

Purification and Characterization of Chitinase Produced by Endophytic *Streptomyces hygroscopicus* Against Some Phytopathogens

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Abstract Microorganisms, which secrete a complex of mycolytic enzymes are considered to be possible biological control agents of plant diseases. Since Chitinases are digestive enzymes that break down glycosidic bonds in chitin. In this present study, an industrial enzyme chitinase produced by endophytic *Streptomyces* was purified and its antifungal activity was investigated against phytopathogens *i.e.* *Rhizoctonia solani*, *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Sclerotinia sclerotiorum*, *Phytophthora parasitica* and *Botrytis cinerea*. A chitinase was produced by endophytic *Streptomyces hygroscopicus* in cultures containing chitin as the sole substrate that degrades chitin in 0.9 mm zone of clearance. The purification steps included ammonium sulfate precipitation, with columns of DEA E-Sepharose anion exchange chromatography and Sephacryl S-400 high resolution gel chromatography. The method gave a 5.4 fold increase of the specific activity and had a yield of 18%. The molecular weight of the chitinase was found to be around 80.8, 78 and 76 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. Chitinase was optimally active at pH of 6.0 to 7.0 and at 30°C. The chitinase were found to inhibit the growth of all phytopathogenic fungi.

Keywords Antifungal Activity, Chitinase, Plant Phytopathogens, *Streptomyces hygroscopicus*

1. Introduction

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants. Chemical fungicides are extensively used in current agriculture. However, excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide. Because of the worsening problems in fungal disease control, a serious search is needed to identify alternative methods for plant protection, which are less dependent on chemicals and are more environmentally friendly.

Microbial antagonists are widely used for the biocontrol of fungal plant diseases [1]. Endophytic micro-organisms have received considerable attention for their potential as biocontrol agents of fungal plant pathogens. Varied enzyme production may result in new biochemical characteristics and in part be responsible for the inherent biodiversity of endophytic micro-organisms. Among the lytic enzymes evaluated as a source of biocontrol agents, chitinases have

been studied largely because these enzymes are produced by a variety of endophytic micro-organisms [2]. Endophytic actinomycetes have been cited as promising biocontrol agents, either acting directly on fungal cell walls or initiating increased plant responses against disease. In addition, research indicates that both mechanisms operate to control fungal pathogens [3]. Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environmental-friendly industrial sectors. With the advancement in biotechnology especially in the area of genetics, protein engineering, developments in bioinformatics, and the availability of sequence data have opened a new era of enzyme applications in many industrial processes [4]. Chitinase, a group of enzymes capable of degrading chitin directly to low molecular weight product and found in a broad range of organisms, including bacteria (*Bacillus*, *Aeromonas*, *Pseudomonas*, *Serratia*, *Enterobacter*, *Actinomycete*), fungi (*Trichoderma* and *Aspergillus*), and higher plants, insects, crustaceans, invertebrates and some vertebrates [5].

Chitinases have received increased attention due to their wide range of biotechnological applications, especially in the biocontrol of fungal phytopathogens [6]. Chitinase have been implicated in plant resistance against fungal pathogens because of their inducible nature and antifungal activities *in vitro*. In viruses, chitinases are involved in pathogenesis [4].

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Due to multiple applications of chitinases in biocontrol, waste management, medicine and biotechnology they become interesting enzymes for study.

In the present study, chitinase production by endophytic actinomycetes, *Streptomyces hygrosopicus* and their anti-fungal activities against phytopathogens. *Rhizoctonia solani*, *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Sclerotinia cleoitorum*, *Phytophthora parasitica* and *Botrytis cinerea* was evaluated

2. Materials and Methods

2.1. Micro-organism Strains and Growth Conditions

Streptomyces hygrosopicus was previously isolated from peanut plants and identified in Plant Pathology Department, National Research Centre, Egypt. Culture was grown and maintained on solid starch medium [7] at 28 °C.

