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Influence of Culture Conditions of *Streptomyces* sp. (Strain S_{242}) on Chitinase Production

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Abstract

The purpose of this study was to determine the influence of growth conditions and medium composition on the production of chitinase by *Streptomyces sp.* (strain S_{242}). Production of chitinase by strain S_{242} was detected on colloidal chitin agar (CCA) medium after 8 days of incubation at 28°C resulting in a clear zone 10 mm around the colony. Chitinase activity was assayed as the amount of *N*-acetylglucosamine released in µmol/ml/min using the dinitrosalicylic acid assay method. The crude enzyme had maximum activity (0.162 U ml/l) after 4 days of incubation at pH 7 and 30°C when the broth medium was supplemented with 1.6% of colloidal chitin. However, enzyme activity was strongly decreased at 40°C and extreme acidic and alkaline pH values. SDS-PAGE and zymogram analysis revealed six distinctive bands that range from 39 to 97 kDa with chitinolytic activity. The findings of this investigation create a possibility for the use of the organism in the commercial production of chitinase. In addition, it can be a source of DNA for cloning the chitinase gene(s) to generate phytopathogen resistant transgenic plants.

Key words: *Streptomyces* sp., chitinase, culture conditions

Introduction

A wide variety of bacteria are known as hydrolytic enzyme producers, with streptomycetes being the best known so for (Vinogradova and Kushnir, 2003). Streptomycetes produce stable mycelia which are capable of secreting an array of different extracellular enzymes including cellulases, chitinases and xylanases. They produce such enzymes to degrade naturally occurring macromolecules, and thus to sustain their growth and survival (Christodoulou *et al*., 2001; Williamson *et al*., 2000).

As chitinolytic microorganisms, streptomycetes are capable of degrading chitin solely by hydrolysis of glycosidic bonds (Goody, 1990). In fact, *Streptomyces* strains are regarded as the major producers of chitinases in soil (Tanabe *et al.*, 2000). Most streptomycetes secrete a number of chitinases hydrolyzing chitin to its oligomers, such as chitooligosaccharides, chitobiose or N-acetylglucosamine. Such oligomers can be utilized as carbon or nitrogen sources (Schrempf, 2001).

Several studies have been conducted on *Streptomyces* sp. isolated from Jordan soil for their potential to produce enzymes of industrial importance. Rawashdeh *et al*. (2005) isolated several *Streptomyces* isolates that were able to grow on tomato pomace. Upon further characterization, the isolates were able to produce cellulase, pectinase and relatively large amount of xylanase. Tahtamouni *et al*. (2006) isolated indigenous *Streptomyces* sp. isolates that were capable of producing chitinase. The isolates exhibited fungicidal activity against sclerotia of the white cottony stem rot pathogen *Sclerotinia sclerotiorum*.

Streptomycetes isolated from Jordanian habitats, are poorly studied especially the chitinase enzyme producers. Therefore, the present investigation was conducted to isolate soil streptomycetes from different habitats in Jordan and screen them for their ability to utilize chitin as a sole source of carbon and nitrogen. The influence of different culture conditions on production of crude chitinase by the most active *Streptomyces* isolate in submerged cultures was also studied. In addition, chitinase produced by the most active strain was characterized by means of enzyme assay, SDS-PAGE and zymogram analysis.

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Experimental

Materials and Methods

Location, sampling, treatment of soil samples and isolation technique. Soil samples were collected from 19 different regions in Jordan. One soil sample was collected from each location. Enrichment of streptomycetes in the soil samples and isolation of *Streptomyces* spp. were performed as described by Saadoun *et al*. (2008). Selected colonies were purified by repeated streaking. *Streptomyces*-like colonies were selected and screened for their ability to produce chitinase enzyme on a specified medium.

