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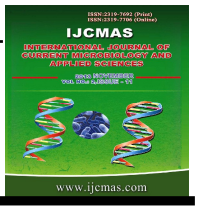

Original Research Article

Optimization process for blastospore production of *Beauveria bassiana* isolates in poly ethylene glycol (peg) supplemented medium

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A B S T R A C T

The entomopathogenic fungus *Beauveria bassiana* is a globally distributed Hyphomycete, strains of which infect a range of insects. Strains of *Beauveria bassiana* have been used as the active agents in a number of biopesticides against a variety of agricultural pests, including whiteflies, beetles, grasshoppers and psyllids. Blastospore production of three different *Beauveria bassiana* isolates *viz*., Bb – 1, Bb – 2 and Bb - 3 in Poly Ethylene Glycol (PEG) supplemented medium was investigated in the present study. The SDA medium was supplemented with various concentrations (1%, 2%, 3%, 4% and control) of PEG 6000 and the growth of *Beauveria bassiana* isolates were estimated. Among the three *Beauveria bassiana* isolates, *Beauveria bassiana* (Bb – 1) showed maximum blastospore production followed by *Beauveria bassiana* (Bb – 2) and least blastospore production was observed in the isolate *Beauveria bassiana* (Bb – 3). Blastospore production was maximum at pH 6, 27°C, 11 hrs incubation period, 175 rpm agitation speed and 8% inoculum size

K e y w o r d s

Beauveria bassiana; Poly Ethylene Glycol (PEG); Blastospore and optimization.

Introduction

The entomopathogenic fungus, *Beauveria bassiana* is of commercial importance as an alternative to chemical insecticides in an agroecosystem (Khachatourians *et al*., 2002). The fungal pathogen *Beauveria bassiana* is a widely used mycoinsecticide for control of several insect pests, providing a biological alternative to synthetic chemical insecticides (Hajek *et al*., 2001). A key advantage for microbial control agents is their potential to replicate and persist in the environment, offering continued suppression of insect pest populations. Exploiting this advantage, however, is commensurate with the need to determine the risks to non - target organisms of mass releasing this fungus. To date, no information is available on the potential for genetic recombination between strains of *Beauveria bassiana* neither in agricultural fields nor on whether this recombination could result in altered virulence and host range.

Beauveria species attack many insect species worldwide. Species range from the ubiquitous insect pathogen *Beauveria bassiana* (Balsamo) Vuillemin to rare species but the entomogenous life - style is prevalent (Glare, 2004; Glare *et al*., 2008; Sevim *et al*., 2010). Currently, six species of this genus are recognized: *Beauveria bassiana*, *Beauveria clade*, *Beauveria brongniartii*, *Beauveria caledonica*, *Beauveria vermiconia* and *Beauveria amorpha* (Glare, 2004; Glare *et al*., 2008; Sevim *et al*., 2010; Rehner and Buckley, 2005). Among these species, considerable effort has been spent to develop *Beauveria bassiana* as a biological control agent in agriculture and forestry in temperate regions and the most widely used species available commercially is *Beauveria bassiana* (Meyling and Eilenberg, 2007; Goettel *et al*., 2005).

Blastospores are produced during the fermentation process in commercial production of spores where as aerial spores are produced on conidiogenous cells on the infected insects. However, the pathogenicity of blastospores and aerial spores is same. The death of insect may result due to non - availability of nutrients, invasion of organs by fungus and toxicosis due to toxins produced by *Beauveria bassiana.* After the death of the insect, fungus grows saprophytically inside the body of the insects and produces metabolites that may not allow other competing microbes to grow in the cadaver. It reproduces sexually in soils throughout the world and asexually in a variety of insect hosts. In its asexual form, it produces spores known as conidia which are wind dispersed. Once they are released they may land upon another insect host, or once again return to the soil where they reproduce sexually retaining the properties which make it an effective pest control and preventing the qualities which cause it to be harmful to beneficial insects (Boucias and Pendland, 1998).

Blastospore production using liquid culture fermentation is vegetative fungal propagules that are the preferred mode of growth for many entomopathogens in the haemocoel of infected insects (Shimuzu *et* a*l*., 1993; Sieglaff *et al.,* 1997; Vestergaard *et al.,* 1999; Askary *el al.,* 1999). Yeast - like growth allows the fungus better access to the nutrients within the insect. Numerous entomopathogens of the genera *Beauveria, Lecanicillium* and *Metarhizium* can be induced to grow in a 'yeast - like' fashion in submerged liquid culture. Blastospore - based mycoinsecticides are currently produced commercially by *Verticillium lecanii* and *Beauveria bassiana* .The impact of nutrition on conidial yields for various fungal entomopathogens in liquid culture was found to be significant (Vega *et al.,* 2003). Poly Ethylene Glycol incorporation in the media increased the blastospores and curtailed the mycelial pellet development (Sreeramakumar *et al.,* 2005).

