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Vol. 8(34), pp. 3164-3170, 20 August, 2014
DOI: 10.5897/AJMR2013.6501
Article Number: A0FCBA347090
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Conidial production of *Beauveria bassiana* on agricultural products and effect of storage temperature on its formulations

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Received 13 November, 2013; Accepted 6 May, 2014

Rice, wheat, maize, sorghum, mini potato tubers, rice bran and rice straw were evaluated for mass production of three *Beauveria bassiana* (Balsamo) Vuillemin strains. Results show rice as the most suitable substrate for examined fungi as it yielded highest conidial count (31.8×10^5 conidia/g) and colony forming unit (30.5 × 10^5 cfu/g) whereas rice straw recorded minimum conidial count (12.2×10^5 cfu/g) and colony forming unit (9.5×10^5 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 upto three months of storage.

Key words: *Beauveria bassiana,* formulations, temperature.

INTRODUCTION

Beauveria bassiana Bals. Vuill. (Ascomycota: Sordariomycetes) is one of the important entomo-pathogenic fungus used as biocontrol agent. Among fungal biopesticides, 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). *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). 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). 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). Both solid and liquid state fermentation are used for the mass production of *B. bassiana* (Pham et al., 2010). The liquid phase provides active growing mycelia and blatospores, while the solid phase provides support for development of the dry aerial conidia (Lomer et al., 1997). The conidia produced by *Beauveria* can be used directly as natural granules or extracted through sieving and formulated as powder,

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granules or oil concentrate or any other suitable formulation depending on the target insect pest (Yadav et al., 2013).

The mycoinsecticides 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 mycoinsecticides. Biological and physical properties of the formulation must remain viable for at least one year for commercialization to take place (Couch and Ignoffo, 1981). According to Moore et al. (2000), 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). 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 Biocontrol Laboratory of Department of Entomology, College of Agriculture, Punjab Agricultural University, Ludhiana.

Microorganisms

Three isolates of *B. bassiana* were used in this study. These isolates, *B. bassiana* (Accession No 6291), *B. bassiana* (Accession No 2028), *B. bassiana* (Accession No 4495) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh. These cultures were maintained at 4°C in potato dextrose agar (PDA) slants and subcultured after every three months.

Substrates

Seven solid substrates such as rice, sorghum, wheat, maize, mini potato tubers, rice straw and rice bran were selected to study the suitability of different substrates for mass production of *B. bassiana*. One hundred gram of each substrate was washed, drained and soaked in water overnight except rice which was soaked for 3 h before starting the experiment. The excess water was drained by decanting and shade drying for half an hour to remove the excess moisture. The substrates were packed separately in individual autoclavable polythene bags which were plugged with cotton wool and autoclaved at 15 psi for 50 min. After autoclaving, the substrates were cooled at room temperature and preserved in the refrigerator till further 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 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 rice, sorghum, wheat, maize, mini potato tubers, rice straw and rice bran 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 polythene bag which was incubated at 25±2°C at relative humidity ≥50% for 15 days (Sahayaraj and Namasivayam, 2008; Rajanikanth et al., 2010). To avoid clumping, after a week of inoculation, the bags were shaken vigorously to separate the grains and to break the mycelial mat. After incubation of 20 days, 1 g of homogenous grain samples were taken from each polythene bag 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 $\times 10^4$

To determine colony forming unit per gram (cfu/g), prepared serial dilutions were plated 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 powder 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. Carboxy 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 Rangeshwarran, 1999).

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). 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.

Table 1a. Conidial count of *B. bassiana* isolates cultivated on different substrates.

