Vol 3 Issue 10 Nov 2013

ISSN No : 2230-7850

Monthly Multidisciplinary
Research Journal

Indian Streams Research Journal

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RNI MAHMUL/2011/38595 **ISSN No.2230-7850**

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Indian Streams Research Journal
Volume-3, Issue-10, Nov-2013
ISSN 2230-7850

Available online at www.isrj.net

PRODUCTION OF HYDROLYTIC ENZYMES BY *Beauveria Bassiana* ISOLATES ON SOLID MEDIUM

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Abstract: The entomopathogenic fungus, *Beauveria bassiana* is of commercial importance as an alternative to chemical insecticides in an agroecosystem. The fungal pathogen *Beauveria bassiana* is a widely used mycoinsecticide for control of several insect pests, providing a biological alternative to synthetic chemical insecticides. The potential *Beauveria bassiana* isolates (Bb – 1, Bb – 2 and Bb – 3) to produce different hydrolytic enzymes such as chitinase, protease, amylase and lipase was determined in the present study by using the specific substrates. Enzyme index value of extracellular enzymes (Chitinase, Protease, Caseinase, Lipase and Amylase) produced by efficient *Beauveria bassiana* isolates was estimated in the present research. Maximum enzyme activity was observed by the isolate Beauveria bassiana (Bb – 1) followed by *Beauveria bassiana* (Bb – 2) and Beauveria bassiana (Bb – 3). Chitinase and protease activity of *Beauveria bassiana* isolates was investigated during Day – 2, Day – 4, Day – 6, Day – 8 and Day – 10. Maximum chitinase and protease activity was observed by the isolate *Beauveria bassiana* (Bb – 1) at Day – 4 followed by *Beauveria bassiana* (Bb – 2) and *Beauveria bassiana* (Bb – 3). Minimum enzyme activity was recorded at Day – 10.

Keywords: *Beauveria bassiana*, Chitinase, Protease, Caseinase, Lipase and Amylase

1. INTRODUCTION

Insecticides are the only tool in the pest management strategy that is reliable for emergency action when insects at the times of blooming. However, insecticidal control has led to several problems in insect management such as appearance of insecticide resistance pests, pest resurgence, undesirable toxic effects to natural enemies of target pests, disruption of the ecosystem, toxic residues in crop plants and environmental problems. Consequently, the research for new environmentally safe method is being intensified.

The entomopathogenic fungus *Beauveria bassiana* is well known as a potential alternative to chemical pesticides for the control of insect pests and is commercially available for such purposes in numerous countries worldwide. As a broad host range insect pathogen, strains of this fungus have been exploited for use against crop and invasive pests as well as for insects that act as human and animal disease vectors such as mosquitoes and ticks (Kirkland et al., 2004; De Faria and Wraight, 2007; Farenhorst, 2009). Aside from its interest as a pest biological control agent, Beauveria bassiana is also an emerging model organism that can be used to examine unique aspects of fungal growth and development including host pathogen interactions (Jin et al., 2010; Lewis, 2009; Wanchoo, 2009). Infection of insects does not require any specialized mode of entry and begins with attachment of fungal spores to the target hosts. In response to cuticle surface cues, the fungus germinates, and the emerging germ tubes produce a variety of enzymes that combined with mechanical pressure begin the process of cuticle penetration. In this regards, the surface characteristics of the infectious fungal spores as well as several genetic determinants of virulence have been characterized (Holder and Keyhani, 2005; Holder, 2007; Fang, 2008; Fang, 2009).

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 (De La Rosa et al., 2000; Brownbridge et al., 2001; Butt et al., 2001). The fungus is a facultative saprophyte and there are reports of Beauveria bassiana growing as a plant endophyte and interacting with plant roots (Wagner and Lewis, 2000; Lewis et al., 2001; White et al., 2002).