Materials and Methods

Blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol (PEG) supplemented medium

Beauveria bassiana cultures regularly grown on Potato Dextrose Agar (PDA) in test tubes at 27°C for ten days were used for the study. The culture was then grown on Sabouraud's Dextrose Agar medium (SDA) and the conidia was harvested using sterilized distilled water using 0.02% Tween 80 as wetting agent (Rombach *et al.,* 1989). Spore load of the suspension was assessed using an

improved Neubauer's haemocytometer. The effect of Polyethylene glycol 6000 on the biomass and spore load and virulence were assessed using this suspension. Polyethylene glycol at different concentrations *viz.,* 1, 2, 3 and 4 per cent (w/v) were prepared separately and dispensed into 500 ml Erlenmeyer flask containing 200 ml of Sabouraud's Dextrose broth per flask and the pH of the medium was adjusted to 6.5 using 0.1 N HC1 or NaOH. The flasks were sterilized in an autoclave at 121°C at 15 lbs pressure for 20 min. Three replications were maintained for each treatment. The conidial suspension (with a spore load of 1 × 10^8 ml^{-1}) prepared from Sabouraud's Dextrose Agar plates were inoculated to the cooled medium at the rate of 3 ml per 100 ml broth and incubated in an orbital rotary shaker at 150 rpm for 7 days at room temperature. After 24 hrs, the shaker speed was increased to 200 rpm to dislodge the mycelial bits. The suspension without polyethylene glycol was treated as control. After incubation, the broth was estimated for the blastospore production using Naubauer's Haemocytometer and the blastospore number was expressed in blastospores ml^{-1} of suspension. The other parameters like pellet number, pellet size, wet, dry weight and blastospore germination were also determined. For estimating the pellet size, 10 fully formed (spherical and loose) pellets per replicate were measured and for pellet number the numbers of such pellets per 10 ml were counted (Sreeramakumar *et al.,* 2005).

Effect of pH on the blastospore production of *Beauveria bassiana* isolates in PEG supplemented medium

The different isolates of *Beauveria bassiana* were tested in Sabouraud's Dextrose Broth supplemented with 4 per cent poly ethylene glycol and the pH was adjusted to 3, 4, 5, 6, 7 and 8 individually using 0.1 N HC1 and 0.1 N NaOH and they were sterilized and inoculated with respective isolates. The inoculated medium was incubated for 7 days and the blastospore production was estimated using Naubauer s Haemocytometer and expressed as number of blastospores ml^{-1} of broth.

Effect of temperature on the blastospore production of *Beauveria bassiana* isolates in PEG supplemented medium

The conical flasks containing Sabouraud's Dextrose Broth supplemented with 4 per cent poly ethylene glycol were inoculated individually with respective isolates and incubated at different temperatures *viz.,* 23°C, 25°C, 27°C, 29°C, 31°C and 33°C. After 7 days of incubation, the respective broths were tested for its blastospore production individually using improved Naubauer's Haemocytometer.

Effect of incubation period on the blastospore production of *Beauveria bassiana* isolates in PEG supplemented medium

The conical flasks containing Sabouraud's Dextrose Broth supplemented with 4 per cent poly ethylene glycol were inoculated individually with respective isolates and incubated at different incubation period *viz.*, 5, 7, 9, 11 and 13 days. After incubation, the respective broths were tested for its blastospore production individually using improved Naubauer's Haemocytometer.

Effect of shaking speed on the blastospore production of *Beauveria bassiana* isolates in PEG supplemented medium

The conical flasks containing Sabouraud's Dextrose Broth supplemented with 4 per cent poly ethylene glycol were inoculated individually with respective isolates and incubated at different shaking speed *viz.*, 75, 100, 125, 150, 175 and 200 rpm individually and after incubation the respective broths were tested for its blastospore production individually using improved Naubauer's Haemocytometer.

Effect of inoculum size on the blastospore production of *Beauveria bassiana* isolates in PEG supplemented medium

The conical flasks containing Sabouraud's Dextrose Broth supplemented with 4 per cent poly ethylene glycol were inoculated individually with respective isolates at different inoculums sizes *viz.,* 2, 4, 6, 8 and 10 per cent and incubated. After incubation, the respective broths were tested for its blastospore production individually using improved Naubauer s Haemocytometer.

Results and Discussion

Blastospores displayed the broadest binding characteristics of the *Beauveria bassiana* single - cell types, and they were able to bind to hydrophobic, weakly polar and hydrophilic surfaces (Jackson *et al.,* 2007). Compared to conidia, these blastospore preparations show great promise as a bioinsecticidal propagule due to their rapid rate of germination on agar and on the cuticle of insects (Thomas *et al.,* 1987; Jenkins and Prior, 1993; Vega *et al.,* 1999; Jackson, 2007). Blastospores can be very infective when applied to the insect host, are not amenable to simple drying techniques. In addition, nutritional and environmental conditions in liquid media have been shown to impact spore yield, stability and biocontrol efficacy (Humphreys *et al.,* 1989; Lane *et al.,* 1991). The blastospore production in the submerged conditions can be increased by addition of polymers.