Screening for chitinase-producing streptomycetes. All pure isolates of streptomycetes were screened for their efficiency of chitinase production. Consequently, each isolate was suspended in sterile vial containing 3 ml distilled water, to give a spore suspension of 10^7 spores/ml, then drop of 0.1 ml volume was cultured in the center of a colloidal chitin agar (CCA) media, which is specified for screening of chitinolytic actinomycetes, according to Hsu and Lockwood (1975). Colloidal chitin was prepared from partially purified chitin from crab shells (Sigma) by blending of 40 g in a blender then dissolving in 400 ml of concentrated HCl by stirring for 30 to 50 min. The chitin was precipitated as colloidal suspension by adding it slowly to 2 liters of water at 5 to 10°C. The suspension was collected by filtration with suction on a coarse filter paper and then washed by suspending it in about 5 liters of tap water and re-filtering. Washing was repeated at least three times or until the pH of the suspension was about 3.5. Water content of the chitin was determined by drying a sample at 100°C. For use sufficient water was added to re-suspend the chitin, and the suspension was blended at high speed for about 10 min. Autoclaved filter cake or aqueous suspension could be stored indefinitely at room temperature. Plates were incubated at 28°C for 8 days. The appearance of chitin clearing zone around those colonies is indicative of the presence of chitinase activity in these isolates, and the difference between clearing zone diameter and the colony diameter is a measure for the enzyme activity. Most active isolates were sub cultured to confirm purity and then stored on CCA agar medium at 4°C. For long-term storage, suspensions of bacteria were stored in glycerol with nutrient broth (20% v/v) at – 20°C.

Characterization of the isolates. *Streptomyces* colonies were characterized morphologically and physiologically according to the International *Streptomyces* project (ISP) (Shirling and Gottlieb, 1966) and as described by Saadoun *et al*. (2008).

Chitinase standard curve. Different concentrations of N-acetyl-D-glucosamine (ACROS, Belgium) (1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 µmol/ml) were prepared by diluting N-acetyl-D-glucosamine in 0.1 M citrate buffer (pH 7.0) to obtain the desired concentration. The reaction mixture, containing 1 m1 N-acetyl-D-glucosamine, 1 m1 citrate buffer and 2.0 m1 of 3, 5-dinitrosalicylic acid reagent (DNS) was incubated in 100°C for 5 min. After cooling the tubes, the colored solution was centrifuged at 4000 rpm for 15 min and the optical density (OD) of the test samples was measured at 575 nm. Each single point in the curve represented the average measurements. A linear relation was plotted between the N-acetyl-D-glucosamine concentration and its corresponding OD at 575 nm to yield the N-acetyl-D-glucosamine standard curve (Priest, 1985).

Chitinase activity assay. This study focused on the assay of chitinase enzyme for the most active *Streptomyces* isolate. Chitinase activity assay was performed according to Miller (1959). Briefly the reaction mixture composed of 1 ml of crude enzyme solution, plus 1 ml 0.5% colloidal chitin in 0.1 M citrate buffer (pH 7.0) was incubated at 37°C in a shaking water bath (GFL, Germany) for 30 min (Miller, 1959). The reaction was then terminated by adding 2 ml DNS reagent. The colour was developed in a boiling water bath for 5 min. The optical densities of samples were measured at 575 nm against a blank containing 1 ml substrate-buffer solution of 0.5% concentration, 1 ml buffer and 2 ml DNS. These optical densities were then compared with controls media inoculated with an inactive chitinolytic streptomycete isolate. The amount of released was determined from an *N*-acetyl-D-glucosamine calibration curve, where one unit (U) of chitinase activity is defined as the amount of enzyme that releases 1 µmol of glucose/min under the above mentioned conditions.

Optimization of growth conditions and chitinase production. Erlenmeyer flasks containing 50 ml of colloidal chitin broth medium were inoculated with 1 ml of spore suspension (10^7 spores/ml) of a 7 days old culture. Cultures were incubated in an orbital shaker incubator for 7 days. Then the chitinase activity was assayed daily by the DNS method as described above.

Effect of incubation time on chitinase activity. The effect of incubation time on the chitinase production was performed using 250 ml Erlenmeyer flasks containing 50 ml of CC broth. Flasks were then incubated at 28°C for 7 days, then the enzyme activity was performed each 24 h in order to determine the optimum incubation time for enzyme production.

Effect of pH and temperature on chitinase activity. To detect the effect of pH and temperature on chitinase production 250 ml Erlenmeyer flasks were prepared containing 50 ml of CC broth, with pH values in the range of (4–10). Flasks were then incubated at 28°C for the optimum incubation time. The pH value

giving the highest enzyme activity was used in further enzyme assay.