Substrate	Conidial count (1×10^5conidia/g) of different *Beauveria bassiana* isolates cultivated on different substrates (Mean*±S.D*)			Mean
	Beauveria bassiana MTCC 2028	*Beauveria bassiana* MTCC 6291	*Beauveria bassiana* MTCC 4495	
Rice	40.5±1.94	36.4±0.87	25.1±1.10	34.0
Wheat	17.7±1.20	25.8±2.35	14.4±2.20	19.3
Sorghum	32.1±1.90	32.6±1.77	21.3±0.75	28.6
Maize	30.5±0.70	27.4±1.50	10.2±1.11	22.7
Mini potato tubers	16.1±1.81	21.4±1.70	9.5±1.10	15.6
Rice bran	14.8±1.44	20.3±1.61	8.8±1.40	14.6
Rice straw	11.0±1.58	17.3±20.1	6.3±1.21	11.5
Mean	23.42	25.88	13.65	
CD (0.05)		CD (5%) substrates = 1.92 CD (5%) isolates = 0.97 CD (5%) substrates × isolates = 2.58		

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

Table 1b. Colony forming unit (cfu) of fungal isolates cultivated on different substrates.

Substrate	Colony forming units (1×10^5cfu/g) of *Beauveria bassiana* isolates cultivated on different substrates (Mean*±S.D*)			Mean
	Beauveria bassiana MTCC 2028	*Beauveria bassiana* MTCC 6291	*Beauveriabassiana* MTCC 4495	
Rice	36.6±6.11	32.3±2.62	22.6±4.50	30.5
Wheat	16.6±4.16	23.6±5.79	12.6±4.04	17.6
Sorghum	30.6±2.08	28.6±3.30	18.6±6.11	25.9
Maize	26.3±6.50	25.0±4.08	12.3±5.85	21.2
Mini potato tubers	14.3±3.05	21.6±3.05	10.0±3.60	15.3
Rice Bran	11.3±1.52	15.3±3.29	6.6±2.51	11.0
Rice Straw	9.6±2.08	13.3±2.62	5.6±3.05	9.5
Mean	20.7	22.8	12.1	
CD (0.05)		CD (5%) substrates = 4.1 CD (5%) isolates = N.S (non-significant CD (5%) substrates × isolates = 7.18		

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

RESULTS AND DISCUSSION

Effect of substrates

Solid substrates rice, sorghum, wheat, maize, mini potato tubers, rice bran and rice straw exhibited significant differences in their utility to yield conidia and colony forming unit. Among these substrates, maximum mean conidial count (34.0×10^5 conidia/g) (Table 1a) and colony forming units (30.5×10^5 cfu/g) (Table 1b) was recorded in rice medium. This was followed by sorghum and maize which were at par with each other. Rice straw gave minimum conidial count (11.5×10^5 conidia/g) and cfu count (9.5×10^5cfu/g) (Figure 1). Among *B. bassiana* strains, the mean conidial count varied. The maximum mean conidial count (25.8×10^5 conidia/g) and cfu count (22.8×10^5 cfu/g) was recorded for *B. bassiana* MTCC 6291 whereas the minimum mean spore count (13.6×10^5 conidia/g) and cfu count (12.1×10^5 cfu/g) was recorded for *B. bassiana* MTCC 4495. The substrates which were rich in nutritional composition produced not only high spore count but also higher colony count (Rajnikanth et al., 2010). Hence, rice was the best substrate followed by sorghum for mass multiplication of *B. bassiana* which may be due to the presence of rich source of carbon and adequate source of nitrogen.

