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Bioconversion of Shrimp Shell Waste by *Bacillus Licheniformis* for the Production of Antifungal Chitinase Enzyme

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ABSTRACT

The enzyme chitinase is produced by *Bacillus licheniformis* from the shrimp shell waste. The shrimp shell is used as a carbon and N_2 source. In this study the enzyme chitinase was partially purified and tested for antifungal activity. The plant pathogenic fungi *Fusarium solani, Rhizconia solani, Fusarium oxysporum* and *Aspergillus sp.* were isolated from some infected plant sample. The antifungal activity of chitinase enzyme against the phytopathogen was studied by agar plate method. *In vitro* antifungal activity of chitinase enzyme showed higher inhibitory action against the selected phytofungal pathogens. Chitinase enzyme production from shrimp shell waste plays a dual role in utilization of chitinous waste and decreasing the production cost of the microbial chitinases. The enzyme tested was considered as an important biocontrol agents against selected phytofungal pathogens.

Keywords: Shrimp shell, Chitinase, *Bacillus licheniformis, Fusarium solani, Rhizconia solani* and Antifungal activity.

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INTRODUCTION

Chitinase are a group of enzymes that degrade chitin, produced by diverse range of life forms such as snails, crustaceans, insects, vertebrates, plants and microorganism [12]. Microbial chitinase attracted the attention for the applications in agriculture, pharmaceutical, waste management, biotechnology and industry [1]. Cody, et al., [6] reported that several bacterial species *Aeromonas, Serratia, Vibrio*, *Streptomycetes and Bacillus* genera are the best known chitinase producers. Among the plant diseases, fungal plant diseases are one of the important concerns to agricultural food production. Gohel, et al., 2006 reported that soil borne pathogenic fungi such as *Phythium, Fusarium, Rhizoctonia* and *Phytophthora* attack most economically important crop plants resulting in loss of yield.

Soil borne pathogen *Fusarium oxysporum* cause Fusarium wilt in crop of solanaceae family. The pathogen infects young root, growing, developing and spreading in root and stem vessel, inhibiting water and nutrient transport [12]. To avoid this problem, the use of chemical fungicides to treat the fungal diseases has adverse environmental effects causing health hazards to humans and other beneficial organisms [17]. So there is a pressing need to control fungal disease in an environmental friendly manner. Most of the fungal cell wall contains chitin, which ranges from 22-40% [13] and other polysaccharides. Boller, [2] stated that chitinase play a protective role against fungal pathogens. Besides its ability to attack the fungal cell wall, it also releases oligo – N – acetyl glucosamine that function as elicitor for the activation of defense related responses in plant cells [16].

In this work, the chitinolytic enzyme was produced by *Bacillus licheniformis* is used to investigate the antifungal activity of some phytofungal pathogens.

MATERIALS AND METHODS

The shrimp shell wastes were collected from marine food processing industry, Thoothokudi, Tamilnadu, India. These wastes were washed with tap water repeatedly, dried in room temperature for further experiments. All other chemicals used in this experiment were analytical grade. *Bacillus liceniformis* chitinolytic bacteria was isolated and characterized from soil.

Mass production of Chitinase enzyme

The production of chitinase enzyme was done by following method by Wang, *et al.,* (2006). 20gm of shrimp waste was washed thoroughly, dried and mixed with 500ml of minimal media. After sterilization, chitinolytic organisms *Bacillus licheniformis* was inoculated and incubated for 5 days at 30-40^0 C on a rotary shaker (150 rpm). The culture broth was centrifuged at 8000 rpm for 10 minutes and the supernatant was allowed for partial purification. The supernatant was precipitated by adding $(NH_4)_2SO_4$ and purified by sephadex column chromatography and followed by sephacryl S-200 gel filtration chromatography. The partially purified chitinase enzyme was used for further studies.