The fungal pathogens *Rhizoctonia solani*, *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Sclerotinia cleoitorum* and *Botrytis cinerea* were isolated originally from different naturally diseased plants collected from different agricultural fields. The isolated fungi were grown on potato dextrose agar (PDA) (Difco, USA) plates and incubated at 28 °C for 4 to 6 days. Purification of the resulting isolates was done using the hyphal tip or single spore techniques to obtain them in pure cultures; the detected isolates were then transferred into slant of PDA and kept at 4 °C for further studies. Pure cultures of the isolated fungi were identified according to the cultural properties, morphological and microscopical characteristics of each fungus [8].

Antagonistic activity assay: Dual culture assay was performed to assess the potential biocontrol activity of *Streptomyces hygrosopicus* against pathogenic fungi. In the dual culture assay, conidial suspension of each pathogen and bacterial suspension of *Streptomyces* were streaked on the surface of LB agar at a distance of 4 cm. The growth of both was examined daily for the formation of inhibition zone and growth reduction.

2.2. Quantitative Determination of Extracellular Chitinase Activity

Streptomyces was grown in ISP-2 broth [9], with continuous shaking at 150 rpm at 27 °C for 10 days. Cell-free supernatants were collected at 1-day intervals by centrifugation at 8,000 rpm for 20 min at 4 °C. Cell pellets were dried at 80 °C and weighed to determine the growth. Chitinase activity was quantitatively determined by measuring the reducing end group of N-acetylglucosamine (NAG) that degraded from colloidal chitin as substrate [10]. The estimation of chitinase activity was carried out by the procedure described by Wang *et al.* [11].

Purification of chitinase: The proteins from the culture were precipitated by ammonium sulphate (75%). The precipitate was collected by centrifuging at 8,000 × g for 20

min, and resuspended in an acetate buffer (50 mM, pH 5.0). It was dialyzed against the same buffer and freeze-dried. The sample was then loaded on a pre-equilibrated DEAE-cellulose column (2 × 20 cm), and washed with the acetate buffer. The proteins were eluted in a stepwise gradient on NaCl (0-1.0 M) at a flow rate of 24 ml/h. Fractions of 3 ml were collected, and the absorbance was read at 280 nm in a spectrophotometer. The fractions with chitinase activity were combined, dialyzed against the acetate buffer, and concentration by lyophilization. The concentrated sample was passed through a Sephadex G-100 column (2 × 40 cm) and eluted with the acetate buffer at the rate of 15 ml/h. The fractions of 3 ml were collected, and the absorbance and chitinase activity were measured.

Chitinase activity measurements A fluorometric assay was used to determine chitinase activity using 4-methylumbelliferyl-N,N',N''-chitotriose (Sigma, St. Louis, USA) as a substrate. The amount of 4-methylumbelliferone (4-MU) released was measured spectrofluorometrically by using a fluorescence spectrophotometer (F-4500, Hitachi) (excitation 390 nm and emission 450 nm). One unit (U) of chitinase activity was defined as the amount of enzyme required to release one μmol of 4-MU per min at 37 °C.

SDS-PAGE analysis: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli procedure [12], using the Mini-Protean II apparatus (Bio-Rad, Hercules, USA). A 10% separating gel containing 0.01% glycol chitin as the substrate of chitinase was used to detect chitinase activity. After electrophoresis, proteins in the gel were renatured in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 at 37 °C. Subsequently, the gel was stained with 0.01% Calcofluor White M2R (Sigma, St. Louis, USA) in 0.5 M Tris-HCl (pH 8.9) and examined for the chitinolytic bands under UV transilluminator [13]. In another gel, proteins were stained with Coomassie Brilliant Blue G-250.

Protein concentration was determined by Bradford's method [14] using bovine serum albumin as standard.

2.3. The Studies of Chitinases Characterization Determination of Optimum Temperature and pH

The reaction was carried out at temperatures ranging from 20 to 80 °C and the chitinase activity at different temperatures were measured to find the optimum temperature. A similar method was applied at varying pH levels from 3.0 to 8.0 to determine the optimum pH.

