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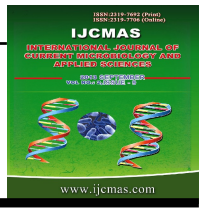

Original Research Article

Effect of process parameters on the enzyme activity of a novel *Beauveria bassiana* isolate

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A B S T R A C T

Enzyme production potential of a native *Beauveria bassiana* (HQ917687) isolate was elucidated through the assessment of effect of incubation period and temperature. Evaluation of enzyme activity of fungal filtrate showed increase in activity with increase in incubation time. Maximum enzyme activity was observed on 5th day [chitinase (5.24 U/ml), Pr1 protease (4.66 U/ml), lipase (2.26 U/ml)] or 4th day [pr2 protease (5.48 U/ml)] of the culture growth. The enzymes showed variation in activity with temperature, with maximum chitinase (5.05 U/ml) and pr1 (4.31 U/ml) activity at 40 ºC, whereas maximum activity of pr2 was observed at 50 ºC (4.52 U/ml). The optimum activity of enzyme was observed between pH 5-7, while beyond this range decrease in enzyme activity was observed with increase in medium acidity or alkalinity. The results obtained from this study are expected to help in experimental design and biotechnological modification using particular strain of *B. bassiana*.

Keywords

Beauveria bassiana; enzyme; incubation period; temperature; activity.

Introduction

Beauveria bassiana infection to the host insects involves penetration of insect cuticle through enzymatic and mechanical degradation (St. Leger *et al.,* 1996; Parani *et al.,* 2011). The insect cuticle is made of chitin framework, protected by protein and lipid. The fungal conidia adhere to the cuticle surface to produce a penetration structure called appresoria, which produces a series of extracellular enzymes; chitinase, protease and lipase, involved in the enzymatic degradation of chitin, protein and lipid layer of insect cuticle, respectively (Inglis *et al.,* 2001; Dhar and Kaur, 2010; Kumar *et al.,* 2011). The enzymes are not only involved in host penetration but have also been positively correlated with entomopathogenic fungal pathogenesis (Mustafa and Kaur, 2010; St. Leger *et al.,* 1996).

Fungal chitinase belongs to family 18 or 20 of glycosyl hydrolases, on the basis of amino acid sequence similarity of the catalytic domains (Xia *et al.,* 2011). In fungus, this catalytic domain contains a chitin-binding site which facilitates efficient degradation of chitin while

leaving aside soluble substrates (Fan *et al.*, 2007). Fungal proteases are primarily, subtilisin-like serine protease (Pr1) and trypsin-like protease (Pr2). Proteases Pr1 is indispensable for cuticle penetration by helping cuticle degradation through peptide bond, whereas Pr2 is involved with regulation of Pr1 activity. Lipase, although not very prominent contributor of extracellular enzyme consortium, has a significant contribution in cuticle penetration through degradation of cuticle lipid.

Production of these extracellular enzymes is governed by environmental and physical factors (Matsumoto, 2006; Dhar and Kaur, 2010; Luis *et al.*, 2012). Temperature, pH and culture age are principal factors influencing their induction and activity. Moreover, variation in the enzyme activity has been accounted for strain variation among *B. bassiana* isolates, which may possibly be involved in their host range determination (Gupta *et al.*, 1992; Dhar and Kaur, 2010; Mustafa and Kaur, 2010). In view of the above facts, the present study evaluated the effect of incubation period, temperature and pH on the enzyme (chitinase, protease Pr1 & Pr2, lipase) activity of *B. bassiana* HQ917687.

Materials and Methods

Fungal strain and culture condition

Beauveria bassiana HQ917687 used in the present study was isolated in our laboratory from soil samples collected from Northern part of Uttar Pradesh, India (Data unpublished). The efficacy of fungal isolate against housefly has been reported earlier (Mishra and Malik, 2012). The fungal isolate was maintained on Potato Dextrose Agar slants at 4°C. Fresh slants cultured for 5 days were used in inoculums preparation for liquid culture.