The optimal temperature for enzyme activity was determined by performing the standard assay procedure that is mentioned before at a range of temperature 25 to 40°C. All further enzyme assays were performed at the optimum temperature.

Effect of different colloidal chitin (CC) concentrations on chitinase production. To detect the effect of various CC concentrations on chitinase production 250 ml Erlenmeyer flasks were prepared containing 50 ml of colloidal chitin broth supplemented with the following CC concentrations in (g/l); 2, 4, 8, 16, 20, 24, 28, 32 and 36, giving a total of 0.5% carbon source. Chitinase assay was performed at the optimum incubation time, pH and temperature.

Chitinase production under optimum conditions. *Streptomyces* sp. (strain S_{242}) was cultivated on CC broth medium with the optimized incubation time, incubation temperature, pH and colloidal chitin concentration. Crude chitinase enzyme produced by this active strain under these optimal conditions was assayed as mentioned before.

Gel electrophoresis and crude chitinase zymogram

SDS-PAGE. SDS-PAGE was performed as described by Trudel and Asselin (1989) with some modifications. Separating gels were made of 12.5% (W/V) polyacrylamide 0.01% (W/V) glycol chitin and 0.1% (W/V) SDS. Stacking gels were made of 5% (W/V) polyacrylamide containing 0.1% (W/V) SDS. Samples from cultured bacteria for different incubation times were boiled for 5 min with 25% (W/V) glycerol and 2% (W/V) SDS in 60 mM Tris-HCl (pH6.8) with 5% (V/V) 2-mercaptoethanol, then loaded into two SDS-PAGE gels, one for chitinase activity and the other for regular protein staining. Bromophenol blue (0.l%), (W/V) was used as a tracking dye. Electrophoresis was run at 80 and 120 volts for the stacking and separating gels, respectively, using a mini protein II (BioRad) system. Wide range protein molecular weight (6,500–200,000 Da) marker (Sigma, USA) was used for the molecular weight determination.

Detection of chitinase activity after SDS-PAGE. After electrophoresis, chitinase activity gel was incubated for 2 h at 37°C with reciprocal shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (V/V) Triton X-100. After that, gels were stained with freshly prepared 0.01% (W/V) Calcofluor white M2R (Sigma, USA) in 500 mM Tris-HCl (pH 8.9). After 5 min, the brightener solution was removed and the gels were incubated for about 1 h at room temperature in distilled water. Lytic zones were visualized by placing the gels on a UV transilluminator (Vilber Lourmat, EU) then photographed with Polaroid camera (Japan).

Glycol chitin synthesis. This was followed as described by Trudel and Asselin (1989). Five grams of glycol chitosan (Sigma, USA) were dissolved in 100 ml of 10% acetic acid by grinding in a mortar. The viscous solution was allowed to stand overnight at 22°C. Methanol (450 ml) was slowly added and the solution was vacuum filtered through a Whatman No 4 filter paper. The filtrate was transferred into a beaker and 7.5 ml of acetic anhydride was added with stirring. The resulting gel was allowed to stand for 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel pieces was discarded. Gel pieces were transferred to a Blender, covered with methanol, and homogenized for 4 min at high speed. This suspension was centrifuged at 20 000 g for 20 min at 4°C. The gelatinous pellet was resuspended in about 1 vol of methanol, homogenized, and centrifuged as described above. The pellet was resuspended in distilled water (500 ml) containing 0.02% (W/V) sodium azide and homogenized for 4 min. This was the final 1% (W/V) stock solution of glycol chitin.

Protein determination and staining of polyacrylamide gels. After electrophoresis, SDS-PAGE gel was stained with silver stain kit (Bio-rad, USA) according to the manufacturer instructions. The gel was photographed with a digital camera (Sony, Japan).

Statistical analysis. Analyses of variance for all data were performed using statistical analysis system (SAS). Means were separated by the least significant differences (LSD) at $\alpha = 0.05$.

