

RESEARCH ARTICLE

ECONOMIC NATURAL BROTH MEDIA FOR SPORE PRODUCTION OF *BEAUVERIA BASSIANA* (BALSAMO)VUILLEMIN AND *BEAUVERIA BRONGNIARTII* (SACC.) PETCH.

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Abstract

Commonly available low-cost natural substrates, including agricultural products and byproducts, were evaluated for blastospore production by the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Beauveria brongniartii*(Sacc.) Petch, two common entomopathogenic fungi in sugarcane ecosystem. Of the resources namely Sesame Seed Cake Extract (SSCE), Groundnut Seed Cake Extract (GSCE), Cotton Seed Cake Extract(CSCE), Coconut Cake Extract(CCE), Rice Bran Extract(RBE), Wheat Bran Extract(WBE), Red gram Husk Extract(RHE) and Jaggery solution tested at three different concentrations viz., 5,10 and 15% infused with 1% peptone and compared with Sabouraud Dextrose Broth(SDB),significant differences in the production of *B.bassiana* spores among the media, with SSCE (2.81×10^8 /ml) proven better than all other media tested including the standard, SD broth (0.77×10^8 /ml) irrespective of concentration were observed. Economics being a consideration, SSCE(1.94×10^8 /ml), GSCE (1.48×10^8 /ml), CSCE (1.46×10^8 /ml) , Jaggery (1.39×10^8 /ml) followed by CCE, RBE at 5% concentration are the best alternative media for effective spore production of *B.bassiana* which can be utilized depending upon availability and season For *B.brongniartii* spore production, irrespective of concentrations tested many media i.e., CCE(11.93×10^7 /ml) , RBE (11.02×10^7 /ml),WBE (11.67×10^7 /ml), RHE (8.27×10^7 /ml)and CSCE (5.8×10^7 /ml) were on par with each other and better than the rest, the standard SD medium, Jaggery, GSCE and SSCE which were on par among themselves. No significant differences due to concentration was observed in blastospore production of *B. brongniartii* and *B. bassiana* except in the case of Jaggery based medium for latter species. SSCE at 10%, 15% and CSCE 5% yielded the maximum number of *B.bassiana* spores per rupee spent on raw material (7.51×10^9 , 4.09×10^9 and 4.08×10^9 respectively) with the SD broth resulting in 0.68×10^9 spores while WBE (15%), RBE (15%) yielded maximum *B.brongniartii* spores (2.92×10^9 , 2.29×10^9) per rupee with the standard SD broth being costlier, producing 10 times lesser spores (1.59×10^8).The intrinsic differences observed in spore production between the two species and possible implications of media on the virulence are discussed.

Key words: Economic medium, sporulation, *Beauveria bassiana*, *B. brongniartii*

Introduction

Fungi are ubiquitous in nature and have evolved over time to colonize a wide range of ecosystems including pest control in agricultural cropping systems (Dinu *et al.*, 2012; Liu *et al.*, 2015). Over 750 different species of entomopathogenic fungi have been identified to date (Scheepmaker and Butt 2010) which act as natural regulators of insect populations and in many cases have some

very species-specific action (Butt 2002).Large-scale practical use of these fungi as microbial control agents for pest management depends on the production of stable fungal propagules (Pham *et al.*, 2009; Gouli *et al.*, 2013) at reasonable cost (Bena-Molaei *et al.*, 2015). While standard synthetic media are available for mass production of entomopathogenic fungi, the search for alternative sources has been continuous. It is vital to evolve and improvise a synthetic or semisynthetic

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medium as it elevates status of availability around the year, the applicability and economics of the agent. Two species of *Beauveria* viz., *Beauveria bassiana* (Balsamo) Vuillemin and *Beauveria brongniartii* (Sacc.) Petch, have shown promise against sugarcane pests in India. Considering the large spore dosage required to achieve reasonable levels of infection as in the case of *B. brongniartii* against the white grub *Holotrichia serrata* (F) (Easwaramoorthy *et al.*, 2004) it is essential to economise the production of the media.