2.4. Inhibition of Fungal Growth by the Purified-Enzyme Extract

A spore suspension of pathogenic fungi was uniformly spread on plates of PDA (potato dextrose agar). Discs that were soaked with the purified enzyme extract (5 U, 10 U) were laid on the seeded plates; the control was disc-soaked in boiled-enzyme extract. Fungal growth was observed over 2 d of incubation at 30 °C.

2.5. Statistical Analysis

All data (chitinase production and antagonistic isolates) were analysed for significance ($P < 0.01$) using the SAS software package.

3. Results

Chitinolytic and antifungal activities

The endophytic *S.hygrosopicus* was screened for chitinase production and inhibition of fungi some phytopathogens *i.e.* *R.solani*, *F.oxysporum*, *A. alternate*, *A. niger*, *A.flavus*, *S. scleotiorum* and *B.cinerea*. *S.hygrosopicus* produced a high concentration of chitinase and inhibited the phytopathogens.

S.hygrosopicus generally induced larger fungal growth inhibition zones for *R. solani*, *S. scleotiorum*, *B.cinerea* and *F. oxysporum* than for *A.alternate*, *Aspergillus* and *P.parasitica* (Fig. 1).

The production of extracellular chitinase enzyme by a potent antagonist

The production of extracellular chitinolytic enzyme in the strain was determined at different growth phases. The level of chitinase was sharply increased during the exponential phase and dramatically declined when the cells entered the stationary phase (Fig.2). The strain produced relatively high levels of chitinase (5.8 U/mg protein) at day 1 of the incubation period.

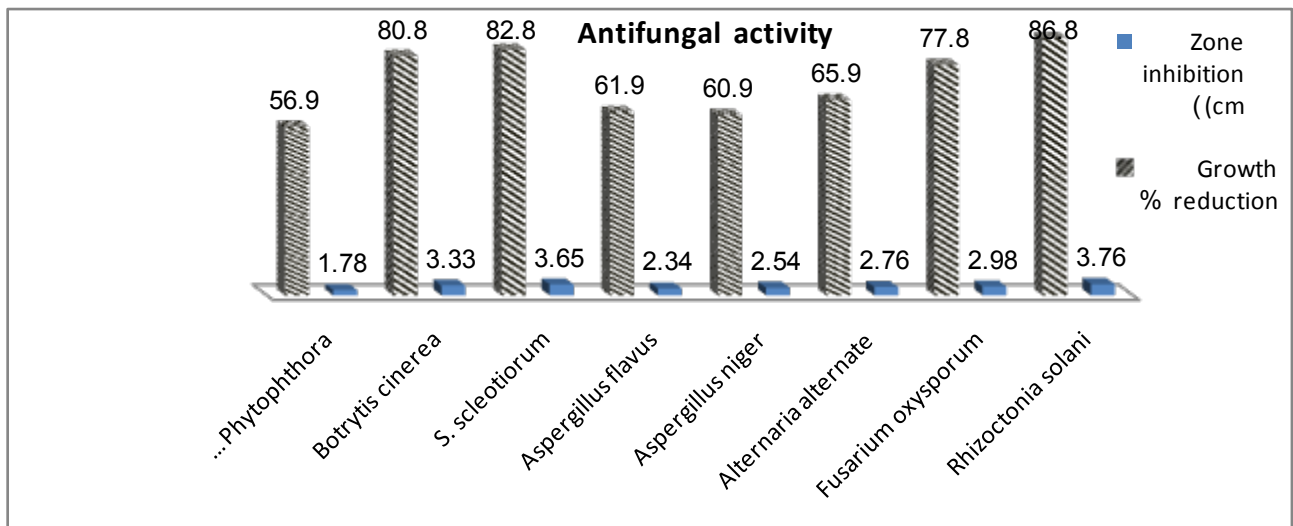


Figure 1. Chitinolytic and antagonistic activities of *Sterptomycesyhygrosopicus*