Preparation of filtrate for enzyme assay ml basal media [containing (per liter) KH_2PO_4 1g, $MgSO_4.7H_2O$ 0.5g, $FeSO_4.7H_2O$ 0.2g, $ZnSO_4.7H_2O$ 0.1g, NaCl 0.5g and chitin (1%)] in a 250 ml conical flak. The flasks were incubated at 28±2 ºC at 180 rpm for 5 days.

Following growth of fungus in basal medium, the mycelium was harvested by centrifugation of cultured broth at 8000 g and 4 ºC for 20 min, followed by filtration (0.2 µm). Supernatant (10 ml) obtained through filtration was incorporated with crystalline ammonium sulfate, through slow addition and gentle stirring until 90% (w/v) saturation and held at 4 ºC for 12 h to obtain the precipitate (Kim and Je, 2010). The precipitate was dissolved in 0.1 M citrate-phosphate buffer (pH 6) to adjust to the original volume (10 ml) of intact supernatant.

Effect of incubation period on enzyme activity

The fungal precipitate was prepared (described above) for each day of culture growth till sixth day. The precipitate from each day was used to determine the enzyme activity.

Effect of temperature on enzyme activity

The precipitate obtained from the fungal culture (described above) was subjected to temperature variation from 10-80 ºC to assess the effect of temperature on enzyme activity (chitinase and protease). The precipitates were kept at different temperature for 30 min, followed by incubation at 37 ºC for 1 h. After the incubation period, precipitates were subjected to kinetic assay using spectrophotometer at 405 nm.

Effect of pH on enzyme activity

The pH effect on enzyme activity (chitinase and protease) was measured by adjusting the pH of the precipitates over a pH range from 4 to 10, using the universal buffer. These precipitates with varied pH were incubated at 37 ºC for 1 h. After 1 h, spectrophotometeric assay of the precipitates was performed at 405 nm.

Enzyme assay for chitinase activity

Chitinase activity assay was done by determining the release of p-nitrophenol from p-nitrophenyl-β-D-acetylglucos aminide (pNG) (Kim *et al.,* 2011). A 100-µl fungal precipitate prepared earlier (described above) was mixed with 100 µl of 10 mM pNG (Sigma-Aldrich) and 300 µl of 0.1 M citrate-phosphate buffer (pH 6). After incubation at 37 ºC for 1 h, 500 µl of 1 M sodium carbonate was added into the solution. The kinetic assay was performed using a spectrophotometer (UV-260, Shimadzu) at 405 nm. One unit of chitinase activity was defined as the amount of enzyme needed to release one millimole of p-nitrophenol per hour per ml. Each treatment was replicated three times while the entire assay was repeated twice using different batches of enzyme pellet.

Enzyme assay for protease activity

The activity of subtilisin-like (Pr1) protease and trypsin-like (Pr2) protease was assessed according to the method described by Lakshmi *et al.,* (2010). The assay of Pr1 protease was done by determining the release of N-Suc-$(Ala)_2$-Pro-Phe-p-nitroanilide (substrate for pr1 assay) while for Pr protease assay, the release of benzoyl-Phe-Val-Arg- p-nitroanilide (substrate for pr2 assay) was determined. The reaction mixture included 15 µl substrate (2µM), 10 µl of fungal precipitate prepared earlier (described in section above) and 75 µl of 50 mM Tris-Hcl (pH 8.0). The kinetic assay was performed using a spectrophotometer (UV-260, Shimadzu) at 405 nm. One unit of protease activity was defined as the amount of enzyme needed to release 100 nanomoles of nitroanilide per ml per hour at 37 ºC.