Several efforts (Sahayaraj and Karthick 2008; Siwachand Jaipal 2004; Sharma *et al.*, 2002; Thodsare *et al.*, 2014) to identify grain or plant product or agricultural byproduct based medium have been made. Complementary nourishment of media with agricultural by-products enhanced chlamydospore production in liquid culture of *Fusarium oxysporum* (Elzein and Kroschel 2004). While there is abundant information on using grains or molasses for blastospore production with (Balakrishnan *et al.*, 2011; Tamilarasi *et al.*, 2002) or without fortification (Nirmala *et al.*, 2005; Joshi *et al.*, 2016) there is far less information with reference to other natural substrates or byproducts.

Materials and Methods

The cultures of *B. bassiana* and *B. brongniartii* were from the original stock being maintained at the ICAR-Sugarcane Breeding Institute, Coimbatore, isolated from naturally infected larvae of shoot borer, *Chilo infuscatellus* Snellen and white grub *Holotrichia serrata* F. of sugarcane. These cultures were grown on SDA (Sabouraud Dextrose Agar) slants (Dextrose 40g/l, peptone 10g/l and agar 15g/l, pH 5.6±0.2) at 25±2°C for 10 days, and stored at 4 °C until use. Germination potential of the colony from the slants (≥90%) was ascertained before the start of the experiments. Profusely sporulating cultures of both species were developed by inoculating the cultures from

the original slants and incubating at 25±2°C for 10 days on SDA medium. Culture plugs of 8mm diameter were drawn with a cork borer aseptically from these cultures for inoculating in the tested liquid media.

Commonly available economic, agricultural products and byproducts were chosen for comparing blastospore production by both species of *Beauveria*. The resources procured from local market were used for preparation of Sesame Seed Cake Extract (SSCE), Groundnut Seed Cake Extract (GSCE), Cotton Seed Cake Extract (CSCE), Coconut Cake Extract (CCE), Rice Bran Extract (RBE), Wheat Bran Extract (WBE), Redgram Husk Extract (RHE) and Jaggery solution. All media were infused with mycological peptone (Himedia®; 10g/l of medium) as a source of organic nitrogen. The substrates were suspended in distilled water at concentrations of 5, 10 and 15% (w/v), boiled and filtered through cheese cloth and autoclaved (121°C) for 15 minutes. The tested media were compared with sterilized Sabouraud Dextrose Broth (Himedia®; 30g/1000 ml distilled water), the standard medium for the production of spores.

Ten-day old culture discs of either of the two species from the mature colony plates of SDA were inoculated aseptically, in 100ml of each test media of a specific concentration in 250 ml Erlenmeyer flasks, replicated twice and incubated as stationary cultures at 25 ± 2 °C. At 30 days post-inoculation, the cultures were homogenized with 0.02% Tween-80 and filtered through cheese cloth. Subsequent to required dilutions in sterile distilled water, the blastospores were counted with a Neubauer' improved haemocytometer (Germany). Cost of the raw materials used in the media was worked out at prevailing local market prices of the ingredients. Cost of peptone and SD was as per the manufacturer' or supplier's catalogue. The economics was worked out in terms of the number

of blastospores produced for every rupee invested in media ingredients.

Data analysis

Data obtained were subjected to $\log(x)$ transformation and factorial CRD analysis in SPSS (version 11.5). The General Linear Model (GLM) was used to perform ANOVA with Tukey HSD test ($\alpha = 0.05$) for overall mean comparison to analyze the effect of media and concentration. In the case of comparisons of means of different media at individual (same) concentration as well as means of different concentrations at individual (one) medium, one-way ANOVA with the means separated by Tukey HSD test ($\alpha = 0.05$) was used.

Results and Discussion

There were significant differences in the production of blastospores among natural media and the standard medium for both species at 30 days post inoculation as indicated by analysis of variance through GLM. Comparisons of different concentrations of a single medium showed that differences were insignificant except in the case of Jaggery based medium for *B.bassiana* (Table 1) blastospore production ($F_{2,3} = 29.24$, $P < 0.05$) while no significant differences due to concentration were observed for *B.brongniartii* (Table 2).