Chitinase assay

Extracellular chitinolytic activity of the cell supernatant of bacteria was determined using colloidal chitin as the substrate at optimum pH of chitinolysis as determined by primary selection. The reaction mixture containing 2 ml of colloidal chitin (3 mg/ml) and 5 ml of crude enzyme was kept at 35^0C and shocked for 1 hr. The amount of reducing sugar formed was measured with dinitrosalicyclic acid (DNSA) using a standard curve of N-acetyl glucosamine (Monreal and Reese, 1969).The unit of chitinase activity is defined as the amount of enzyme required for producing 1.0 µM of N-acetyl glucosamine per minute.

Antifungal activity of crude chitinase enzyme

The antifungal activity of chitinase enzyme was estimated using a growth inbibition assay described Wang *et al.,* 2006. The pertidishes were poured with 15 ml of Rose Bengal agar and pre-cooled at 45° C it can divide into two groups. In the experimental plate, an enzyme solution of 5 ml was added. The control group was added with an equal amount of sterile minimal medium instead of crude chitinase enzyme solution. After solidification, the fungal agar plug was placed onto the agar surface. Both groups were incubated for 5 days at 30°C the fungal growth was observed.

The minimal inhibitory concentration (MIC) of chitinase enzyme against the *Fusarium solani*

The antifungal activity of minimal inhibitory concentration of crude chitinase enzyme was performed by agar plate method. In this method, Rose Bengal agar was prepared and sterilized. After sterilization, various concentration of crude chitinase enzyme of 1ml, 2ml, 3ml, 4ml, 5ml were inoculated to the petriplate. Plates were rotated in clockwise and anticlockwise for proper mixing of crude enzyme and medium. The plates were allowed to solidify. Then, the equal size of agar plug of *Fusarium* was inoculated into all the plates. Simultaneously, the Rose Bengal agar plate with boiled crude chitinase enzyme was used as a control. After incubation, the diameters of the fungal colonies were recorded. The inhibition ratios were calculated with the following formula.

Inhibition ratio (%) =C-E/C*100

E=Experimental group
C=Control group

Measurement of antifungal activity - Agar diffusion test

A spore suspension of 0.5 cm agar plug of *Fusarium solani* was uniformly spread on plate of Potato dextrose agar (PDA). After 5 minutes, 5 wells were punched in the PDA agar plate with a help of the sterile well puncher. Then various concentrations of 0.1, 0.2, 0.3, 0.4 ml of chitinase enzyme were inoculated into the wells respectively. The fifth well was used as a

control. Fungal growth was observed over 4 days of incubation at 30°C.

Microscopic observation - Inhibition of fungal growth using chitinase enzyme

Fusarium oxysporum was cultured on Potato Dextrose agar. A mycelial disk of 6 mm diameter was transferred on to a glass slide and incubated at 30°C in a moist chamber. After 48 hours growth, the chitinase enzyme was overlaid and incubated at 37°C for 12 hours in moist chamber. Morphological modification of the mycelia structures of the fungus were observed under a light microscope (40 x) and compared with control (boiled chitinase enzyme) [18].

Field application of crude chitinase enzyme against *Sclerotium rolfsii*

In this study, two sets *of Chrysanthemum segetum* plants were collected. The set one was experimental and another set was control each sets consist of five plants. The experimental plant root was dipped in partially purified crude enzyme for 5 minutes. Spores of *Sclerotium rolfsii* were inoculated in both plant roots. The plants were transplanted into separate pots containing sterile soil. After 3 weeks of inoculation, the roots were uprooted and observed visually.

Crude chitinase enzyme for seed protection

Tomato seeds were collected in the sterile petridishes. In the experimental plate, seeds were soaked with crude chitinase enzyme and control plate was soaked with sterile medium and dried. After 5 minutes, spores of *Fusarium* were inoculated into both the plates. After 5 days of incubation, results were observed.