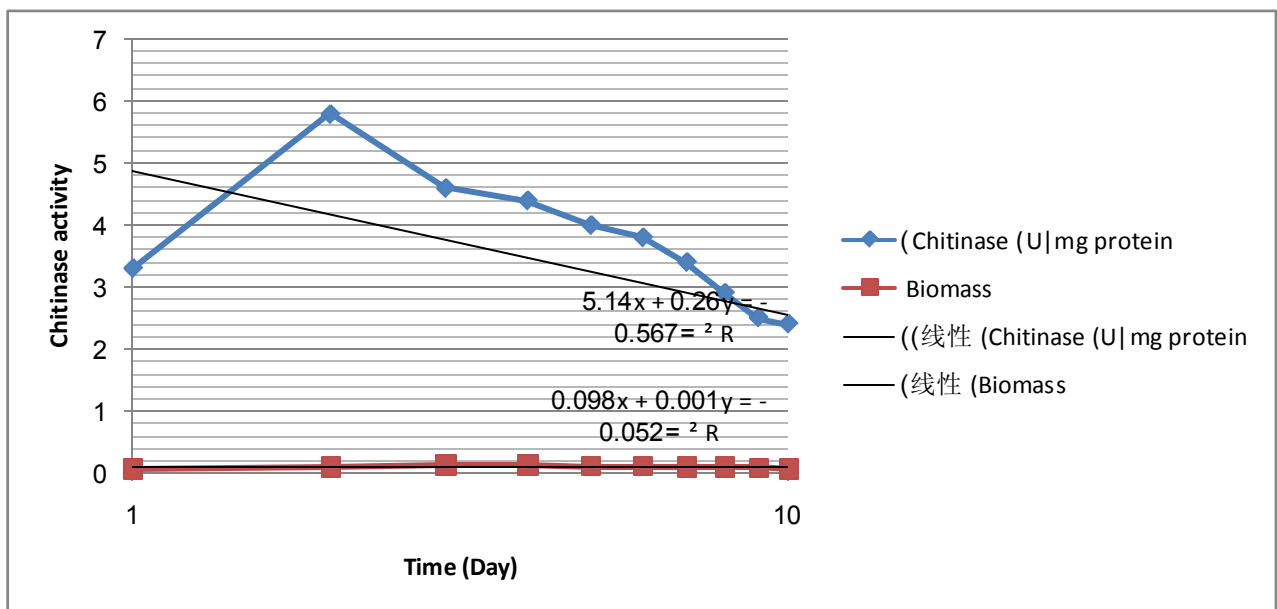


Figure 2. Time-course experiments related to growth, chitinase activity in the *Sterptomycesyhygrosopicus* strain

Purification of the chitinase

The purification procedures of the chitinase secreted by the *S. hygrosopicus* are summarized in Table 1. The results showed that, the enzyme was purified 1.9-fold with a specific activity of 38.7 U/mg protein after ammonium sulfate fractionation. The enzyme was then purified with DEAE-sepharose fast flow and showed 4.7-fold enzyme purification with a specific activity of 7.5 U/mg protein. The final purification step presented 5.4-fold enzyme purification with a specific activity of 3.1 U/mg protein. These results indicated that the effectiveness of the purification method applied in this research and the chitinase was purified to 5.4-fold with a 18% yields (Table 1).

The extract was purified through fractional precipitation with ammonium sulphate, column chromatography, and non-denaturing PAGE, and was then checked using SDS-PAGE. The result indicates high chitinase activity, which showed as a three band, at a molecular weight of about 80.8, 78 and 76 kDa (Fig.3).

Effect of temperature

The chitinase activity was most active at 30°C. Above 40°C, the activity decreased and was lost completely at 60°C. When the enzyme was kept at various temperatures for 30 min in an acetate buffer (pH 5.0), it was significantly inactivated above 50°C and completely at 65°C (Fig. 4).