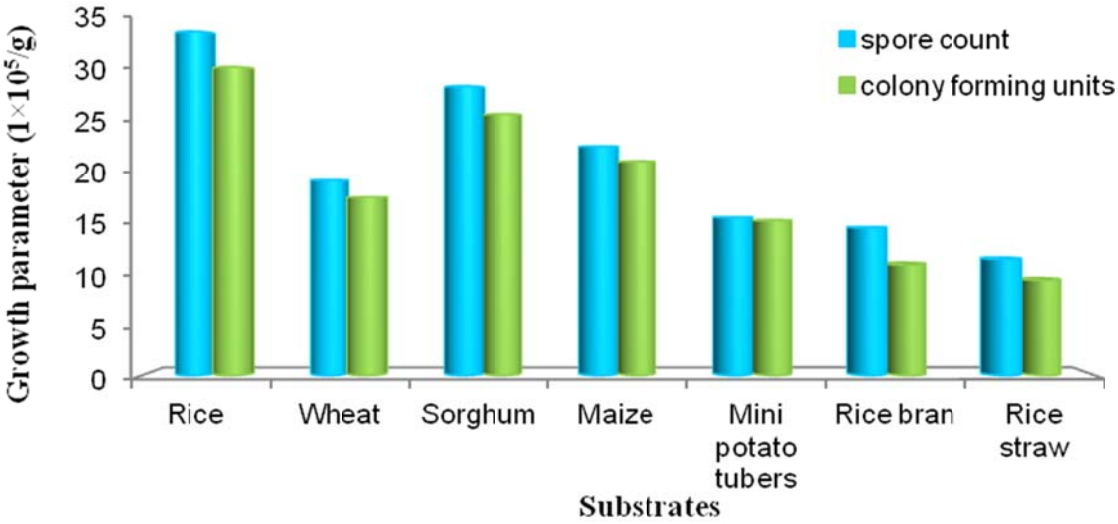

Figure 1. Conidial count and colony forming unit of *Beauveria bassiana* cultivated on different solid substrates.

It has been reported that the rice grain consists of 75-80% starch, 7% protein and sorghum contains 75% starch, 25% amylase, which are rich sources of carbon and nitrogen that enhanced the growth and sporulation (Oko et al., 2012). Mar and Lumyong (2012) also reported that *B. bassiana* showed maximum growth on rice. Sahayaraj and Namasivayam (2008) found sorghum to be the suitable substrate for large multiplication of *B. bassiana*. The rice straw and rice bran were the weakest supportive for *B. bassiana* growth. The growth in rice bran was less as compared to rice and sorghum. 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). The reason why rice straw 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).

All cereals 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) also investigated the effect of broken rice, maize, machicha and maize husk for mass production of *B. bassiana* isolate (Bb575) and two *Metarhizhium 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 MTCC 6291 and *B. bassiana* MTCC 2028 biomass produced on rice were formulated with talc, charcoal and oil. These formulations were stored at 4 and 25°C upto 90 days and their viability was evaluated fortnightly. In *B. bassiana* MTCC 6291 formulation, maximum cfu count (44.4×10^5 cfu/g) was recorded at 4°C on 15th day of storage in talc based formulation, which was at par with count recorded initially (control) (44.6×10^5 cfu/g). This was comparably at par with the count recorded upto 90th day at the same temperature. However, significantly lower count (12.3×10^5 cfu/g) was recorded at 25°C after 90 days of storage (Table 2a).

Hence, it can be concluded that in *B. bassiana* MTCC 6291 formulation, viable count of a *Beauveria* was at par with initial count upto three months at refrigeration temperature. Charcoal based formulation stored at refrigeration temperature recorded maximum mean colony forming units upto 30th day of storage and were at par with the control which recorded 39.3×10^5 cfu/g. However, significantly least count (6.6×10^5 cfu/g) was recorded at 25°C after 90 days of storage. In oil based formulation, colony forming unit ranged from 7.6 to 35.6×10^5 cfu/g at different temperatures. Colony forming unit (cfu) recorded upto 75th day of storage at 4°C were at par with control (36.0×10^5 cfu/g). However, significantly least cfu count (7.6×10^5 cfu/g) was recorded at 25°C on 90th day of storage. Similar results were recorded in *B. bassiana* MTCC 2028 formulation (Table 2b).

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 remain viable for more time as compared to 25°C. This supports reported findings, that decrease in temperature increases the longevity of *B. bassiana*

Table 2a. Effect of storage temperature on *B. bassiana* MTCC 6291 formulations.