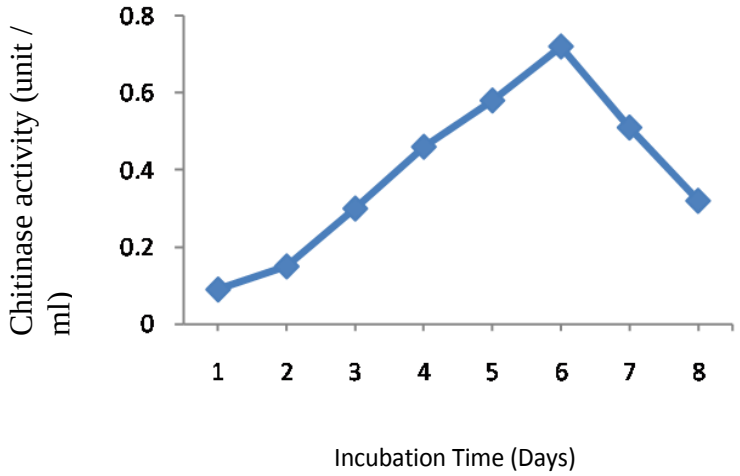
RESULTS

The chitinase enzyme was produced using minimal media with prawn shell as a chitin source. At the beginning of incubation days, chitinase enzyme production increased gradually. The highest chitinase enzyme production (0.72 U / ml) was observed on the 6^{th} day of incubation (Graph-1).

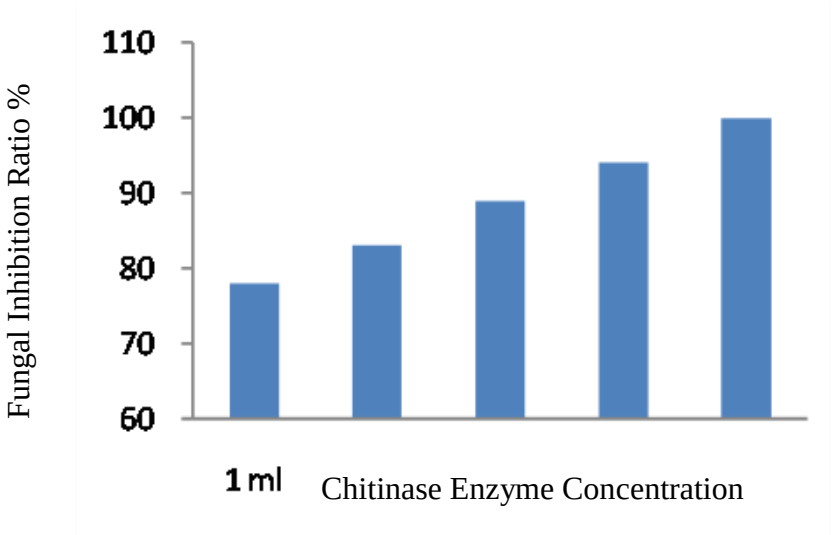
The plant pathogenic fungi were isolated from the infected plant sample. The fungal morphology and staining procedure identified isolates as *Rhizoctonia solani, Fusarium oxyspoum, Aspergillus flavus and Fusarium solani.* The *In vitro* antifungal activity of chitinase enzyme against phytopathogenic fungi was found to be 100% after 5 days of incubation, when compared with control. Therefore, inhibitory activity gradually decreased with the increased incubation time because stability of enzyme decreased in the increased incubation time.

The minimal inhibitory activity of crude enzyme was observed in *Fusarium solani*. The result showed that highest enzyme concentration (5ml) revealed no growth of fungi on the plate, but below 5ml of crude enzyme added plate showed gradual growth of fungus. In the

agar diffusion test, 0.4ml of chitinase exhibited highest antifungal activity compared to low concentration of enzyme (Graph-2).



Graph 1 In Chitinase assay the highest chitinase enzyme production (0.72 U / ml) was observed on the 6th day of incubation



Graph-2. The minimal inhibitory concentration (MIC) of chitinase enzyme against the *Fusarium solani*

Morphologically changes of fungi were observed in the staining procedure. Chitinase treated fungus showed no growth in spore germination. Fungal cell wall lysis was observed in the experimental plate but defined morphology of fungi was observed in the control.