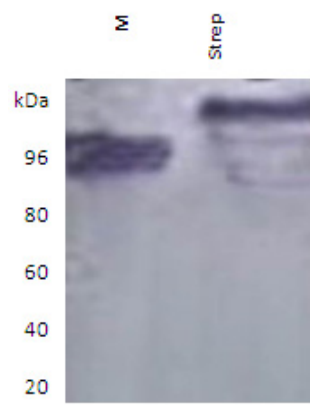


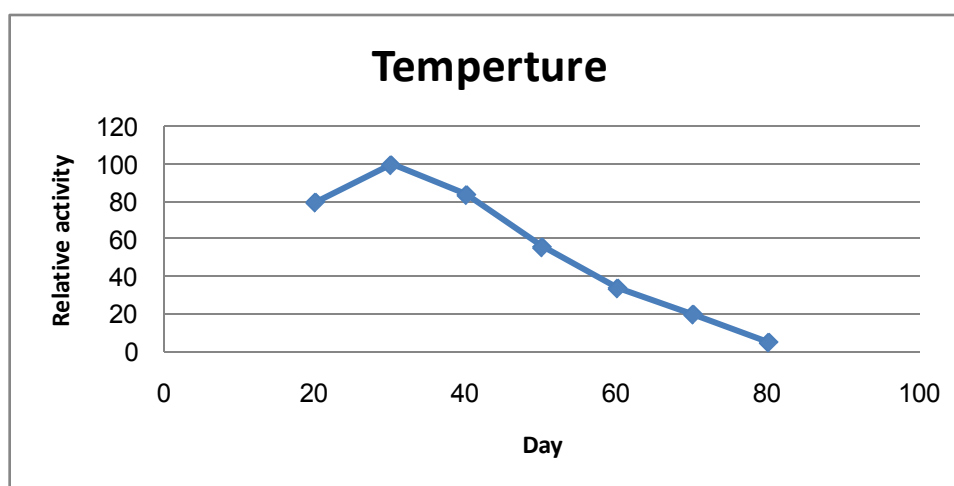
Figure 3. Activity gels of extracellular lytic chitinase enzyme secreted by *Streptomyces hygrosopicus* showing detection of lytic enzyme activities on Native gel; M = Marker; Streptomyces = Strep

Effect of pH

The optimal pHs for chitinase activity and stability of the chitinase were examined. The enzyme was most active between pH 5.0 and 6.0. It was relatively stable at pHs between 4.0 and 8.0, when kept at 4°C. However, beyond these pH ranges, it rapidly lost its activity (Fig. 5).

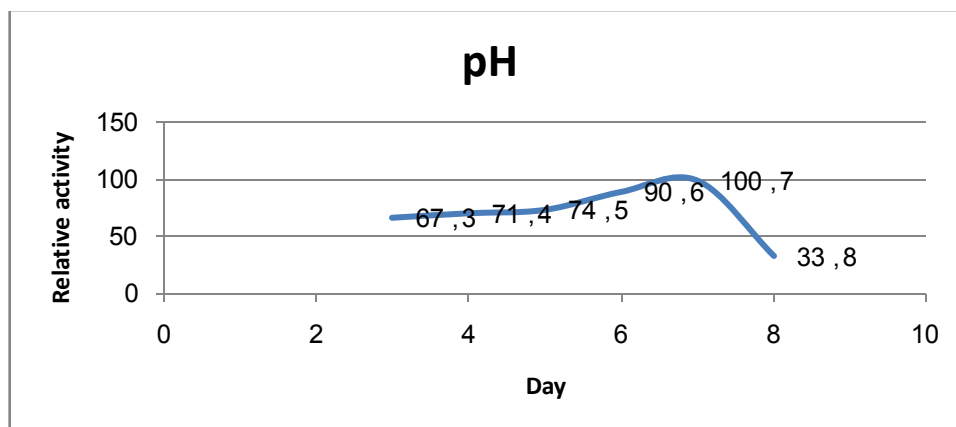
Table 1. Purification of thermal chitinase from *Streptomyces hygrosopicus*

| Step | Total protein (mg) | Total activity (Unit) | Specific (U mg ⁻¹) | Purification (fold) | Yield (%) |
|---|--------------------|-----------------------|--------------------------------|---------------------|-----------|
| Culture supernatant | 81.3 | 87.6 | 14.6 | 2 | 100 |
| (NH ₄) ₂ SO ₄ | 38.7 | 64.1 | 29.8 | 1.9 | 76 |
| DEAE-Sephadex fast flow | 7.5 | 33.5 | 56.9 | 4.7 | 41 |
| Sephadex | 3.1 | 12.4 | 66.9 | 5.4 | 18 |