Day	Colony forming units (1×10^5cfu/g) of *B. bassiana* MTCC 6291((Mean*±S.D*) (% decrease)					
	Talc		Charcoal		Oil	
	4°C	25°C	4°C	25°C	4°C	25°C
0 (Control)	44.6±8.02		39.3±11.50		36.0±8.18	
15	44.4±8.14 (0.8)	40.0±7.54 (10.3)	39.0±12.00 (0.7)	36.0±7.93 (8.3)	35.6±6.50 (1.1)	31.6±13.20 (12.2)
30	43.3±10.4 (2.9)	34.6±7.50 (22.2)	38.6±7.02 (1.7)	33.6±7.63 (14.5)	34.3±5.68 (4.7)	27.3±8.02 (24.1)
45	42.6±7.02 (4.4)	28.6±4.50 (35.8)	35.6±5.85 (8.6)	28.0±7.54 (28.7)	34.0±8.50 (5.5)	25.6±10.69 (28.8)
60	43.0±9.00 (3.5)	20.6±3.05 (47.5)	34.3±7.50 (12.7)	20.6±2.08 (47.5)	33.6±6.42 (6.6)	23.3±4.16 (35.2)
75	42.3±6.11 (5.1)	14.6±5.03 (67.2)	33.6±4.04 (14.5)	12.3±1.52 (68.7)	33.3±5.50 (7.5)	13.6±2.51 (62.2)
90	41.3±9.07 (7.3)	12.3±2.51 (72.4)	33.3±4.04 (15.2)	6.6±3.21 (83.2)	32.3±7.50 (10.2)	7.6±1.52 (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)

Table 2b. Effect of storage temperature of *B. bassiana* MTCC 2028 formulations.

Days	Colony forming units (1×10^5cfu/g) *B. bassiana* MTCC 2028 (Mean*±S.D*) (% decrease)					
	Talc		Charcoal		Oil	
	4°C	25°C	4°C	25°C	4°C	25°C
0 (Control)	35.3±9.29		34.6±5.68		35.6±14.04	
15	35.0±4.04 (0.8)	32.0±7.21 (9.3)	34.0±3.60 (1.7)	31.0±2.64 (10.4)	35.3±5.68 (0.8)	33.6±8.02 (5.6)
30	35.0±3.60 (0.8)	29.0±7.54 (17.8)	33.6±6.80 (2.8)	28.6±6.11 (17.3)	35.0±5.00 (2.7)	31.3±9.07 (12.0)
45	34.6±6.02 (1.9)	26.0±9.64 (26.3)	33.6±6.50 (2.8)	22.6±4.04 (34.6)	34.3±6.42 (3.6)	28.3±6.02 (20.5)
60	34.0±5.56 (3.6)	22.3±8.73 (36.8)	33.0±4.58 (4.6)	18.6±5.03 (46.2)	33.6±8.32 (6.6)	22.6±3.05 (36.5)
75	34.3±4.16 (2.8)	16.6±3.21 (52.9)	32.3±6.50 (6.6)	14.6±4.04 (57.8)	32.6±5.68 (8.4)	15.3±3.21 (57.0)
90	32.6±4.12 (7.6)	10.6±5.03 (69.9)	31.6±9.16 (8.6)	8.6±4.16 (75.1)	32.3±4.72 (10.2)	10.3±2.51 (71.0)
CD (5%)	CD (5%) Temperature = 3.51					

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

conidia (Hong et al., 1997). Morley-Davies et al. (1995) 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) 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) who reported 82.47% conidial viability of *Normuraea rileyi* formulations stored for 180