Results

By employing enrichment methods, a total of 231 different *Streptomyces* isolates were recovered from 19 soil samples that were collected from different habitats in Jordan. All of these isolates matched the genus description as reported by Shirling and Gottlieb (1966), Nonomura (1974) and Williams *et al*. (1983).

Screening for chitinase-producing *Streptomyces* isolates. Data showed that only 35 of all screened isolates (15.15%) were chitinase producers as indicated by a clear zone formed around the colonies after 8 days of incubation on CC agar plates (Fig. 1A). Colonies not forming the clearing zone were considered as negative chitinase producers (Fig. 1B). Active chitinase producing isolates were categorized into 3 groups: strong (group 1), moderate (group 2), and week (group 3) with the clear zone diameter of 5 – 10 mm, 2.1 – 4 mm and 1 – 2 mm, respectively (Table I). Group 1 represented only 14.28% of the active isolates; however the other 30 isolates were divided equally between group 2 and group 3 with 42.86% for each. Our results show that most of the isolates (84.84%) did not show any activity, even after incubation of the colonies for 20 days.

Table I
Distribution of the most active chitin-degrading *Streptomyces* isolates

Chitinolytic activity (Δ X[a])					
Group 1 Total: 5 (5–10 mm)		Group 2 Total: 15 (2.1–4 mm)		Group 3 Total: 15 (1–2 mm)	
Isolate	ΔX	Isolate	ΔX	Isolate	ΔX
S_{242}	10	A_{142}, M_{132}, K_{331}, M_{141}, Ju_{232}, Kr_{132}, De_{332}, S_{342}, Bo_{132}	4.0	Ma_{242}, Hm_{242}, M_{151}, M_{142}, A_{231} K_{232}, N_{241}, Hm_{331}, A_{151}, Mr_{242}, N_{151}, Ma_{231}, S_{142}, M_{152}	2.0
M_{342}, Bo_{241}, T_{232}, M_{241}	5.0	Hm_{242}, A_{431}, M_{531}, K_{231}, Hm_{442}, Ju_{132}	3.0	M_{251}	1.0

[a] Δ X (chitinase activity) = the clearing zone diameter-colony diameter

The isolate S_{242} was chosen as the most active chitin-degrading isolated *Streptomyces*, which exhibited a 30 mm diameter of clear zone on CCA (Fig. 1A). Morphological and physiological characterization of this strain, revealed that it belongs to the white colony colour series with a distinctive reverse side colour, did not produce diffusable and melanin pigments and had a rectiflexible (RF) sporophore arrangement. The isolate was unable to utilize sucrose, I-inositol, rhaminose and raffinose. However, it utilizes D-glucose, L-arabinose, D-xylose, D-fructose and D-mannitol.

Optimal conditions for production of chitinase enzyme by S_{242} isolate. Strain S_{242} started to produce the enzyme after 3 days of incubation with 91% relative activity when compared to the maximum activity (0.0456 U/ml) that is considered as 100% at day 4 (Fig. 2A). It was further noticed that the enzyme production declined gradually after 5 days of incubation and decreased to less than 38% at day 7 and 8. Data revealed that the isolate S_{242} optimally produced the enzyme at day 4 which was significantly different ($p<0.05$) from the amount produced after day 1, 2, 6, 7 and 8 of incubation. However, it was not significantly different ($p>0.05$) when compared to 3 and 5 days of incubation.

Determination of optimum pH for chitinase production. The crude chitinase from strain S_{242} was active over a pH range of 6–10 and most active at pH 7–8, with maximum activity at pH 7 (0.0416 U/ml) which was significantly different ($p<0.05$) from the control and the pH values of 5 and 10. The relative activity at pH values of 4, 5 and 6 was 4%, 4% and 26%, respectively; at pH values of 8, 9 and 10, the relative activity was 72%, 43% and 24%, respectively (Fig. 2B). Enzyme activity at pH 7 was not significantly different ($p>0.05$) from the activity at pH values of 4, 6, 8 and 9.

