

Enhancement and Detection of Chitinase Enzyme of Fungus for the Eradication of Pests

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Abstract

Chitinase is an enzyme having the ability to degrade chitin to a low molecular weight chito oligomers which have a wide range of applications. In the present study, chitinase was produced from the entomopathogenic fungi such as *Pochonia chlamydosporia*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* using chitin as a carbon source. The enzyme was partially purified by ammonium sulphate precipitation and the activity was detected using colorimetric method. The enzyme showed the maximum activity in *Trichoderma harzianum* among the four fungal species at 40°C and pH 6.5. This enzyme could be used directly to control of pathogenic fungi and various pests in the egg stage itself.

Keywords: Chitinase, Chitosan, *Pochonia Chlamydosporia*, *Beauveria Bassiana*, *Metarhizium Anisopliae*, *Trichoderma Harzianum*

I. INTRODUCTION

Dendrobium is a diverse genus of orchids which is easily discernible from other plant species in the wild by its very distinguishable and unique flower, which comes in many colours, size. It is very expensive. This plant is attacked by the pest - *Dendrobium blossom midge*, *Contanaria maculipennis* and the pest epicuticle is made up of chitin; it acts as a physical barrier to the pest. There is no effective biological or chemical control against blossom midge [1]. Chitin is a β -1, 4 N-acetyl glycosamine polymer which is 2-acetamido-2-deoxy-D-glucose linked by β -(1-4) glycosidic bonds and is the second highest occurring biopolymer [2]. It exists in nature in three different forms α -chitin, β -chitin and γ -chitin [3] and it is found in various species such as marine vertebrates, insects, fungi and algae [4], it is mainly obtained from marine vertebrates for commercial purpose and more than 80,000 metric tons of chitin is produced per year from marine waste [5]. Alkaline hydrolysis of chitin chains give rise to chitosan, which is relatively nontoxic and has antimicrobial properties.

Chitinases (E.C.3.2.2.14) are glycosyl hydrolases, having the ability to degrade chitin as a renewable source [4]. It is classified into two main classes, endochitinase and exochitinase. The endochitinase randomly splits chitin at internal sides, thereby forming the di-cetylchitotriose and low molecular mass multimers of GlcNAc. The exochitinase cleave chitin from its nonreducing end, releasing dimers (GlcNAc)₂ [6]. Chitinase plays an effective role in the controlling of pests and insects that can be an alternative to chemical pesticides and it is present in a wide range of organisms such as bacteria, virus, protozoa, fungi, plants and animals to reshape their own chitin [7]. Among all the biological control agents, entomopathogenic fungi play a major role in controlling pest due to its broad host range.

The entomopathogenic fungi like *Pochonia chlamydosporia*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* are more efficient for the control of pathogens by the production of chitinase which are secreted by fungi in liquid culture supplemented with chitin as a carbon source [8]. The enzyme could either be used directly in the biological control or through gene manipulation [9]. Apart from application of chitinase as biopesticides, the chitinase have also been used for production of single cell proteins [10], and for the production of fungal protoplasts [11]. The present study was aimed to detect chitinase enzyme in entomopathogenic fungi for the eradication of pests employing chitin as a carbon source.

II. MATERIALS AND METHODS

A. Preparation of Chitin

Chitin was obtained from Genewin Biotech, R&D Division, Hosur, Tamil Nadu. 1 g of chitin powder was added very slowly in 1% acetic acid and was shaken vigorously for 15 minutes at room temperature.

B. Microorganisms and Culture Condition

The entamopathogenic fungi such as *Pochonia chlamydosporia*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* were obtained from Genewin Biotech, R&D division, Hosur, Tamil Nadu. The fungal species were grown in 200mL Erlenmeyer flasks containing 100 ml of the optimal medium. *Pochonia chlamydosporia*: Sabouraud Dextrose Broth (pH 6); *Beauveria bassiana*: Sabouraud Dextrose Broth (pH 5.6 ± 0.2); *Metarhizium anisopliae*: Sabouraud Maltose Broth (pH 5.6 ± 0.2); *Trichoderma harzianum*: Production media (pH 6).

C. Production of Enzyme

The flasks were inoculated with different fungal species and incubated for 5 days at 28°C on a rotary shaker at 200rpm. After 5 days, the culture medium was centrifuged at 10,000 rpm.

D. Partial Purification of Enzyme

The crude enzyme extract was precipitated with ammonium sulphate (90%) at room temperature and incubated overnight. The precipitate was collected by centrifugation at 10,000 g for 10 minutes and then the precipitate was allowed to dissolve in 20 ml of 0.1M sodium phosphate buffer at pH 6. It was dialyzed against the same buffer overnight.

E. Chitinase Assay

Chitinase activity was determined using colorimetric method [12]. One unit of the chitinase activity was defined as the amount of enzyme that catalysed the release of 1mM of β -1, 4-N-acetyl-D-glucosamine/min at 25°C.

F. Effect of Temperature on Enzyme Activity

The optimum temperature was determined by subjecting the crude enzyme extract to various temperatures viz., 30°C, 35°C, 40°C under standard assay conditions using chitinase as the substrate.

G. Effect of pH on Enzyme activity

The effect of pH on enzyme activity was measured by incubating the crude enzyme extract at different pH (5.5, 6.5, and 7) under standard assay conditions using chitosan as a substrate.

III. RESULTS AND DISCUSSION

The four fungal species were grown aerobically in 100 ml of production medium at 30°C for 5 days. The chitinase production increased progressively and reached the maximum level after 5 days (Table. 1).