Although, a sexual stage is now known (Li et al., 2001) most *Beauveria bassiana* exist as asexual organisms, reproducing mainly through the production of single cell conidia. *Beauveria bassiana* produce three single cell forms, aerial conidia, in vitro blastospores and submerged conidia in different conditions (Jeffs et al., 1999). Aerial conidia are produced on the surface of solid medium by a process of hyphal extension, formation of phialides (rachis) and spore production. Aerial conidia usually are used for biological control agents because they are relatively resistant to varying

environmental conditions and can be formulated to prolong shelf life. Aerial conidia contain a rodlet layer that results in a hydrophobic property (Holder and Keyhani, 2005). Blastospores are produced in nutrient liquid medium. They are hydrophilic, and they germinate and grow at much higher rate than aerial conidia. Submerged conidia are produced in defined liquid medium (Thomas et al., 1987). They are also hydrophilic, showing a rough surface morphology. Submerged conidia represent an important developmental stage for growth in a limited nutrient medium.

2. MATERIALS AND METHOD

2.1. Inoculum multiplication

Seven to ten days old culture on SDA slant was used for preparing spore suspension in 0.02% Tween 80 solution at 1 × 106 spores ml^{-1}. 200 µl of 106 spores was poured on SDA medium individually and was incubated for 3 days at 28°C. At the end of 3 days, 5 mm mycelial disc with agar was retrieved with help of cork borer and placed in middle of fresh test substrate plates and incubated at 27°C for 8 days. The control plates did not have the substrate on them, they just had minimal media solidified on 2 per cent agar. Enzyme activities were calculated as an index of the total diameter of the colony + halo divided by the diameter of the colony. Enzymatic index value >1.0 indicates enzyme activity minimal media solidified on 2 per cent agar. Enzyme activities were calculated as an index of the total diameter of the colony + halo divided by the diameter of the colony. Enzymatic index value > 1.0 indicates enzyme activity

2.2. Test for chitinase production

2.2.1. Colloidal chitin

Colloidal chitin was prepared by a modified method suggested by Shimahara and Takiguchi (1988). Ten Grams of practical - grade crab shell chitin was mixed with 100 ml 12 N hydrochloric acid with a continuous stirring for about 2 hrs at 4°C. The suspension was mixed with 1 litre of water and filtered. The step was repeated 4 - 5 times and then the pH of the suspension was brought to neutralization by addition of 5 N NaOH and again colloidal suspension was washed several times with double distilled water for desalting.

2.2.2. Chitinase production test

Two per cent colloidal chitin was used to prepare chitin agar plates and inoculated with the mycelial disc over the medium and incubated at 28°C for 8 days. At the end of incubation period, a thin layer of 0.002% Calcofluor white was spread on the culture plates and left for incubation for few more hours after which they were observed. Presence of a zone of clearance indicated chitinase activity. The diameter of the fungal colony was measured both exclusively and inclusively of the surrounding halo and enzymatic index values were ascertained.

2.3. Test for protease production

One per cent gelatin extract in minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% KPO_4) and pH was adjusted to 7.0 before autoclaving, was used for in vitro estimation of protease. The plates were inoculated with a mycelial disc over the medium and incubated at 28°C for 8 days. At the end of the incubation period, the culture plates were flooded with a solution of 1.5 per cent mercuric chloride in 2 N HC1. A distinct transparent zone of clearance could be seen around the colony while the rest of the plate appeared translucent white in colour.

2.4. Test for caseinase production

Milk Agar @ 2.4 g per 100 ml was used as substrate (pH 7.2 at 25°C) for caseinase activity. The plates were inoculated with the mycelial disc over the medium and incubated at 25°C for 8 days. At the end of the incubation period, a clear transparent halo could be seen around the colony while the rest of the plate appeared opaque white in colour.

2.5. Test for Lipase production

One per cent Tween 20 was used for in vitro lipase estimation. The other components of the media included 1 per cent peptone, 500 mg NaCl, 10 mg CaCl2 and 2 per cent agar (for solidifying), pH adjusted to 6.0 before autoclaving. The plates were inoculated with the mycelial disc over the medium and incubated at 28°C for 8 days. At the end of the incubation period, formation of lipolytic enzymes by a colony was seen as either a visible precipitate due to the formation of crystals of the calcium salt of the lauric acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid.