Determination of optimum temperature for chitinase production. Chitinase activity was present over the temperature range 25–35°C and most active at 30°C (0.0461 U/ml) and 35°C (100% and 99%, respectively) (Fig. 2C). However, the activity declined

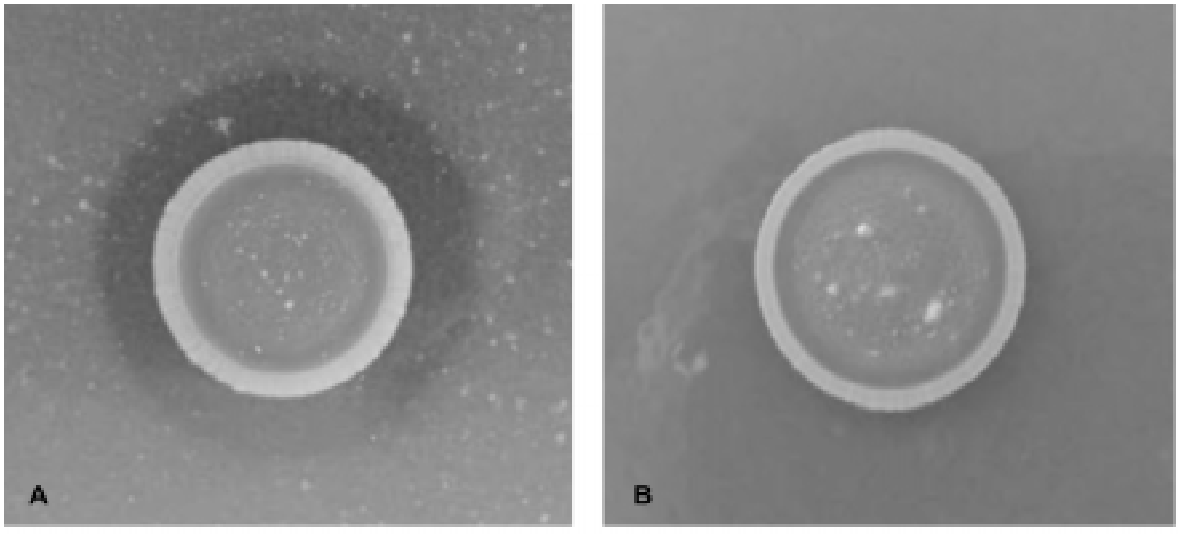

Fig. 1. Chitinolytic activity of *Streptomyces* isolates on colloidal chitin agar; (A) clearing zone surrounding S_{242} most active chitinase producing isolate, (B) negative control; no clearing zone indicating inactivity.