The overall impact of different media on the blastospore production of both species (Table

Table 1. Production of *Beauveria bassiana* spores at different concentrations of tested media ($\times 10^8$ /ml)

Low-cost media	Sporulation ($\times 10^8$ /ml) at different concentration of media			Effect of medium
	5%	10%	15%	
Sesame Seed Cake Extract (SSCE)	1.94 \pm 0.42 Ab	4.16 \pm 0.01 Ab	2.32 \pm 0.68 Ab	2.81 \pm 1.12 d
Groundnut Seed Cake Extract (GSCE)	1.48 \pm 0.40 Ab	0.76 \pm 0.06 Aa	1.14 \pm 0.20 Aab	1.13 \pm 0.38 abc
Cotton Seed Cake Extract (CSCE)	2.22 \pm 0.08 Ab	1.82 \pm 0.03 Aab	2.20 \pm 0.23 Ab	2.08 \pm 0.23 cd
Coconut Cake Extract (CCE)	1.46 \pm 0.93 Aab	1.08 \pm 0.34 Aa	1.0 \pm 0.22 Aab	1.18 \pm 0.51 abc
Rice Bran Extract (RBE)	1.28 \pm 0.05 Aab	1.22 \pm 0.08 Aa	1.50 \pm 0.14 Aab	1.33 \pm 0.15 bcd
Wheat Bran Extract (WBE)	0.72 \pm 0.34 Aab	1.55 \pm 0.04 Aab	1.54 \pm 0.04 Aab	1.27 \pm 0.45 bc
Redgram Husk Extract (RHE)	0.40 \pm 0.05 Aa	0.86 \pm 0.65 Aa	0.64 \pm 0.34 Aa	0.63 \pm 0.39 a
Jaggery	1.39 \pm 0.04 Bb	1.26 \pm 0.03 Bab	0.83 \pm 0.10 Aa	1.18 \pm 0.26 abc
SD broth	0.77 \pm 0.06 ab	0.77 \pm 0.06 a	0.77 \pm 0.06 a	0.77 \pm 0.06 ab
Mean of concentration	1.29 \pm 0.63 A	1.50 \pm 1.04 AA	1.33 \pm 0.63	

Means are separated by Tukey HSD test ($P = 0.05$). Means with the same upper case letters across a row and lower case letter in a column are not significantly different at $p = 0.05$. *SD was standard medium and thus is of single concentration and thus the same result has been utilized for comparison with media tested at various concentrations.

Table 2. Production of *Beauveria brongniartii* spores at different concentrations of tested media(x 10⁷/ml)

Medium based on <i>B. brongniartii</i>	Sporulation (x 10 ⁷ /ml) at different concentration of media			Mean of medium*
	5%	10%	15%	
Sesame Seed Cake Extract (SSCE)	3.2±0.56Aab	7.0±0.85Abcde	5.0±3.67Aab	5.07± 2.41abcd
Groundnut Seed Cake Extract (GSCE)	3.4±1.41Aab	2.8±1.13Aabc	5.2±2.83Aab	3.8±1.87abc
Cotton Seed Cake Extract (CSCE)	5.4±1.4Aab	4.0±0.00Aabcd	8.0±1.70Aab	5.8±2.07bcd
Coconut Cake Extract (CCE)	9.8±0.14Ab	11.78±8.77Ae	8.2±0.08Aab	11.93±6.09d
Rice Bran Extract (RBE)	9.0±5.37Aab	8.4±2.26Acde	13.30±0.99Ab	11.02±3.56cd
Wheat Bran Extract (WBE)	6.6±1.98Aab	11.2±2.83Ade	17.2±1.24Ab	11.67±7.48cd
Redgram Husk Extract (RHE)	3.4±2.82Aab	9.8±5.37Acde	11.6±0.40Aab	8.27±4.88bcd
Jaggery	2.6±1.97Aab	1.9±0.42Aab	7.0±2.55Aab	3.83±2.87ab
SD broth	1.8±0.28a	1.8±0.28a	1.8±0.28a	1.8±0.28a
Mean of concentration	5.02±3.21A	7.19±0.58A	8.59±5.74A	

Means are separated by Tukey HSD test (P=0.05). Similar letters (upper case) across a row and letters (lower case) in a column are not significantly different at p=0.05. *SD was standard medium and thus is of one concentration and thus the same result has been utilized for comparison with media tested at various concentrations.