Enzyme assay for lipase activity

Activity of lipase was determined by titrimetric method (Cordenons *et al.*, 1996). In this procedure, native substrate (triacylglycerols) are hydrolyzed to yield fatty acids. Reactivity of the reactive mixtures was quenched by the addition of ethanol. The amount of fatty acids released during the reaction was determined by direct titration with NaOH to a thymolphthalein end point. The method includes addition of supernatant (5 ml) into olive oil/gum arabic emulsion substrate (20 ml; 5% w/v) which was preincubated for 15 min in a 37 ºC water bath along with magnetic stirring. 5 ml of this reaction mixture was removed and transferred to titration cocktail containing 10 ml of 95% (v/v) ethanol and 2 to 3 drops of 1% (w/v) thymolphthalein indicator. The contents were titrated with 0.05 N NaOH using a burette until a light blue color appears. Quantity of fatty acids liberated in samples was determined by equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following equation:

$$\mu\text{mol fatty acid/ml subsampl} = \frac{[(\text{ml NaOH for sample} - \text{ml NaOH for blank}) \times N \times 1000]}{5\ \text{ml}}$$

where *N* is the normality of the NaOH titrant used (0.05 in this case). Lipase activity (U/ml) was calculated by

determining the amount of supernatant that produces 1 µmol of fatty acid per minute under the specified assay conditions.

Statistical analysis

In shake-flask experiments, all treatments were run in triplicate and all experiments were repeated at thrice. Data calculated were the mean of values from different replicates. For data not suitable for ANOVA, standard error values were calculated and provided as a measure of variance using StatPlus (2007).

Result and Discussion

Effect of incubation period on enzyme activity

The culture precipitate of *B. bassiana* was tested for enzyme activity during 6 days of culture growth. Substantial activity for tested enzymes was observed during first day of fungal growth (Table 1). Activity of chitinase increased with increase in incubation time, reaching its maximum activity of 5.24 U/ml on 5^{th} day of culture growth, after which decrease in chitinase activity was recorded. For pr1 and pr2 proteases, activity showed steady increase with incubation time. Pr1 protease showed maximum activity of 4.66 U/ml, on 5^{th} day of incubation while maximum activity for pr2 protease was recorded to be 5.48 U/ml on 4^{th} day of incubation. The lipase activity varied between 0.24-2.26 U/ml for different incubation period, with maximum activity observed on 5^{th} day of incubation.

Effect of temperature on enzyme activity

Effect of temperature on enzyme activity is represented in Fig. 1. Maximum chitinase activity (5.05 U/ml) was obtained at 40 ºC, followed by activity at 30 ºC (5.00 U/ml). Beyond 40 ºC, the enzyme showed steady decrease in its activity. Maximum activity of protease, pr1 was observed at 40 ºC (4.31 U/ml), followed by at 50 ºC (3.98 U/ml). For pr2 protease, maximum activity was observed at 50 ºC (4.52 U/ml). Minimum activity for both, pr1 (2.77 U/ml) and pr2 (2.21 U/ml) protease was observed at 10 ºC.

Effect of pH on enzyme activity

Effect of pH on enzyme activity is represented in Fig. 2. Each enzyme showed optimum activity between pH 5-7, while beyond this range decrease in enzyme activity was observed with increase in medium acidity or alkalinity. Chitinase showed maximum activity at pH 5 (4.78 U/ml), followed by that at pH 6 (4.56 U/ml). Maximum activity of protease, pr1 and pr2 was observed at pH 6 (4.88 U/ml) and pH 5 (4.29 U/ml), respectively. Activity of both the protease showed drastic decrease with increase in acidity below pH 5, while decrease in activity was gradual beyond pH 7.

Extracellular enzymes produced by entomopathogenic fungi are correlated to their pathogenic activity. The role of chitinase activity in pathogenesis of entomopathogenic fungi is well established (Kang *et al.,* 1999; Kim and Je, 2010; Mustafa and Kaur, 2010). Similarly, proteases have been reported for their role in insect pathogenecity (Namasivayam *et al.,* 2010). The production and activity of these enzymes in *B. bassiana* have said to be influenced by variation in process parameters; viz. environmental and physical factors. Activity of protease (Pr1 and Pr2) after 24 h of culture growth was reported from