2.6. Test for Amylase production

Starch Agar @ 30 g per 1000 ml, containing starch as the sole carbon source was used as substrate with pH 6.4 at 25°C. About 20 ml of sterile starch agar media was poured in 9 cm petriplates. The plates were inoculated with the mycelial disc over the medium and incubated at 28°C for 8 days. Colony growth of the fungus implies that starch has been degraded. At the end of the incubation period, the culture plates were flooded with Lugol's Iodine solution (1 g iodine and 2 g potassium iodide in 300 ml distilled water) and a yellow colored halo could be seen around the colony in an otherwise blue medium indicating amylolytic activity. After each assay, the enzymatic index values were calculated using the formulae.

$$\text{Enzymatic Index Value} + \frac{\text{Total diameter of the colony} + \text{halo}}{\text{Diameter of the colony}}$$

2.7. Estimation of Chitinase and Protease produced by *Beauveria bassiana* isolates

2.7.1. Chitinase estimation

Chitinase assay was done by the method of Yanai et al (1992). 500 µl of respective culture supernatant was incubated with 300 ml of 10 per cent (w/v) colloidal chitin and 200 ml of 0.2 M acetate buffer (pH 4) at 37°C for 2 hrs. The reaction product N - acetyl glucosamine was determined by using para – dimethyl - Amino benzaldehyde reagent. Absorbance at 585 nm was taken using UV visible Spectrophotometer (Elico Ltd., India) against water as blank. Sampling was done every two days till tenth day of culture incubation. One unit of chitinase activity was defined by the

amount of enzyme that produced 1 µM of N- acetyl glucosamine per minute under the above conditions.

2.7.2. Protease estimation

Proteolytic activity was assayed by a modified method of Kunitz (1947). 100 µl enzyme samples were incubated with 400 µl of 0.5 per cent (w/v) gelatin in 50 mM Tris - HCl buffer, pH 10.0, at 50°C for 20 min. The enzyme reaction was terminated by addition of 500 µl 10 per cent (w/v) trichloroacetic acid and kept at room temperature for 10 min. The reaction mixture was centrifuged at 10,000 g for 10 min at 4°C and the absorbance measured against water as blank at 280 nm. One unit of proteases was defined as the amount of enzyme releasing the equivalent of 1 µM of tyrosine per minute under the defined assay conditions.

3. RESULTS AND DISCUSSION

The fungus produces several classes of cuticle degrading enzymes such as proteases, chitinases and lipases upon germination. The lack of production of these enzymes in some strains of Beauveria bassiana may delay the infection process in certain insect species (Bidochka and Khachatourians, 1990; Hallsworth and Magan, 1996; Fargues et al., 1997; Boucias and Pendland, 1998).

Apart from cell wall degrading enzymes secreted by a wide variety of saprophytic and phytopathogenic micro organisms (Bailey and Pessa, 1990), the pectinases also play an important role in the entry of the plant pathogen intra and inter cellular into the host tissues thus blocking the conducting vessels resulting in the development of wilt. Most pathogens have the capacity to produce more cellulolytic than pectolytic enzymes (Sadik and Payak, 1983). The correlation between stalk rot and cellulase production was significant in maize (Chambers, 1987). Lipases and proteases are important enzymes in pathogenesis which attack the plasmalemma after the degradation of cell wall by proteases along with pectolytic and cellulolytic enzymes (Hameed et al., 1994). Not much work seems to have been reported on the enzyme production by pathogens causing corn stalk rot disease. Experiments were therefore carried out to study the production of enzymes by fungal pathogens associated with corn stalk rot disease and the role of these enzymes in pathogenesis.

The potential of the isolates *Beauveria bassian*a (Bb – 1), *Beauveria bassiana* (Bb – 2) and *Beauveria bassiana* (Bb – 3) to produce different degradative enzymes namely chitinase, protease, caseinase, lipase and amylase in medium containing respective substrates were studied in the present research and the results are presented in Table - 1. All the isolates produced the various degradative enzymes studied in the present research work. However, the quantity of each enzyme produced between the isolates varied significantly. The enzyme index worked out for chitinase, protease, caseinase, lipase and amylase is highest for the isolate *Beauveria bassiana* (Bb – 1) and the values were 2.80, 2.56, 3.45, 2.96 and 1.90 respectively. The isolate *Beauveria bassiana* (Bb – 3) showed least enzyme index value of 1.23 for chitinase, 1.63 for protease, 2.98 for caseinase, 2.89 for lipase and 0.033 for amylase.

