose Agar. Sahayaraj and Namasivayam (2008) [20] found

Saboured Dextrose Agar to be the suitable substrate for large multiplication of *B. bassiana*. The Potato Dextrose Agar and Saboured Dextrose Agar were the weakest supportive for *B. bassiana* growth. The reason may be that medium becomes compact mass after sterilization which does not allow *B. bassiana* to ramify with the medium (Puzari *et al.*, 1997) [17]. The reason why Czepeck Dox Agar did not support the

growth of the fungus may be due to the presence of lignin (20-47%) and cellulose (30-45%) which act as binding material and results in hardness. Secondly, less nitrogen content (3.27%) and high silica content (94%) inhibits the fungal growth (Siwach and Jaipal, 2004) [22].

All used in this study, were coarsely broken to provide enough surface area to volume ratio for aeration and formation of conidia. Similarly, Karanja *et al.* (2010) [7] also investigated the effect of broken rice, maize, machicha and maize husk for mass production of *B. bassiana* isolate (Bb575) and two *Metarrhizium anisopliae* isolates (M58 and M60) and reported that enough surface provides better conditions for fungal growth, sporulation and such form of the substrate is a source of assimilable nutrients.

Effect of storage temperature on formulations

B. bassiana NFCCI 3319 biomass produced on Potato Dextrose Agar were formulated with talc, charcoal and oil. These formulations were stored at 4 and 25°C up to 90 days and their viability was evaluated fortnightly. In *B. bassiana* NFCCI 3319 formulation, maximum cfu count (44.4×10⁹cfu/g) was recorded at 4°C on 15th day of storage in talc based formulation, which was at par with count recorded initially (control) (44.6×10⁹cfu/g). This was comparably at par with the count recorded up to 90th day at the same temperature. However, significantly lower count (12.3×10⁹ cfu/g) was recorded at 25°C after 90 days of storage (Table 3).

Table 3

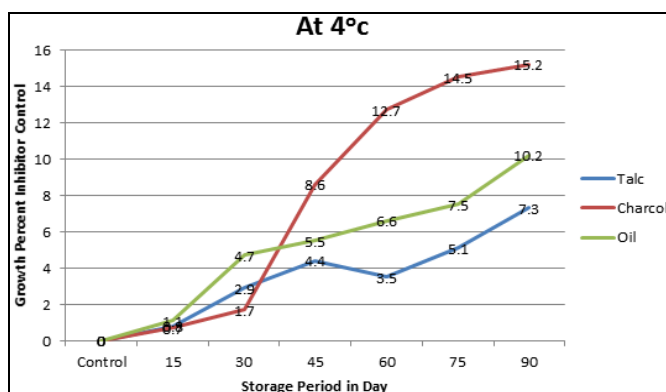
Day	Colony forming units (1×10 ⁵ cfu/g) of <i>B. bassiana</i> NFCCI 3319 (Mean±S.D*) (% decrease)					
	Talc		Charcoal		Oil	
	4°C	25°C	4°C	25°C	4°C	25°C
Control	44.02±8.02		39.3±11.20		36.1±8.18	
15	44.4 (0.8)	40.7 (10.3)	39.0 (0.7)	36.0 (8.3)	35.6 (1.1)	31.6 (12.2)
30	43.3 (2.9)	34.6 (22.2)	38.6 (1.7)	33.6 (14.5)	34.3 (4.7)	27.3 (24.1)
45	42.6 (4.4)	28.6 (35.8)	35.6 (8.6)	28.0 (28.7)	34.0 (5.5)	25.6 (24.1)
60	43.0 (3.5)	20.6 (47.5)	34.3 (12.7)	20.6 (47.5)	33.6 (6.6)	23.3 (35.2)
75	42.3 (5.1)	14.6 (67.2)	33.6 (14.5)	12.3 (68.7)	33.3 (7.5)	13.6 (62.2)
90	41.3 (7.3)	12.3 (72.4)	33.3 (15.2)	6.6 (83.2)	32.3 (10.2)	7.6 (78.8)
CD 5%	CD (5%) Temperature = 3.45					