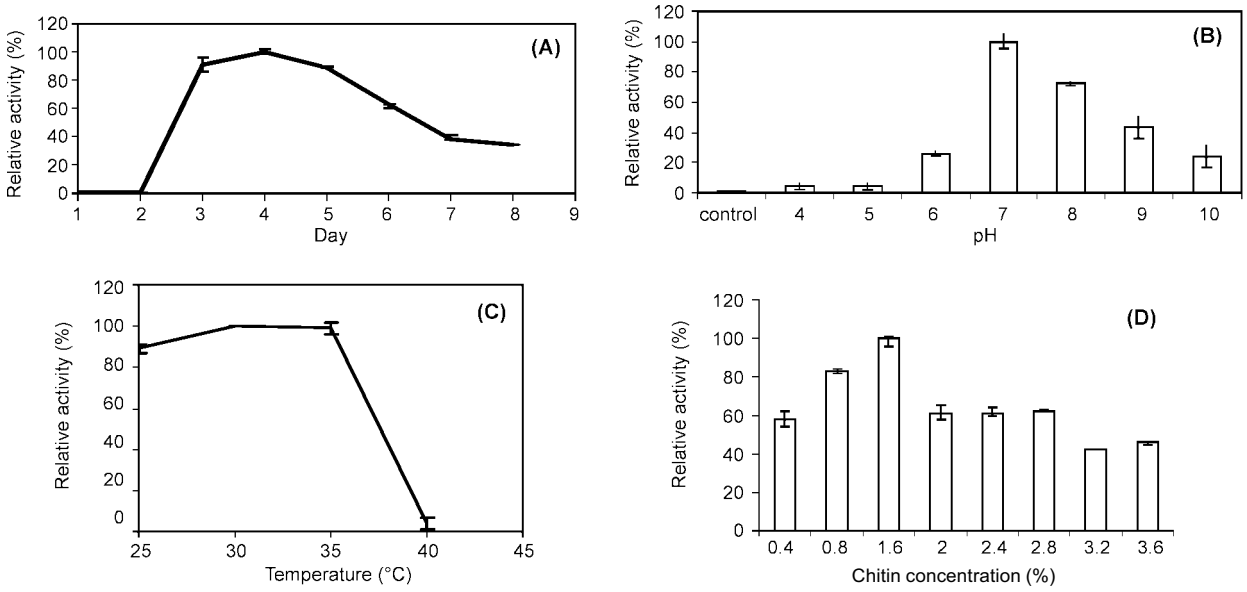

Fig. 2. Determination of optimum (A) time; (B) pH; (C) temperature; (D) colloidal chitin concentration for chitinase production by the S_{242} isolate.

Enzyme activities under the different conditions are compared to the highest value, considered as 100%.

to 4% at 40°C. Data showed that the amount of chitinase enzyme produced at 30 and 35°C was significantly different ($p<0.05$) from that produced at 40°C, but not significantly different ($p<0.05$) from that produced at 25°C.

Effect of different colloidal chitin concentrations on chitinase production. Figure 2D shows that the maximum enzyme activity was obtained when strain S_{242} was cultured in broth of 1.6% colloidal chitin concentration with 0.0774 U/ml. Data indicated that when strain S_{242} was cultured in broth with 0.4 to 2.8% CC, the relative activity of the enzyme was more than 58%; however, the activity was less than that when cultured in broth of 3.2 and 3.6% CC. Enzyme activity achieved at 1.6% of CC was significantly different ($p<0.05$) from all other CC concentrations.

SDS-PAGE electrophoresis and zymogram analysis. Crude chitinase profile of the *Streptomyces* (S_{242}) strain during the incubation period (day 1 to day 4) shows an increase in the number of bands as the incubation time increases with the observation of 6 bands at day 4 (Fig. 3, lane 4). Data indicated that the increase in the number of chitinase bands during the incubation period corresponds to the increase in the crude enzyme activity. Some bands appeared more abundant than the others with MW ranged from 51 to 55 kDa. After applying the crude chitinase from the culture filtrate of the *Streptomyces* (S_{242}) strain, six distinctive bands exhibited a chitinase activity in the zymogram with MW ranged from 39 to 97. These bands corresponded to the M.Wt of 39, 44, 51, 55, 64 and 97 kDa (Fig. 3).

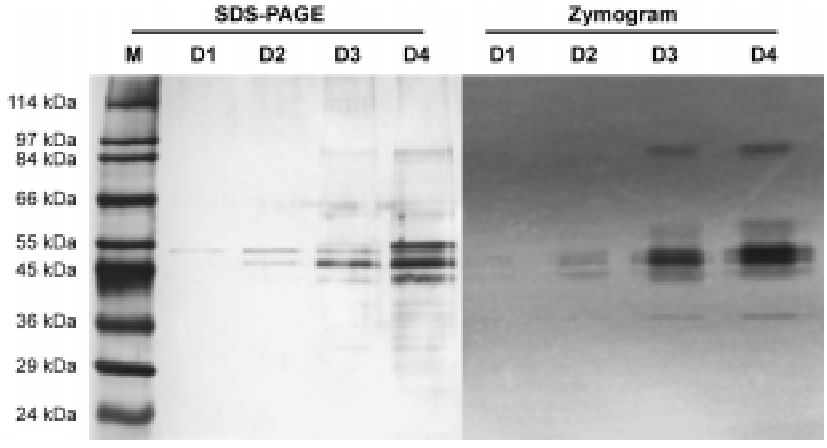

Fig. 3. 12% SDS-PAGE and zymogram analysis of crude chitinase from the culture filtrate of the active isolate (S_{242}) followed from day 1 to day 4 of incubation.

M: broad range protein marker (kDa), D1: day 1, D2: day 2, D3: day 3, D4: day 4.