Polyethylene glycol (PEG), a versatile osmoticum has been used as an ingredient in culture media for the mass production of several fungal species that have biocontrol potential. Polyethylene glycol has been shown to have varied effects on biomass characteristics in addition to its influence on the shelf - life and field performance of different fungal species. In any entomopathogenic culturing medium, the addition of Polyethylene glycol (PEG) increased more hyphal bits and thereby increased blastospore formation (Inch and Trinci, 1987). Studies have shown that the medium water activity can be lowered with the addition of Polyethylene glycol (PEG) to get increased conidial density and colony forming units (Jin *et al.,* 1991).

Blastospore production of three different *Beauveria bassiana* isolates *viz*., Bb – 1, Bb – 2 and Bb - 3 in Poly Ethylene Glycol (PEG) supplemented medium was investigated in the present study and the results were furnished in Table – 1. The SDA medium was supplemented with various concentrations (1%, 2%, 3%, 4% and control) of PEG 6000 and the growth of *Beauveria bassiana* isolates were estimated. Among the three *Beauveria bassiana* isolates, *Beauveria bassiana* (Bb -1) showed maximum blastospore production followed by *Beauveria bassiana* (Bb – 2) and least blastospore

production was observed in the isolate *Beauveria bassiana* (Bb – 3). Blastospore production was maximum at 4% concentration followed by 3%, 2% and 1%. Least blastospore production was observed in the control. At 4% PEG 6000 concentration, blastospore production by *Beauveria bassiana* (Bb – 1) was 37.09 × 10^9/ml broth, *Beauveria bassiana* (Bb – 2) produced 37.09 × 10^9/ml broth and blastospore produced by *Beauveria bassiana* (Bb – 3) was 30.70 × 10^9/ml broth.

The addition of Polyethylene glycol suppressed the formation of pellets in liquid cultures of certain entomopathogenic fungi having commercial value has been reported (Humphreys *et al.,* 1989; Kleespies and Zimmermann, 1992). The Polyethylene glycol at 2 per cent also favoured both higher biomass and blastospore in *Beauveria bassiana* and it also acts as water activity depressor (Geetha and Balaraman, 2001). Bidochka *et al.* (1997) reported production of blastospores of *Beauveria bassiana* on liquid media containing peptone, peptone-glucose, glucose peptone yeast extract. Results showed four-fold higher production of blastospores in peptone - glucose as compared to glucose-peptone yeast extract. Geetha and Balaraman (2001) reported that Polyethylene glycol (2%) favoured both higher biomass and blastospores in the case *of Beauveria bassiana.* Polyethylene glycol at 6 per cent concentration in Sabouraud's Dextrose Agar influenced both quality and quantity of the biomass of *Hirsutella thompsonii* (nonsynnematous) and *Hirsutella thompsonii* var. Synnematosa (synnematous) fungi in submerged culture (Sreeramakumar *et al.,* 2005).

In the present study, the effect of pH, temperature, incubation period, shaking speed and inoculum size on the blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol supplemented medium. The blastospore production at various pH *viz*., pH - 3, pH - 4, pH - 5, pH - 6, pH - 7 and pH – 8 was investigated and maximum blastospore production was observed in *Beauveria bassiana* (Bb – 1) at pH – 6 (62.25 × 10^9/ml broth) followed by pH – 5 (40.86 × 10^9/ml broth), pH – 7 (28.12 × 10^9/ml broth), pH – 4 (13.10 × 10^9/ml broth) and pH – 8 (10.08 × 10^9/ml broth) and least blastospore production was observed at pH – 3 (07.60 × 10^9/ml broth) (Table – 2). Among the various temperatures tested, blastospore production was maximum in the *Beauveria bassiana* (Bb – 1) at 27°C (60.66 × 10^9/ml broth), 29°C (23.53 × 10^9/ml broth), 25°C (18.35 × 10^9/ml broth), 23°C (12.45 × 10^9/ml broth) and 31°C (07.13 × 10^9/ml broth). Minimum blastospore production was recorded at 33°C (05.88 × 10^9/ml broth) (Table – 3). Jin (2010) also reported the pH and other parameters influenced the spore yield of *Beauveria bassiana.*

Blastospore production was studied at various incubation periods *viz*., 5 days, 7 days, 9 days, 11 days and 13 days. Maximum blastospore production was recorded by the *Beauveria bassiana* (Bb – 1) after 11 days (68.25 × 10^9/ml broth) followed by 13^{th} day (42.85 × 10^9/ml broth), 9^{th} day (33.17 × 10^9/ml broth) and 7^{th} day (21.65 × 10^9/ml broth). Least blastospore production was observed after 5 days (10.45 × 10^9/ml broth) (Table – 4). Among the various shaking speed, maximum blastospore production was recorded by the *Beauveria bassiana* (Bb – 1) at 175 rpm (69.98 × 10^9/ml broth) followed by 125 rpm (50.35 × 10^9/ml broth), 100 rpm (33.86 × 10^9/ml broth) and 75 rpm (21.25 × 10^9/ml broth).

