

# Isolation of Novel Marine Chitinolytic Actinomycete *Streptosporangium Sp.* and Production Optimization of Extracellular Chitinase

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## ABSTRACT

Chitin is the second most abundant natural polymer and widely distributed as a structural component of crustaceans, insects, and other arthropods, as well as a component of the cell walls of most fungi and some algae. Chitinase (EC 3.2.11.14) enzyme is capable of hydrolyzing insoluble chitin to its oligo and monomeric components found in a variety of organisms including viruses, bacteria, fungi insects, higher plants and animals. In the present study, a rare marine actinomycete strain *Streptosporangium sp.* was isolated from the marine sediments and screened for its ability to degrade chitin. The effects of media composition and various fermentation conditions were optimized. Maximum chitinase activity was observed in the CCMB medium with 0.1% of colloidal chitin. The optimum pH value was found to be 7 and the temperature range was 35<sup>o</sup>C. Highest chitinase activity was observed on the 14<sup>th</sup> day of incubation, where the growth of the organism was found to be maximum.

**Keywords:** Chitin, Chitinase, Actinomycete, *Streptosporangium*.

## I. INTRODUCTION

Chitinase is an enzyme used by insects to degrade the structural polysaccharide “chitin” during the molting process (Zhang *et al.*, 2002) and it is an important biocontrol agent against insects (Reguera and Leschine, 2001) and fungal pathogens (El-Tarabily *et al.*, 2000 and El-Tarabily, 2003). Chitin is an important component of the cell wall of fungi and occurs in combination with other polymers, such as proteins. The increase in the accumulation of chitinous wastes demands the need of their proper management. Their utilization as substrate for chitinase production might serve as an effective method for management of chitinous waste (Brzezinska *et al.*, 2013). Chitinases are a class of antifungal proteins, and are of particular interest owing to their resistance against both insects as well as fungal pathogens (Vogelsang and Barz, 1993; Ye *et al.*, 2005). Among the chitinolytic bacteria, several Actinobacteria and *Streptomyces* species are thought to degrade the chitinous cell wall of plant fungal pathogens through the production of chitinases and antibiotics (Kawase *et al.*, 2006; Yu *et al.*, 2008).

The antifungal activity and highly biocompatible quality make the chitin and its derivatives particularly useful for biomedical applications such as wound healing, cartilage tissue engineering, drug delivery and nerve generation. Chitin's biodegradable and antifungal properties are also useful for environmental and agricultural uses and food technology and cosmetics. Chitinases are produced by several bacteria as well as Actinomycetes and some of them are reported to produce multiple forms of chitinases with different molecular masses. Several chitinolytic bacteria and fungi have been reported to be potential biological agents. For example, *Serratia mercrescens* is used in control of *S. rolfsii* (Ordentlich *et al.*, 1988), *Paenibacillus illinoisensis* is for against *Rhizoctonia solani* Kuhn (Jung *et al.*, 2003), and *Trichoderma harzianum* is used in control of *Botrytis cinerea* (Tronsmo, 1991).

The present study is designed to screen the chitinolytic activity of a rare marine actinomycete *Streptosporangium sp.* SETB16 isolated from the marine sediments along the south east coasts of Chennai. Several species of actinomycetes have been isolated from these coast lines by many workers with tremendous

pharmacological significance. Among the strains isolated, the genus, *Streptomyces* has been found predominantly in large numbers and has yielded commercially valuable bio-active compounds. Moreover, several species of *Streptomyces* have shown chitinase activity and they are investigated for their antifungal and bio-control potentialities. Therefore, the present investigation is focused on the screening of chitinase activity by a rare actinomycete belonging to the genus *Streptosporangium*. The genus *Streptosporangium* was first described by Couch (Couch, 1955). This genus was named so because of its special “spore – case” shape. It is closely related to *Streptomyces* but belongs to a group of rare actinomycetes (Goodfellow et al., 1990; Embley and Stackebrandt, 1994).

The apparent diversity of the genus *Streptosporangium* seems to be very limited, and hence the exploration of novel metabolites from this rare genus is on the budding stage. This interesting genus was chosen for investigations because it is very heterogeneous (Embley and Stackebrandt, 1994), but closely related to *Streptomyces* (Kemmerling et al., 1993, Embley and Stackebrandt, 1994). However, the difficulty in the isolation strategies from *Streptosporangia* regarding chemical diversity is not surprising, since most papers of natural products are referred to *Streptomyces* and therefore, production conditions are often similar or very closely related to those of *Streptomyces*. So, it can be concluded that a larger amount of bioactive compounds can be found after an optimization of the growth and production conditions of ‘rare actinomycetes’ like *Streptosporangium*.

In par with the *Streptomyces*, the research into non-streptomycetes is gaining upper hand in the novel drug discovery pipelines and the production of industrially significant enzymes. The study is aimed to screen the chitinase activity of *Streptosporangium* sp. SETB16, and optimization of extracellular chitinase production.

## II. METHODS AND MATERIAL

### Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Hsu and Lookwood, 1975 with slight modifications. 5 gms of chitin powder were added slowly to 60 ml of

conc. HCl and left at the room temperature overnight with vigorous stirring. The mixture was added to 200ml ice cold 95% ethanol and incubated overnight at room temperature with vigorous stirring. The precipitate was collected by centrifugation at 5000g for 20 minutes at 4°C and transferred to a glass funnel with filter paper (80gm). The colloidal chitin was washed with the sterile distilled water until colloidal chitin became neutral (pH 7.0), the colloidal chitin retained on a filter paper was removed, weighed and stored in a dark place at 4°C.