After 3 weeks of incubation, the roots were uprooted. The control plant root was infected with *Sclerotium sp*. But the experimental root did not show any infection because chitinase enzyme showed very good response for inhibition of fungal growth (Fig-1). Seed treatment with crude chitinase enzyme showed more fungal inhibition, but fungal growth was observed on control plate. (Fig-2)

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Fig 1 The effect of chitinase enzyme treated *Chrysanthemum segetum* plants root infected with the spores of *Sclerotium rolfsii* were inoculated

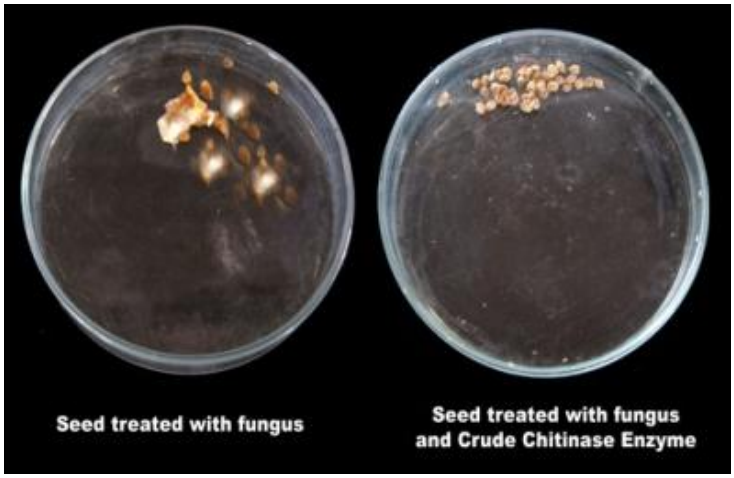

Fig 2 The tomato seed treated with crude chitinase enzyme showed more fungal inhibition compared with untreated

DISCUSSION

Chitinase are glycosyl hydrolases that catalyse the degradation of chitin. There is an increasing interest in the use of chitinase for the control of moulds, insects, nematodes and production of different chitin oligomers [10]. Bowyer (1999) Stated that among the plant diseases, one third is due to fungal infections. They reduce the crop yield and constant supply of food to ever increasing world population. Treating plant diseases are important in agricultural food production. Use of chemical fungicide is an increasing concern towards the toxicity and biomagnification in agriculture.

Recently, the use of biocontrol has increased in plant pathology. Chitinase have been isolated from variety of bacteria and used as a biocontrol of fungal plant pathogens [5]. The

present study revealed that the chitinase produced by *Bacillus licheniformis* from shrimp shell waste acts as an excellent enzyme inducer and carbon source. The same work supported by Wang *et al.,* 2006 *Bacillus subtilis* W-118, a strain excreted chitinase enzyme when cultured in a medium containing shrimp and crab shell powder as the major carbon source. It is an inexpensive method for the production of chitinase enzyme. In addition, the oligosaccharides prepared by acidic hydrolysis might be toxic because of chemical changes during conversion [20]. Therefore, there is a growing interest for enzymatic hydrolysis of chitin from shrimp shell wastes.

Our study reported that bacterial chitinase in crude (or) partially purified form possessed antifungal activities against selected phytofungal pathogens. Ordentlich *et al.,* 1988, found that crude chitinase of *S.marcescens* caused lysis of hyphal tips of *Sclerotium rolfsii.* Pleban *et al.,* 1997 also found that crude extracellular chitinase of an endophytic bacterium *B.cereus* 65 decreased spore germination of *F.oxysporum*. Chitinolytic enzymes have been considered as important biocontrol agents of soil borne pathogens because of its ability to degrade fungal cell walls, whose major component is chitin [9].

Microscopic slide observation of chitinase treated fungal cell wall showed apparent changes in hyphal cleavages of the *Fusarium* mycelia. Similar phenomena were seen when plant pathogenic fungi were treated with purified endochitinase from *Bacillus cereus* YQ 308 [4]. From our study, seeds protected with chitinase enzyme showed very good response in the inhibition of fungal growth. The potential use of chitinase enzyme from *Bacillus thuringiensis var israelensis* in the protection of bean seeds infested with phytopathogenic fungi [7].

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