Table.1 Effect of incubation period on enzyme activity of *B. bassiana*

Days	Activity* (U/ml, mean ± SD)			
	pr 1	pr 2	Chitinase	Lipase
1	1.85^{a} + 0.21	1.84^{a} + 0.18	1.42^{a} + 0.10	0.24^{a} + 0.07
2	3.88^{b} + 0.09	3.69^{b} + 0.09	3.11^{b} + 0.21	0.94^{b} + 0.09
3	4.09^{c} + 0.12	3.98^{c} + 0.17	3.86^{c} + 0.14	1.35^{c} + 0.13
4	5.48^{d} + 0.14	4.28^{cd} + 0.33	4.62^{d} + 0.16	1.82^{d} + 0.13
5	4.73^{e} + 0.11	4.66^{d} + 0.19	5.24^{e} + 0.12	2.26^{e} + 0.14
6	4.23^{cf} + 0.13	4.04^{c} + 0.24	4.31^{f} + 0.15	1.96^{df} + 0.09

*Values within a column followed by the same superscript letter are not significantly different ($P < 0.05$). SD, standard deviation

Figure.1 Effect of temperature on enzyme activity (mean±SD, n=3) of *B. bassiana*

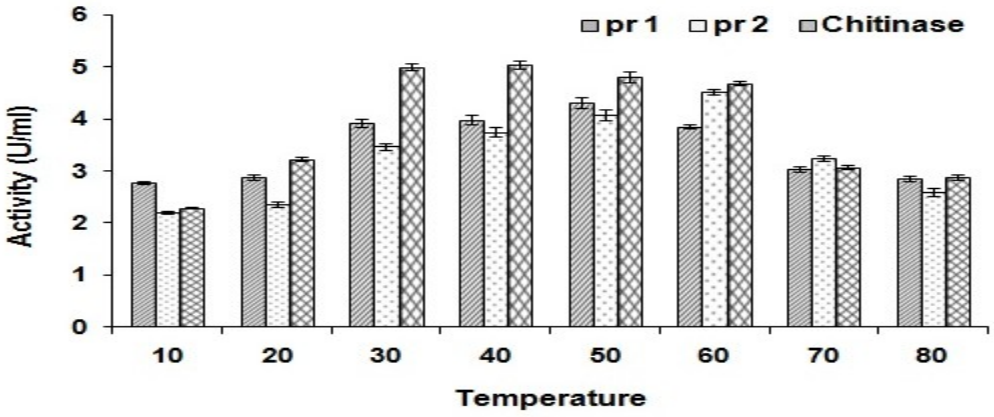
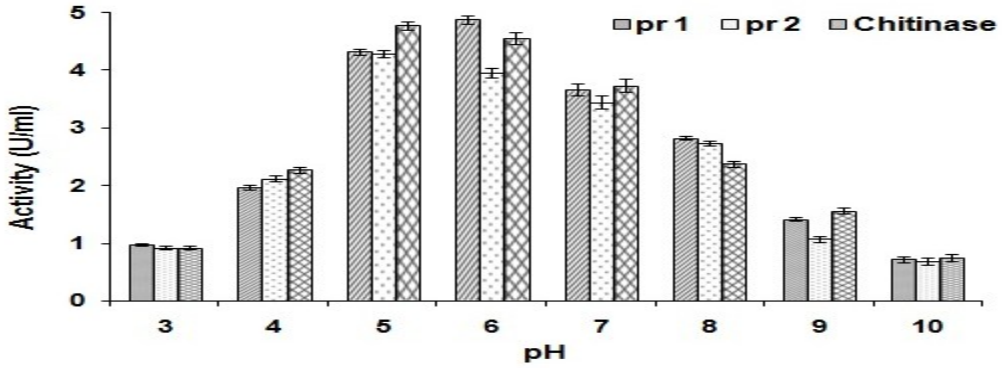


Figure.2 Effect of pH on enzyme activity (mean±SD, n=3) of *B. bassiana*



B. bassiana in the Presence of cuticle containing medium (Donatti *et al.,* 2008). Authors' reported high level of Pr1 (0.258 U/µg protein) in the medium supplemented with methionine, and attributed this to regulatory role of amino acid on protease secretion. Ito *et al.,* (2007) demonstrated maximum protease activity (0.489 U/ml) on 5th day of culture in *B. bassiana*. In the study by Mustafa and Kaur (2010), different isolates of *B. bassiana* showed maximum chitinase activity of 6.96 to 46.49 U/ml on 4th day of fungal growth in a SDA (Sabouraud dextrose Agar) media. The activity in their study was determined after incubation of culture supernatant with 10 % (w/v) colloidal chitin for 2 h at 37 ºC.