Discussion

The major producers of chitinases in soils are *Streptomyces* (Tanabe *et al*., 2000) and this capability makes them as valuable organisms in fields of pollution abatement, and basic and commercial biology. Because chitinases can degrade chitin containing cell wall of many fungi, this property makes it most valuable in fields of biological research and phytopathogenic fungi control (Felse and Panda, 2000). Therefore many studies have been performed in order to locate sources of these enzymes and isolate chitinases of useful activity in different organisms.

Data showed that only thirty five (15.15%) out of the total 231 isolated streptomycetes recovered from nineteen soil samples, were considered as efficient producers of chitinase. Although *Streptomyces* strains considered as major producers of chitinases, their efficiency in production differs greatly. The isolate S_{242} was selected because of its elevated chitinase productivity in comparison to all other chitinolytically active isolated streptomycetes. Such contrast was reported by Gupta *et al.* (1995) who found *S. viridificans* to be a good chitinase producer among nine species of *Streptomyces* screened.

Enzyme activity after incubation at 30 and 35°C was significantly different from the control which was inoculated with a non-chitinolytic *Streptomyces* isolate. This is in agreement with Nampoothiri *et al*. (2004) findings, who obtained maximum chitinase activity from the soil fungal isolate *Trichoderma harzianum* after 4 days of incubation at 30°C. Also it is somehow similar to what Mahadevan and Crawford (1997) found for *S. lydicus* WYEC108 chitinase, of which maximal levels were achieved at 30°C after 5 days of incubation. However, Gupta *et al*. (1995) maximally produced chitinase from *S. viridificans* at 30 after 6 days of incubation.

Maximum chitinase production was found with the media adjusted to pH 7. This maximal activity compared to the control was significantly different ($p<0.05$); also this applies to activity obtained at pH values of 5 and 10. This indicates that the pH of the medium strongly affects the growth and activity of the microorganisms. In general, microbial enzymes are produced in higher yield at a pH near to the maximal for enzyme activity. However, the maximum enzyme production at pH 7 had no significant difference compared to pH values of 6, 8 and 9. Nampoothiri *et al*. (2004) obtained maximum chitinase production with the substrate adjusted to pH 4.5.

Maximum activity of Chitinase from strain S_{242} was observed after the addition of 1.6% colloidal chitin (100% relative activity) and was significantly different ($p<0.05$) from all other tested CC concentrations. Similarly, Gupta *et al*. (1995) achieved maximum levels of *S. viridificans* chitinase by addition of 1.5% CC, what indicates that the chitinase is an inducible enzyme. According to Nampoothiri *et al.* (2004) chitinase production was the highest when CC was used as the source material, compared to other supplements, chitin powder and chitin flakes were not as effective as CC.

Streptomyces isolate S_{242} producing most active chitinase, was considered a valuable chitin degrading isolate that utilizes it as a sole source of carbon and nitrogen. Strain S_{242} constantly produced chitinase after 3 to 8 days of incubation with relative activity of more than 89% between days 3 to 5. Optimum conditions for chitinase production were at pH 7, 30°C and addition of 1.6% CC. Enzyme production was strongly decreased at higher temperatures and extreme acidic and alkaline pH values.

After subjecting the crude chitinase from the culture filtrate of the *Streptomyces* (strain S_{242}) to zymogram analysis, six bands (39–97 kDa) exhibited a chitinase activity. The size of the chitinase active bands exhibited in the zymogram is relatively large compared to that observed by Trudel and Asselin (1989) who reported the molecular mass of chitinase from *Streptomyces griseus* and *Serratia marcescens* to be 24–72 kDa and 40.50–73 kDa, respectively. Tanabe *et al*. (2000) also reported a chitinase from *S. griseus* HUT 6037 of 49 kDa. Similarly, six chitinases from *Bacillus circulans* WL-12 were reported by Watanabe *et al*. (1990) with a molecular mass ranging from 38 to 73 kDa.

The results of the findings of present study suggest an avenue for the potential use of the organism in commercial production of chitinase using chitin and chitin derivatives as raw materials. Another possibility is production of resistant transgenic plants with recombinant DNAs having antifungal or chitinase genes cloned from this biologically active *Streptomyces* sp. (strain S_{242}), which may be a promising candidate for agriculturally important crop with increased resistance to phytopathogens.

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