1 and 2) was highly significant (*B. bassiana*: $F_{8,41}=9.71$, $P<0.001$; *B. brongniartii*: $F_{8,41}=6.16$, $P<0.001$). Within an individual concentration also, the variation due to media was highly significant (For 5% concentration: *B. bassiana*: $F_{8,9}=6.16$, $P<0.01$; *B. brongniartii*: $F_{8,9}=3.93$, $P<0.05$; For 10% concentration: *B. bassiana*: $F_{8,9}=6.60$, $P=0.01$; *B. brongniartii*: $F_{8,9}=11.69$, $P<0.005$). For 15% concentration (*B. bassiana*: $F_{8,9}=7.57$, $P<0.005$; *B. brongniartii*: $F_{8,9}=3.96$, $P<0.05$).

Comparison of general means indicated significant differences in the production of *B. bassiana* spores

among the media, with SSCE (2.81×10^8 /ml) proven better than several media tested including the standard, SD broth (0.77×10^8 /ml). There were not differences due to the concentrations tested across the medium. Within a medium, when concentrations were compared differences were observed only in jaggery based medium with 5% (1.39×10^8 /ml) and 10% (1.26×10^8 /ml) jaggery faring significantly better than 15% jaggery (0.83×10^8 /ml) solution indicating that higher nutrition need not translate to productivity (Table 1). Increasing nutrient concentration of wheat bran

extract and rice bran extract didn't result in higher blastospore production and it was speculated that decreasing oxygen content could be a reason for this (Bena-Molaei *et al.*, 2015). Our finding of the production of *B. bassiana* in SDB as low as 0.77×10^8 /ml, was in accordance with that by Pandey and Kanaujia (2010) who reported a production of 0.5×10^8 /ml but contrary to Joshi *et al.* (2016) who obtained 2.43×10^8 spores/ml with SD medium that was comparable with several synthetic media.

While no variation in spore production of both species due to the concentration of raw material used in media could be observed for most media, 15% Jaggery had resulted in lower production of *B. bassiana* which may be the result of depletion of oxygen affecting *B. bassiana* but not *B. brongniartii* which responded linearly and positively to the increment in concentration of jaggery. So, higher sporulation in increased concentration of a particular media may be species specific response. It may be hypothesized that requirement and utilization efficiency of dextrose by *B. brongniartii* is higher than *B. bassiana*. The lower insignificant variations shown in the production of blastospores due to change in the concentrations of most media indicate the efficient utilization of the low level carbon source aided by available sufficient nitrogen from peptone and possible plateau effect on luxurious usage nullifying linear response to nutrients. Increasing the concentration of wheat bran extract and rice bran extract from 4 to 12% didn't lead to higher blastospore production but increasing to 20% led to decrease in blastospore production of *B. bassiana* (Bena-Molaei *et al.*, 2015).

Determination of the most effective medium at the lowest concentration (5%) showed that all media except RHE were significantly better than SD broth with which the latter was on par with. At 10% concentration, the trend changed to reveal that SSCE and CSCE were better than most media

i.e., the GSCE, CCE, RBE, RHE as well as SD broth while WSE and Jaggery were on par with all (Table 1). At the highest concentration under evaluation (15%), it could be seen that SSCE, CSCE were consistently significantly better than RHE, jaggery, SD broth in terms of *B. bassiana* sporulation while the rest of media were on par with all media. Thus it could be derived that most of these media *viz.*, SSCE (1.94×10^8 /ml), GSCE (1.48×10^8 /ml), CSCE (1.46×10^8 /ml), Jaggery (1.39×10^8 /ml) followed by CCE, RBE are the best alternative media which can be used at 5% (Table 1) for effective economic spore production of *B. bassiana* depending upon availability and season. For example, while jaggery is available round the year in sufficient quantity, supply of sesame seed cake and redgram husk may be restricted to production zones or not available in large quantity.