*Mean = Mean of three replications

*S.D = standard deviation. Figure in parentheses are percentage decrease. (Decrease (%) = Initial count - final count/Initial count x 100)

Thus, from the above results discussed, we conclude that storage of these formulations at refrigeration temperature recorded minimum decrease of viable count in both talc based *Beauveria* formulations (Figure 2). As at refrigeration temperature, the fungus undergoes the stationary phase of growth and the growth rate is decreased therefore, the nutrients requirement is low, so culture remains viable for more time as compared to 25°C. This supports reported findings that decrease in temperature increases the longevity of *B. bassiana* conidia (Hong *et al.* 1997) [6]. Morley-Davies *et al.* (1995) [12] reported that too low temperature can harm the conidia of *M. anisopliae* and *B. bassiana* fungi and concluded that temperature of long-term storage should be maintained preferably between 0 and 20°C. Shimizu and Mitani (2000) [21] reported that high-temperature treatment killed *B. bassiana* conidia in oil formulations. Results of the present investigations are also similar to findings of Ramegowda (2005) [19] who reported 82.47% conidial viability of

Nomuraea rileyi formulations stored for 180 days in refrigerated condition, as compared to 63.23% under room temperature formulation with talc at 4°C.



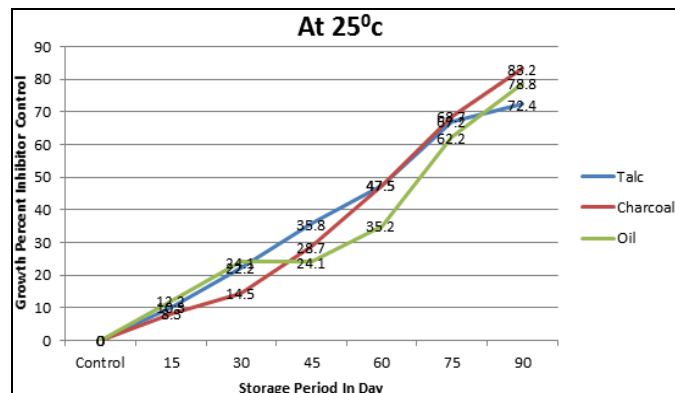


Fig 2: Growth Percent reduction over control of *B. bassiana* NFCCI 3319a) at 4°C b) at 25°C

Described experimental work showed that for mass production of *B. bassiana* formulation on solid substrate, Potato Dextrose Agar is the best substrate. Studies of effect of carriers on the formulations have shown that viability of formulations on storage was optimum in talc based formulation as compared to charcoal and oil at refrigeration temperature over the period of 90 days of storage.