Dhar and Kaur (2010a) performed enzyme activity assay with 17 isolates of *B. bassiana* on four different substrate medium, and observed highest chitinase activity (2.64-35.08 U/ml) in 4-6 days of culture growth. The enzyme activity in their study significantly varied with variance in substrate and type of isolates. In their study, increase in enzyme activity was reported with increase in incubation period for upto 6 days, where after further incubation led to decrease in activity, probably due to nutrient limitations or autolysis of the culture. Dhar and Kaur (2010b) reported variation (0.11-0.90 U/ml) in maximum protease activity (days 4-8) with different isolates of *B. bassiana* cultured on basal salts medium containing 1% casein. The above discussion reflects dependence on medium type for enzyme activity. This presents a difficulty for comparison between results of two studies owing to different culture medium, and also due to variation in enzyme activity assessment methodology. However, this presents a scope for further improvement in enzyme activity with change in growth medium for entomopathogenic fungi. Further, variation in enzyme activity is also subjected to fungal strain (Gupta *et al.,* , 1992; Paterson *et al.,* 1993; Donatti *et al.,* 2008; Dhar and Kaur, 2010b).

For protease activity, preincubation at temperatures between 30-50 °C caused little loss in activity for Prl, while no loss in activity was reported for Pr2 (St. Leger *et al.,* 1987). In the same study, activity of enzyme was shown to be heat denaturated at the incubation temperatures over 50°C, while at 60°C <20% of initial activities of Prl or Pr2 was observed. Compared to these results, the protease activity in the current study showed better temperature tolerance.

St. Leger *et al.,* (1998) observed regulation of virulence genes expression at ambient pH in *M. anisopliae*. The optimum pH for the activity of protease, Prl and Pr2 of various entomopathogenic fungi was found to be 8.0 and 9.0 U/ml, respectively (St. Leger *et al.,* , 1987). Similarly, Pr1 production by *M. anisopliae* showed derepression at alkaline pH (St. Leger *et al.,* 1998). The phenomenon has been attributed to the facilitation in the utilization of proteinaceus substrates by alkalinization (St. Leger *et al.,* 1999). Bidochka and Khachatourians (1988) reported *B. bassiana* protease stability and activity between pH values 5.5 and 8.5. Coudron *et al.,* (1984) reported maximum chitinase activity of entomopathogenic fungi between pH 4.0 and 5.2. They suggested the requirement of an ionized acidic group and a protonated basic group for chitinase activity, emphasizing pH regulation for enzyme activity. From the discussion of the above studies, it could be observed that enzyme activity from entomopathogenic fungi encompassed a broad range of pH. However, in the present study, the optimum enzyme activity was observed only between pH 5-7.

Although, previously there have been several studies evaluating effect of substrate, temperature and pH on enzyme activity (St. Leger *et al.,* 1998; Dhar and Kaur, 2010a, 2010b; Mustafa and Kaur, 2010), present study holds significance with its evaluation of enzyme activity in a new strain of *B. bassiana*. The fungal strain investigated here has earlier been proven for its significant pathogenecity against housefly (Mishra and Malik, 2012). The extracellular enzymes discussed in this study acts locally to damage host cells and have the immediate effect on facilitating the growth and spread of the pathogen by affecting physical properties of host tissue matrices and intercellular spaces. Although, they may not actually kill cells as part of their range of activity, even so, their role in colonization or invasion plays a significant role during early stages of an infection and establishment of pathogenesis. Thus, the interrelationship between enzyme activity and process parameters obtained in this study could in general be used for making a logical inference on pathogenecity of a particular fungi based on substrate used for incubation, time period for incubation and environmental parameters. Further, as enzyme activity varies with variation in fungal strain, the results from present study would helps in deducing enzyme activity profile for the particular fungi. The results obtained are expected to help in experimental design and biotechnological modification using *B. bassiana* isolate.

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