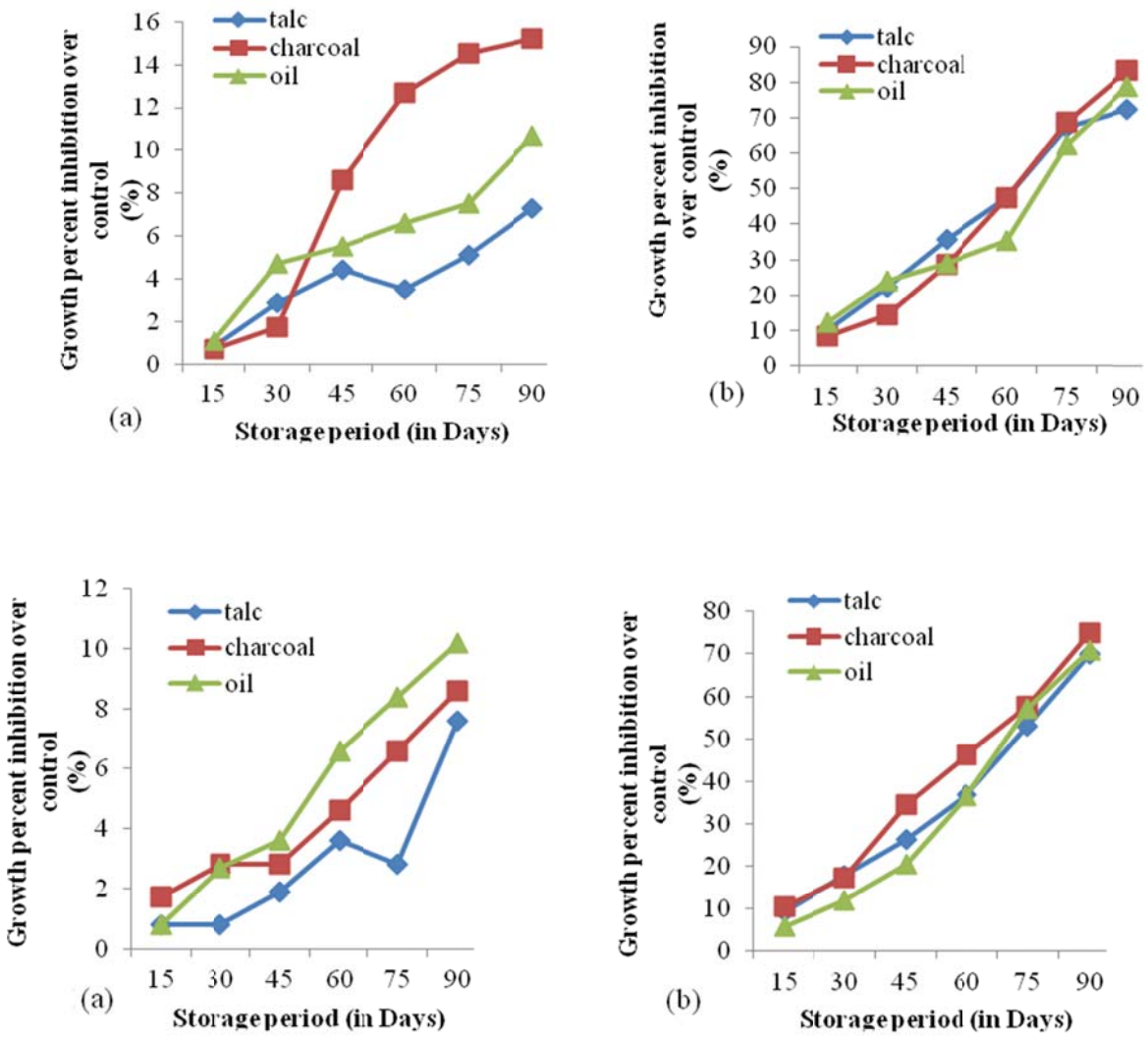

Figure 2. Growth percent reduction over control of (I) *B. bassiana* MTCC 6291 (a) At 4°C (b) At 25°C (II) *B. bassiana* MTCC 2028 (a) At 4°C (b) At 25C in different formulation (talc, charcoal and oil).

days in refrigerated condition, as compared to 63.23% under room temperature formulation with talc at 4°C.

Described experimental work showed that for mass production of *B. bassiana* formulation on solid substrate, rice 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.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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