Table.1 Blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol (PEG) supplemented medium

S. No	Concentration of PEG 6000 (%)	Blastospores ml $^{-1}$ of broth (× 10^9/ml broth)		
		Beauveria bassiana (Bb – 1)	*Beauveria bassiana* (Bb – 2)	*Beauveria bassiana* (Bb – 3)
1	1.0	09.32	07.21	06.52
2	2.0	11.65	08.64	07.69
3	3.0	13.58	11.15	10.25
4	4.0	37.09	33.30	30.70
5	Control	03.35	02.22	01.95

Table.2 Effect of pH on the blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol (PEG) supplemented medium

S. No	Isolates	Blastospores at different levels of pH (× 10^9 ml^{-1} broth)					
		pH - 3	pH - 4	pH - 5	pH - 6	pH - 7	pH - 8
1	*Beauveria bassiana* (Bb – 1)	07.60	13.10	40.86	62.25	28.12	10.08
2	*Beauveria bassiana* (Bb – 2)	04.88	08.62	27.75	46.32	17.72	06.17
3	*Beauveria bassiana* (Bb – 3)	02.20	05.00	18.15	38.23	10.33	04.52

Table.3 Effect of temperature on the blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol supplemented medium

S. No	Isolates	Blastospores at different temperature levels (× 10^9 ml^{-1} broth)					
		23°C	25°C	27°C	29°C	31°C	33°C
1	*Beauveria bassiana* (Bb – 1)	12.45	18.35	60.66	23.53	07.13	05.88
2	*Beauveria bassiana* (Bb – 2)	10.53	15.75	53.55	20.18	05.25	03.15
3	*Beauveria bassiana* (Bb – 3)	07.30	10.32	45.35	17.90	04.44	02.78

Table.4 Effect of incubation period on the blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol supplemented medium

S. No	Isolates	Blastospores at different incubation period (× 10^9 ml^{-1} broth)				
		5 days	7 days	9 days	11 days	13 days
1	*Beauveria bassiana* (Bb – 1)	10.45	21.65	42.85	68.25	33.17
2	*Beauveria bassiana* (Bb – 2)	06.10	18.33	36.95	60.18	27.18
3	*Beauveria bassiana* (Bb – 3)	03.88	13.60	28.88	54.60	22.71

Table.5 Effect of agitation speed on the blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol supplemented medium

S. No	Isolates	Blastospores at different agitation speed (rpm) (× 10^9 ml^{-1} broth)				
		75 rpm	100 rpm	125 rpm	150 rpm	175 rpm
1	*Beauveria bassiana* (Bb – 1)	12.53	21.25	33.86	50.35	69.98
2	*Beauveria bassiana* (Bb – 2)	09.16	16.95	25.78	42.15	62.75
3	*Beauveria bassiana* (Bb – 3)	05.80	11.50	22.69	37.38	58.32

Table.6 Effect of inoculum size on the blastospore production of *Beauveria bassiana* isolates in Poly Ethylene Glycol supplemented medium

S. No	Isolates	Blastospores at different inoculum size (%) (× 10^9 ml^{-1} broth)				
		2%	4%	6%	8%	10%
1	*Beauveria bassiana* (Bb – 1)	19.95	36.16	72.25	69.50	58.16
2	*Beauveria bassiana* (Bb – 2)	12.33	30.95	65.95	62.30	51.09
3	*Beauveria bassiana* (Bb – 3)	08.52	26.70	61.08	58.78	49.53

Minimum blastospore production was observed at 50 rpm (12.53 × 10^9/ml broth) (Table – 5). Blastospore production by *Beauveria bassiana* isolates were tested at different inoculum levels *viz*., 2%, 4%, 6%, 8% & 10%, and maximum blastospore production was recorded at 6% (72.25 × 10^9/ml broth) inoculum level followed by 8% (69.50 × 10^9/ml broth), 10% (58.16 × 10^9/ml broth) and 4% (36.16 × 10^9/ml broth). Minimum blastospore production was observed at 2% inoculum level (19.95 × 10^9/ml broth) (Table – 6). Herta *et al.* (2005) observed that different growth parameters influenced the spore yield.

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