The activity assay was carried out at different temperatures using 1% (w/v) colloidal chitin (dissolved in 50 mM pH 7.2 Tris-HCl buffer) as substrate

Figure 4. Effects of temperature on the chitinase activity of *Streptomyces hygrosopicus*.



The activity assay was carried out at 60°C using 1% (w/v) colloidal chitin (dissolved in 50 mM different pH buffer) as substrate

Figure 5. Effects of pH on the chitinase activity of *Streptomyces hygroscopicus*

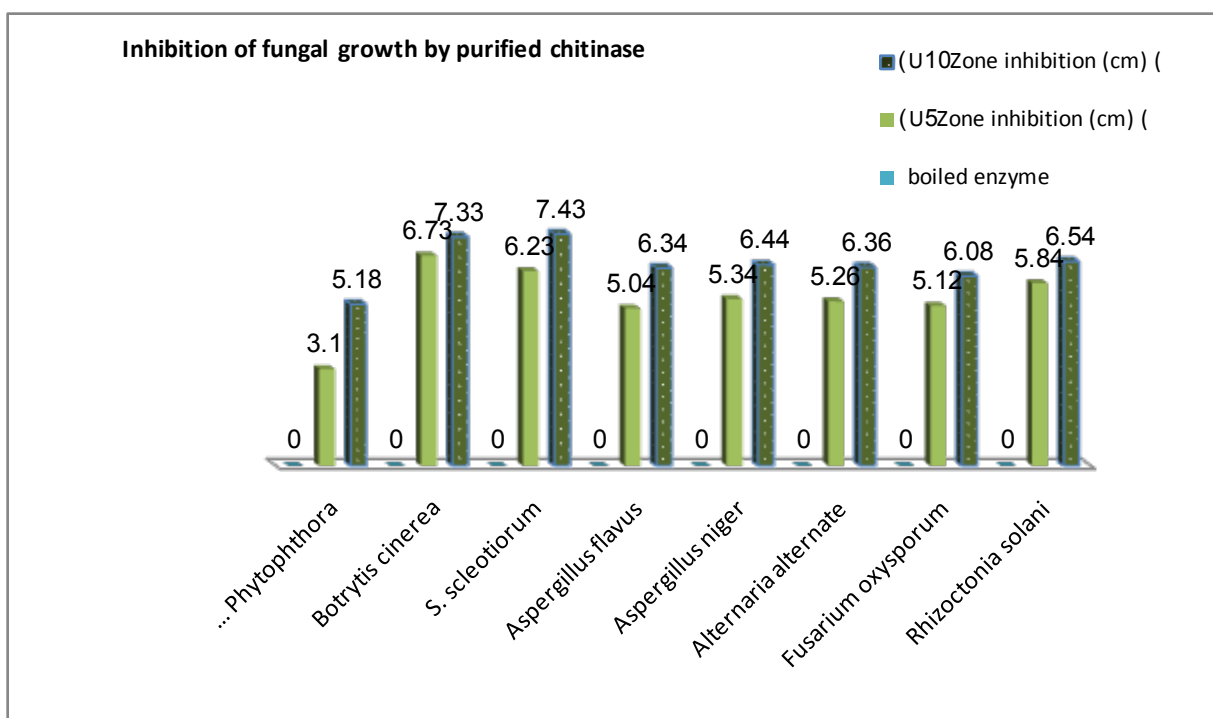


Figure 6. Antifungal activity of chitinase from *Streptomyces hygroscopicus* against some phytopathogens

Inhibition of fungal growth by purified chitinase

The inhibition of the fungi phytopathogens i.e. *R. solani*, *F. oxysporum*, *A. alternate*, *A. niger*, *A. flavus*, *S. sclerotiorum* and *B. cinerea* growth was observed on agar plates with paper discs that were coated with the purified chitinase enzyme (Fig. 6). Antifungal activity of the purified chitinase of *Streptomyces* was confirmed. The inhibition diameter of the 10 U enzyme was greater than that of the 5 U enzyme, and the boiled enzyme had no inhibitory activity against all pathogens.