For the economic production of *B. brongniartii* spores, the best medium irrespective of concentrations tested was CCE (11.93×10^7 /ml) which was on par with many media *i.e.*, RBE (11.02×10^7 /ml), WBE (11.67×10^7 /ml), RHE (8.27×10^7 /ml), CSCE (5.8×10^7 /ml) and SCE (5.07×10^7 /ml) while SD medium supported least sporulation (1.8×10^7 /ml) that was on par with Jaggery (3.83×10^7 /ml), GSCE (3.8×10^7 /ml) and SSCE (Table 2). It could be seen that concentration of the resource material irrespective of the medium did not affect the production of *B. brongniartii* spores (Table 2). However, within a chosen concentration of medium, significant differences among media were observed. Several media were better than SD medium (1.8×10^7 /ml) at 5% with CCE (9.8×10^7 /ml) faring significantly better than the former with the means of the rest of the media overlapping. At 10%, CCE continued to produce highest number of *B. brongniartii* spores (11.78×10^7 /ml) and was significantly better than GSCE (2.8×10^7 /ml), CSCE (4×10^7 /ml), Jaggery (1.9×10^7 /ml) and the standard SD while being

on par with the rest. Similar trend with slight variation was observed at 15% with WBE ($17.2 \times 10^7/\text{ml}$), RBE ($13.30 \times 10^7/\text{ml}$) being significantly better than SD broth but on par with RHE ($11.6 \times 10^7/\text{ml}$), CCE ($8.2 \times 10^7/\text{ml}$) and CSCE ($8.0 \times 10^7/\text{ml}$) that were on par with SD broth. Hence any of natural substrates tested as the alternative media, could be used for *B. brongniartii* culturing without radical impact on spore production.

It could be observed that in general the spore production of *B. bassiana* was 3-5 fold higher than *B. brongniartii* irrespective of the medium tested. *B. bassiana* has been reported to have a higher metabolism than *B. brongniartii* on a wide range of substrates, paralleled by higher biomass production (Canfora *et al.*, 2017). However since *B. brongniartii* could more efficiently produce blastospores than *B. bassiana* in some of the media for example, RHE and jaggery wherein it bridged or overcame the natural variation in the quantum of spore production (*B. bassiana* usually was higher in spore production compared to

B. brongniartii in standard or most mediums) it could be metabolizing those resources successfully. Few C-sources, mainly amino acids, promoted the growth of *B. brongniartii* over *B. bassiana* (Canfora *et al.*, 2017) and media including rice bran and wheat bran are good sources for starch and cheese permeate and molasses are very rich in sugar (Kamyab, 2009).

It has earlier been shown that isolates of the two *Beauveria* species have a very different metabolic profile displaying very little overlap in carbon source use when grown *in vitro* (Canfora *et al.*, 2017). This probably is the reason why *B. brongniartii* grew the best on CCE while *B. bassiana* thrived well on SSCE and CSCE. In the present study all media performed equally or better than the standard SDB. In an earlier study, rice bran extract, wheat bran extracts (4, 12 and 20%) and cheese permeates showed higher blastospore production than molasses and SDB (Bena-Molaei *et al.*, 2015). Puzzari *et al.*, (1997) reported that 3.9×10^8 conidia/ml of *B. bassiana*

Table 3. Cost effectiveness of media tested for *Beauveria bassiana*

Low-cost media	Blastospores ($\times 10^9$) produced Rupee ⁻¹ at concentrations of media		
	5%	10%	15%
Sesame Seed Cake Extract(SSCE)	3.58	7.51	4.09
Groundnut Seed Cake Extract (GSCE)	2.69	1.33	1.93
Cotton Seed Cake Extract (CSCE)	4.08	3.25	3.83
Coconut Cake Extract(CCE)	2.65	1.89	1.69
Rice Bran Extract(RBE)	2.34	2.16	2.57
Wheat Bran Extract (WBE)	1.31	2.72	2.61
Redgram Husk Extract (RHE)	0.72	1.51	1.08
Jaggery	2.48	2.14	1.34
SD broth	0.68	0.68	0.68

were produced using a medium of rice hulls and saw dust whereas a maximum of 1.5×10^8 conidia/ml was produced using RBE.