References

- Bhadoria BP, Puri SA, Singh PK. Mass production of entomopathogenic fungi using agricultural products Int. Q. J Life Sci. 2012; 7:229-232.
- Consolo VF, Salemo GL, Beron CM. Pathogenicity, formulation and storage of insect pathogenic hyphomycetes fungi tested against *Diabrotica speciosa*. Bio control, 2003, 705-712.
- Couch TL, Ignoffo CM. Formulation of Insect Pathogens. In: H. D Burges (editor). Microbial Control of Pests and Plant Diseases. Academic Press, London, 1981, 621-634.
- Devi VPS, Hari PP. Identification of a virulent isolate of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin, its mass multiplication and formulation for development into a Mycoinsecticide for management of *Helicoverpa armigera* (Hubner) J Biol. Control. 2009; 23:137-44.
- Feng MG, Poprawski TJ, Khachatourians GG. Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: Current status. Bio control Sci. Technol. 1994; 4:3-34.
- Hong TD, Ellis RH, Moore D. Development of a model to predict the effect of temperature and moisture on fungal spore longevity. Ann. Bot. 1997; 79:121-128.
- Karanja LW, Phiri NA, Oduor GI. Effect of different solid substrate on mass production of *Beauveria bassiana* and *Metarrhizium anisopliae* entomopathogens. In: 12th KARI Biennial Science Conference proceedings, 2010, 789-97.
- Lomer CJ, Prior C, Kooyman C. Development of *Metarrhizium* sp. for the control of grasshoppers and locusts. In: Goettel MS, Johnson DL, editors, Microbial Control of Grasshoppers and Locust, Memoirs of the Entomological Society of Canada. 1997; 71:265-286.
- Mar TT, Lumyong S. Conidial production of entomopathogenic fungi in solid state fermentation. K. K. U. Res. J. 2012; 17:762-768.
- McCoy CW. Entomogenous fungi as microbial pesticides. In: Baker RR and Dunn PE (Eds) New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases. New York: Alan R. Liss. 1990, 139-59.
- Moore D, Lord JC, Smith SM. Pathogens. In: B Subramanyam and D W Hag strum (eds.), Alternatives to pesticides in stored-product IPM. Kluwer Academic Publishers, Boston MA. 2000, 193-225.
- Morley-Davies J, Moore D, Prior C. Screening of *Metarrhizium* and *Beauveria* spp. conidia with exposure to simulated sunlight and a range of temperatures. Mycol. Res. 1995; 100:31-38.
- Nadeau MP, Dunphy GB, Boisvert JL. Development of *Eryniaconica* (Zygomycetes: Entomophthorales) on the cuticle of the adult black flies *Simulium Rostratum* and *Simulium decorum* (Diptera: Simuliidae) J Invertebrate. Pathol. 1996; 68:50-58.
- Oko AO, UbiBE Efisue AA, Dambaba N. Comparative Analysis of the Chemical Nutrient Composition of Selected Local and Newly Introduced Rice Varieties Grown in Ebonyi State of Nigeria. Int J Agric. For. 2012; 2:16-23.
- Pham TA, Kim JJ, Kim K. Optimization of Solid-State Fermentation for Improved Conidia Production of *Beauveria bassiana* as a Mycoinsecticide. Microbiology. 2010; 38:137-43.
- Prasad RD, Rangeshwaran R. Granular Formulation of *Trichoderma* and *Gliocladium* spp. in Bio control of *Rhizobium solani* of Chickpea J Mycol. Plant. Pathol. 1999; 29:222-26.
- Puzari KC, Sharma DK, Hazarika LK. Medium for mass production of *Beauveria bassiana* (Balsamo) Vuillemin J Biol. Control. 1997; 11:97-100.
- Rajanikanth P, Subbaratnam GV, Rahaman SJ. Evaluation of economically viable substrates for mass production of *Beauveria bassiana* (Balsamo) Vuillemin J Biol. Control. 2010; 24:322-26.
- Ramegowda GK. Aerobiology, Epizootiology and Utilization of *Nomuraea rileyi* (Farlow) Samson [PhD Thesis]. Dharwad, India: University of Agricultural Sciences, 2005.
- Sahayaraj K, Namasivayam SKR. Mass production of entomopathogenic fungi using agricultural products and by products. Afr. J. Biotechnology. 2008; 7:1907-10.
- Shimizu S, Mitani T. Effects of temperature on viability of conidia from *Beauveria bassiana* in oil formulations. Jap J Appl. Entomol. Zool. 2000; 44:51-53.
- Siwach P, Jaipal S. Evaluation of industrial wastes for mass production of *Beauveria bassiana* and their effects against *Chilo auricillus*. Ann. Plant Prot. Sci. 2004; 12:193-95.
- Strasser H, Vey A, Butt TM. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarrhizium*, *Tolypocladium* and *Beauveria* species? Bio control Sci. Technol. 2000; 10:717-35.

24. Wraight SP, Jackson MA, De Kock SL. Production, stabilization and formulation of bio control agents. In: Butt TM, Jackson C and Magan N (editors). *Fungi as Bio control Agents, Progress, Problems and Potential*. CABI Publishing, New York, 2001, 253-88.
25. Yadav S, Tandan N, Kumar K. Mass production of entomopathogens *Beauveria bassiana* and *Metarrhizium anisopliae* using rice as a substrate by diphasic liquid-solid fermentation technique. *Int J Adv. Biol. Res.* 2013; 3:331-35.