4. Discussion

Microbial production of chitinase had great attention of both industrial and scientific environments, not only because

of its wide spectrum of applications but also for the lacuna of an effective production method. Microorganisms, which secrete a complex of mycolytic enzymes are considered to be possible biological control agents of plant diseases.

Endophytes are micro-organisms that inhabit plant interior tissues. These microorganisms cause no harm to the host and do not develop external structures, which exclude nodulating bacteria and mycorrhizal fungi as endophytic micro-organisms [15]. Chitinolytic activity has been implicated in the biocontrol activity of several bacteria, including *Streptomyces* spp. Owing to the innocuous nature of these organisms, delivery systems to introduce endophytic bacteria, such as *Streptomyces* spp., into plants have been developed, and the potential for endophytes as biocontrol agents has been explored [16]. In a recent study,

an endophytic actinomycetes suppressed fungal pathogens, including *R.solani*, *F.oxysporum*, *A. alternate*, *A.niger*, *A.flavus*, *S. sclerotiorum* and *B.cinerea*, indicating their potential use as biocontrol agents [1]. Shekhar *et al.* [1] purified a bioactive compound from endophytic *Streptomyces violaceusniger* that showed a strong antagonism towards various wood-rotting fungi, and chitinase enzymes were associated with this inhibition. In general, the higher chitinase activity was correlated with higher fungal inhibition.

In the present study, the chitinolytic strain, *S.hygroscopicus* showed high inhibition levels against fungi, and the fungal hyphae exhibited a degraded appearance after chitinolytic strain culture treatment. In this study on chitinase production, extra cellular chitinase activity was determined. The purification steps included ammonium sulfate precipitation, with columns of DEAE-Sepharose anion exchange chromatography and Sephacryl S-400 high resolution gel chromatography on AKTA purifier 100 protein liquid chromatography. The method gave a 5.4 fold increase of the specific activity and had a yield of 18%.

Enzyme production was increased in the stationary phase and more amounts were detected in the stationary growth phase. Chitinases are fairly stable over a broad pH range. Chitinase was optimally active at pH of 6.0 to 7.0 and at 30°C. The molecular weight of the chitinase was found to be around 80.8, 78 and 76 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. The chitinase were found to inhibit the growth of all phytopathogenic fungi i.e. *R.solani*, *F.oxysporum*, *A. alternate*, *A.niger*, *A.flavus*, *S. sclerotiorum* and *B.cinerea*, respectively. Since the cell walls of fungi consist largely of chitin [17], it was postulated that chitinase produced by the antagonists could be involved in disease control. The production of these enzymes was therefore used as the criteria for selection of potential biocontrol agents against pathogens.

Streptomyces strains are a likely choice as potential biological control agents. Antagonistic activity of several *Streptomyces* sp. against a number of fungal pathogenic species has been known for a long time [18, 19 & 20]. Also, in greenhouse experiments, *Streptomyces* sp. has conferred various degrees of protection on different plant species against soil-borne pathogenic fungi [2]. Very high protein content of chlamydospores of *Fusarium* sp. (7 - 28%) may be responsible for their ability to resist lysis in soil.

Studies on chitinolytic microorganisms have yielded a large increase in knowledge regarding their role in inhibition of growth of fungal plant pathogens (20 & 21). Still this knowledge is not sufficient enough to formulate a preparation based on these agents that can work efficiently in all different environmental conditions. The biocontrol agents are also affected due to the geological and environmental conditions. Studying the environmental conditions of the area where biological control agent has been employed and statistical based formulation approach techniques will increase life span of microorganisms in different ecological

conditions. The results of this study led us to conclude that chitinase produced by endophytic *Streptomyces* has the potential for control of plant pathogenic fungi. This study provides a quantitative assessment of *Streptomyces* chitinolytic and antifungal activities.