Among the four fungi species, *Trichoderma harzianum* has the maximum activity after 120 hours of incubation (6.4 U/ml). The results of *Beauveria bassiana* was in accordance with the findings Sapna Mishra et al., (2013), [13], who reported a maximum enzyme activity(5.28 U/ml) after 120 hours of incubation. Cirano and John, (1991), [14] performed enzyme activity in *Trichoderma harzianum* and observed highest chitinase activity (2.06U/ml) in second day of culture growth.

Table – 1
Chitinase activity of four different fungus

Fungal species	Specific activity (U/ml)
<i>Pochonia chlamydosporia</i>	3.5
<i>Beauveria bassiana</i>	5.08
<i>Metarhizium anisopliae</i>	1.26
<i>Trichoderma harzianum</i>	6.4

The partial purification of crude enzyme extract was done by ammonium sulphate precipitation. Saturation of 90% was obtained by adding 15 ml ammonium sulphate to 100 ml of crude enzyme extract (Figure.1). After centrifugation, the precipitate was dissolved in Phosphate Buffer Saline (PBS) and then purified by dialysis against the same buffer overnight.

The effect of temperature on enzyme activity of four microorganisms was represented in Fig.2. The Maximum enzyme activity was found at 30°C (4.9 U/ml), 35°C (5.7 U/ml), 40°C (2.3 U/ml), and 40°C (6.2 U/ml) for *Pochonia chlamydosporia*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* respectively. The results showed that the fungi *Trichoderma harzianum* had the highest chitinase activity (6.2 U/ml) at 40°C compared to the other three microorganisms.



Fig. 1: Result for precipitation of crude enzyme extracts (*Trichoderma harzianum*)

Jenifer *et al.*, (2014), [15] performed the enzyme activity in *Trichoderma viride* using chitin as a substrate and observed the maximum enzyme activity at 40°C. The results of *Beauveria bassiana* was partially in agreement with the findings of Sapna Mishra *et al.*, (2013), [13] who reported a maximum enzyme activity (5.05 U/ml) at 40°C.

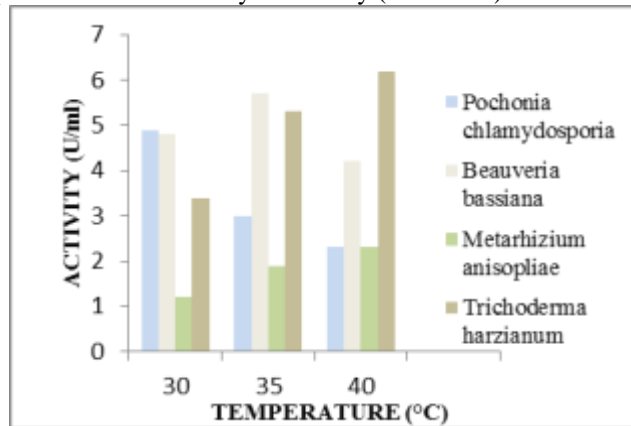


Fig. 2: Effect of temperature on chitinase enzyme activity

The effect of pH on enzyme activity of four species is shown in Fig.3. The fungal strain *Pochonia chlamydosporia* showed maximum enzyme activity (4.2 U/ml) at pH 6.5 while *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* had maximum activity of 3 U/ml at pH 5.5, 2.2 U/ml (pH 5.5) and 8 U/ml (pH 6.5) respectively. The results showed that the fungi *Trichoderma harzianum* had the higher chitinase activity (8 U/ml) at pH 6.5 compared to the other three microorganisms.

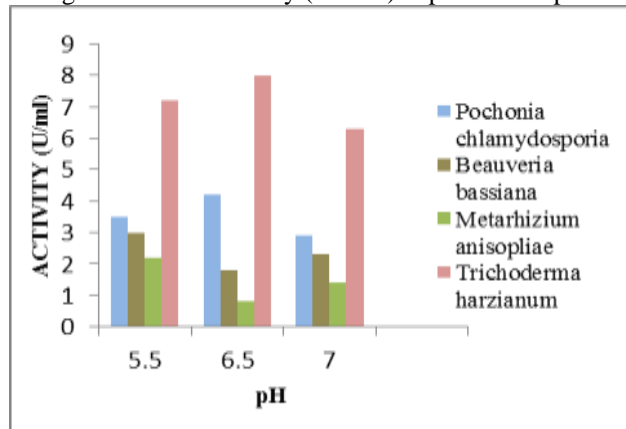


Fig. 3: Effect of pH on enzyme activity of four different species

Rajasekhar *et al.*, (2014), [16] reported that maximum chitinolytic activity (0.06μmol/ml) of *Beauveria bassiana* at pH 5. Madhavan *et al.*, (2004), [17] who reported the maximum chitinase activity at pH 4 for *Trichoderma harzianum*.

The chitinase activity in filamentous fungi such as *Pochonia chlamydosporia*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* generally increases with culture time of incubation, although it also depends on other factors such as pH, temperature. The results obtained in the present study depend on substrate used for inoculation, incubation period, species used and environmental parameters. Further, the enzyme activity may vary with variation in temperature, substrate used, rate of reaction and environmental factors.

IV. CONCLUSION

The selected four entamopathogenic fungi, *Pochonia chlamydosporia*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* have chitinase enzyme activity. *Trichoderma harzianum* has the maximum enzyme activity at temperature of 40°C (6.2 U/ml) and pH 6.5 (8 U/ml) after 5 days of incubation followed by *Beauveria bassiana*, *Pochonia chlamydosporia*, and *Metarhizium anisopliae*. This method is eco-friendly in nature and cost effective. Further, the present study will be continued as a field application for the eradication of blossom midge which affects the dendrobium class of orchid plants.

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