Enzymes such as proteases, lipases and chitinases are detected on germ rubes (Pekrul and Grula, 1979; Smith and Grula, 1981). Lipases and proteases could aid growth of Beauveria bassiana by solubilizing tissue such as fat body or any host tissue that is fatty or proteinaceous. Successful infection depends on the percentage of spore germination, rate of germination (dependent on temperature and humidity), aggressiveness of the fungus and host specificity.

Feng et al (1994) stated that chitinase enzyme *Beauveria bassiana* led to an increase in its virulence potential. Several workers have reported a positive correlation between production of extracellular enzymes and vimlence potential. Specific enzymes or several of them in an enzyme cascade may serve as key virulence determinants. Since enzymes were differentially expressed in test media, it is also possible that the enzymes may be involved in host range determination. In the present study, chitinase production by *Beauveria bassiana* isolates was studied at every 48 hrs intervals upto 10 days of incubation and the results obtained are presented in Table - 2. The chitinase activity of the isolates was higher on 6th day of incubation and there after decreased. The chitinase activity of *Beauveria bassiana* (Bb – 1) isolate was higher than that of *Beauveria bassiana* (Bb – 2) and *Beauveria bassiana* (Bb – 3). On the 4th day of incubation, the isolate *Beauveria bassiana* (Bb – 1) recorded the highest chitinase activity of 50.25 U ml-1 followed by *Beauveria bassiana* (Bb – 2) (43.12 U ml-1) and *Beauveria bassiana* (Bb – 3) (41.33 U ml-1).

Proteases are considered the major cuticular degrading enzyme, and their activity appears to precede the action of chitinases (Smith et al., 1981; Pascual et al., 2002). In the present study, protease production by *Beauveria bassiana* isolates was studied upto 10 days of incubation and the results were given in Table – 3. The protease activity was higher in the 8th day of incubation. The protease production by *Beauveria bassiana* isolates varied significantly between the isolates and period of incubation. The isolate *Beauveria bassiana* (Bb – 1) produced the highest value of 1.25 U ml-1 for protease followed by *Beauveria bassiana* (Bb – 2) (1.20 U ml-1) and *Beauveria bassiana* (Bb – 3) (1.08 U ml-1) on 8th day of incubation and the protease production decreased with increase in incubation period.

Table – 1: Enzyme index value of extracellular enzymes produced by efficient *Beauveria bassiana* isolates

S. No	Isolates	Enzymes				
		Chitinase	Protease	Caseinase	Lipase	Amylase
1	*Beauveria bassiana* (Bb – 1)	2.80	2.55	3.45	2.96	1.90
2	*Beauveria bassiana* (Bb – 2)	2.52	2.30	3.20	2.89	1.53
3	*Beauveria bassiana* (Bb – 3)	2.15	2.13	3.02	2.66	1.38

Enzymatic Index Value = (total diameter of the colony + halo) / diameter of the colony

Table – 2: Chitinase activity of *Beauveria bassiana* isolates

S. No	Isolates	Chitinase activity (U ml^{1}) as on day				
		Day - 2	Day – 4	Day - 6	Day - 8	Day - 10
1	*Beauveria bassiana* (Bb – 1)	21.68	50.25	49.53	22.65	13.65
2	*Beauveria bassiana* (Bb – 2)	19.52	43.12	42.15	20.18	12.52
3	*Beauveria bassiana* (Bb – 3)	18.32	41.33	40.25	17.75	09.85

Table – 3: Protease activity of *Beauveria bassiana* isolates

S. No	Isolates	Protease activity (U ml^{1}) as on day				
		Day - 2	Day – 4	Day - 6	Day - 8	Day - 10
1	*Beauveria bassiana* (Bb – 1)	0.69	0.75	1.05	1.25	0.98
2	*Beauveria bassiana* (Bb – 2)	0.60	0.68	0.95	1.20	0.91
3	*Beauveria bassiana* (Bb – 3)	0.57	0.59	0.88	1.08	0.83

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