All media were found to be as good as or better than the standard medium, SD for a single batch. The suitability of these media may be due to availability of polysaccharides supportive enough for these facultative fungi as suggested by Bena-Molaei *et al.* (2015) who indicated that carbon sources such as dextrose needed by *B. bassiana* can be replaced by polysaccharides such as starch or lipids. However, the impact of such resources on the virulence over generations is to be tested as the type of growth medium and the nutritional and physical conditions of the mass production system greatly affect the number, type, stability, durability and virulence of fungal propagules (Ibrahim *et al.*, 1993; Feng *et al.*, 1994; Fargues *et al.*, 2002) and availability of reports of growth medium having an impact on virulence (Ibrahim *et al.*, 2002; Bena-Molaei *et al.*, 2011) as well as

not impacting virulence (Bena-Molaei *et al.*, 2015 ; Joshi *et al.*, 2016) necessitates such a check.

It was found that SSCE at 10%, 15% and CSCE 5% yielded the maximum number of *B. bassiana* spores per rupee spent on raw material with the SD broth being the least economic with 0.68×10^9 (Table 3). Similarly, WBE at 15% produced the highest number of *B. brongniartii* spores (2.92×10^9) for every rupee spent on material closely followed by RBE at 15% CCE (2.29×10^9) while SD broth was most expensive with 1.59×10^9 /ml (Table 4). Economy of components with reference to spore yield gains importance while meeting the requirement of a huge load of *B. bassiana* spores @ 5×10^{13} /ha (Legaspi *et al.*, 2000; Visalakshi and Bhavani, 2014) and 1×10^{14} /ha of *B. brongniartii* spores (Srikanth *et al.*, 2010) for field application against sugarcane pests. In the current study the cost of the natural substrates was meagre (Table 3 and 4) for production of blastospores of *B. bassiana* and *B. brongniartii* but some of the materials like CSKE involved more

Table 4. Cost effectiveness of media tested for *Beauveris brongniartii*

Medium based on <i>B. brongniartii</i>	Blastospores ($\times 10^9$) produced Rupee ⁻¹ at concentrations of media		
	5%	10%	15%
Sesame Seed Cake Extract (SSCE)	0.59	1.26	0.88
Groundnut Seed Cake Extract (GSCE)	0.62	0.49	0.88
Cotton Seed Cake Extract (CSCE)	0.99	0.72	1.39
Coconut Cake Extract (CCE)	1.79	2.07	1.39
Rice Bran Extract (RBE)	1.65	1.49	2.29
Wheat Bran Extract (WBE)	1.20	1.97	2.92
Redgram Husk Extract (RHE)	0.62	1.72	1.87
Jaggery	0.47	0.32	1.19
SD broth	0.16	0.16	0.16

man-hours in extraction which might require semi automation or training. Analogous notion of time consumption and extraction cost had been expressed for rice bran and wheat bran based media by Bena-Molaei *et al.* (2015).

In the present study, the cost of alternative media escalated due to addition of peptone *i.e.*, by 95-98% indicating that if peptone can be dispensed with, the materials used were 12 (jaggery -15%) to 75 (CSCE-5%) times cheaper than the standard, SDB. Peptone was added at 1% level to ensure adequate N, since Siwach and Jaipal (2004) suggested that inadequate amount of N in media had led to poor growth. While 2% glucose medium produced no blastospores and 1% peptone produced lowest number, in peptone (1%)-glucose (2%) medium, the yield of blastospores of *B.bassiana* was four-fold higher than in glucose (2%) -peptone (1%)-yeast extract (0.2%) . This indicates importance of peptone and that addition of more nitrogen (yeast) need not enhance production (Bidochka *et al.*, 1987). Nitrogen supplements were not found to affect the sporulation of *B.bassiana* and *B.brongniartii* on a molasses based broth (Tamilarasi *et al.*, 2002) but their study did not validate the virulence of such spores. Low nutrition reservoir of blastospores or conidia of *B.bassiana* can be a probable factor for low virulence (Bena-Molaei *et al.*, 2015). It has been indicated that virulence against insects can be improved by culturing *B.bassiana* on selected culture media through higher insect cuticle degradation enzymes and toxins production which would improve its virulence (Safavi *et al.*, 2007).

However experimentation with lower quantum of peptone or alternate source of nitrogen would definitely render these media cheaper. The media were chosen based on their practicality as, though coconut water media, molasses, rice cooked water and rice wash water in a previous work were found to be fit media for production (Joshi *et al.*, 2016) but may be expensive or uncommon or impractical

to be procured in large quantum for production of fungi at cottage industry level. .

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