REFERENCES

- [1] Shekhar, N., Bhattacharya, D., Kumar, D. and Gupta, R.K. (2006). Biocontrol of wood-rotting fungi with *Streptomyces violaceusniger* XL2. *Can J Microbiol* 52, 805–808.
- [2] El-Tarabily, K.A. and Sivasithamparan, K. (2006) Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biol. Biochem* 38, 1505–1520.
- [3] Coombs, J.T., Michelsen, P.P. and Franco, C.M.M. (2004) Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis var. tritici* in wheat. *Biol Control* 29, 359–366.
- [4] Dahiya, N., Rupinder Tewari, Gurinder Singh Hoondal. (2006). Biotechnological aspects of Chitinolytic enzymes: a review. *Applied Microbiol Biotechnology*, 71: 773-782
- [5] Yong, T., Jin Hong, Long Zhangfu, Zhang Li, Ding Xiuqiong, Tao Ke, Ge Shaorong, Liu Shigui (2005). Purification and characterization of an extracellular chitinase produced by bacterium C4. *Annals of Microbiology*, 55 (3): 213-218.
- [6] Nawani and Kapadnis. (2005). Diversity of Chitinases and their Industrial Potential. *Current Perspectives and Potential Applications*: 719-740.
- [7] Kuster, E. and Williams, S. (1964). Selection of media for isolation of *Streptomyces*. *Nature*, 202: 928-929.
- [8] Domsch KW, Gams W, Anderson TH (1980). *Compendium of soil fungi*. Vol. 1, Academic Press, London, p. 589.
- [9] Shirling EB, Gottlieb D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol*. 16:313–40.
- [10] Imoto T. and Yagishita K. (1971) .A simple activity measurement of lysozyme. *Agri. Biol Chem*. 35:1154–6.
- [11] Wang SL, Yieh TC and Shih IL. (1999). Production of antifungal compounds by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as a carbon source. *Enzyme Microb Technol*. 25:142–8.
- [12] Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- [13] Trudel, J. and A. Asselin. (1989). detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem*. 178: 362_366
- [14] Bradford, M. M. (1976) .A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72, 248-254.
- [15] Azevedo, J.L. and Araújo, W.L. (2007). Diversity and applications of endophytic fungi isolated from tropical plants.

In Fungi: Multifaceted Microbes ed. Ganguli, B.N. and

3899-3905.

- [16] Bhattacharya, D., Nagpure, A. and Gupta, R.K. (2007). Bacterial chitinases: properties and potential. Crit Rev Biotechnol 27, 21–28.
- [17] El-Tarabilya, K. A. ; Solimana, M. H. ; Nassara, A. H. ; Al-Hassania, H. A.; Sivasithamparam C., K. ; McKennad, F. and Hardy, G. E. St. J. (2000). Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. Plant Pathology 49, 573±583
- [18] Crawford DI, Lynch JM, Whipps JM, Ousley MA (1993). Isolation and characterization of actinomycetes antagonists of fungal root pathogen. Appl. Environ. Microbiol. 59: 3899-3905.
- [19] Anitha, A. and Rabeeth, M. (2010).“Degradation of fungal cell walls of phytopathogenic fungi by lyticenzyme of *Streptomyces griseus*”, Afr. J. Plant Sci., 4, 61-66.
- [20] Subramaniam1, S., Ravi1, V., Narayanan1, G.. (2012). Studies on Production of Enzyme Chitinase from *Streptomyces sp.* and its anti-fungal activity. Journal of Pharmacy Research 2012,5(3),1409-1413.
- [21] Khamna S, Yokota A, Peberdy JF, Lumyong S (2009). Antifungal activity of *Streptomyces* spp. isolated from rhizosphere of Thai medicinal plants.; Int. Integr. Biol., 6(3): 143-147.