### Primary Screening for chitin hydrolysis

Primary screening was performed by single line streak of spores in the center of CCA media containing colloidal chitin and incubated at room temperature. The zone of clearance due to chitin hydrolysis was recorded up to 5 days. The isolates producing clear zones over 0.5 cm alone were selected and subjected to secondary screening.

### Secondary Screening for chitin hydrolysis

The isolate was grown in yeast malt extract (ISP-2) broth containing 0.1% chitin. 3% of the inoculum was suspended into the medium and incubated at 150 rpm in a rotary shaker at 35°C. After 14 days of incubation, the cultures were harvested, centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatant was collected. Colloidal chitin (0.1%) agar plates were prepared and wells were made using 9mm sterile cork borer. 100 ml of culture filtrate of each isolate was suspended in each well and incubated at 37°C. After 12 hours the development of clear zone around the well was observed.

### Optimization of culture conditions Medium

Production medium designed by (Mitsutomi et al 1995) was used with slight modification: colloidal chitin - 0.1gm, NaNO<sub>3</sub> – 2.0 gms, K<sub>2</sub>HPO<sub>4</sub> – 1.0 gm, Mg SO<sub>4</sub>. 7H<sub>2</sub>O – 1.0 gm, CaCO<sub>3</sub> – 1.0 gm, FeSO<sub>4</sub>.7H<sub>2</sub>O -0.01 gm, KCl – 0.5 gm, H<sub>2</sub>O – 1000ml pH 7.0. 3% of inoculum was inoculated into the medium and incubated at 150 rpm at 35°C. After 8 days of incubation, the cultures were harvested and centrifuged at 10,000 rpm for 15 minutes and the supernatant was used for the chitinase assay.

## Effect of pH, temperature, nitrogen sources and metal ions

The effects of the pH, temperature and the metal ions on the chitinolytic enzyme production by *Sterptosporangium sp.* were studied by growing cultures at temperatures between 15 and 40 degrees centigrade. Initial pH between 5.0 and 8.0 and the metal ions such as Mn<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Na<sup>+</sup>, Hg<sup>+</sup> etc; . All experiments were carried out in 500 ml Erlenmeyer flasks containing 100ml culture medium incubated at 35°C (except for temperature experiments) at 150 rpm for 36 hours. Effects of different nitrogen sources on the enzyme production by *Streptosporangium sp.* were investigated. Results showed maximum chitinase activities obtained from optimized medium (9.36 units/ml). Chitinase secretion was increased using 0.2% colloidal chitin, as a sole nitrogen and carbon source (7.19 units /ml). Inorganic nitrogen source (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was not suitable for enzymes production.

## Chitinase Assay

The reaction mixture contained 0.5ml of 0.01% colloidal chitin in sodium acetate buffer (0.05M pH 5.2) and 0.5ml culture filtrates was incubated at 37°C for 2 hours in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by the colorimetric method (Reissig et al 1955). The reaction was terminated by adding 0.1 ml of 0.08M potassium tetra borate, pH 9.2 to 0.5 ml of reaction mixture and then boiled in a water bath for 3 minutes. Then 3ml of diluted pdimethylaminobenzaldehyde ( p- DMAB sigma chemicals company, USA ) reagent was added and again incubated at 37°C for 15 minutes. The released product in the reaction mixture was read at 585nm in a spectrophotometer (Hitachi, Japan). Chitinase activity was determined using N-acetylglucosamine (Sigma chemicals company, USA) as the standard. One unit of chitinase activity was defined as the amount of enzyme, which produces 1C mole of N- actetylglucosamine in 1 ml of reaction mixture under the standard assay condition (Mathivannan *et al.*, 1998).

## III. RESULT AND DISCUSSION

Chitin is a major source of natural organic compounds (Yang *et al.*, 2009) and it is a long chain polymer of N-acetyl-D-glucosamine (GLcNAc) monomers forming covalent ( $\beta$ -1,4 linkages (Kim *et al.*, 2007, Xayphakatsaa *et al.*, 2008). The degradation and recycling of chitin constitute an important phase in maintaining the cycling of carbon and nitrogen (Hong *et al.*, 2011). The enzymatic hydrolysis of chitin to free N-acetylglucosamine proceeds with the participation of chitinolytic enzymes which catalyze the hydrolytic depolymerization of chitin (Gohel *et al.*, 2006; Saks and Jankiewicz, 2010). There are many reports of chitinolytic activities of actinobacteria. Among the actinobacteria, genus *Streptomyces* has been screened for its chitin degrading capability (Pranee *et al.*, 2012; Anand *et al.*, 2012; Priya *et al.*, 2011 and Santhi, 2006). However, there are reports that show that the species belonging to the genus other than *Streptomyces* could also be effectively screened for its chitinolytic activities.

In the present study, the strain SETB16 was screened for its chitinase activity. On the basis of colloidal chitin degradation, (Fig 1) a maximum of (1 cm) zone of clearance was observed, in the primary screening. The secondary screening was done in a suitable production media and the culture conditions were optimized (Graphs 1 to 3).



Figure 1: Primary screening of hydrolysis of chitin

## Optimization of culture conditions for chitinase production

Three different culture media were tested for maximum chitinase activity namely: CCMB, CGMB and CMMB. Among the three media studied maximum activity was found in the CCMB medium (6.24 units/ml) when compared to CGMB (1.76 units/ml) and CMMB (4.12 units/ml), respectively. Three different concentrations of colloidal chitin were tested for maximum chitinase activity ranging from 0.1% to 0.3%. 0.1% concentration of colloidal chitin exhibited maximum activity, whereas limited activity was observed at 0.2% concentration and beyond 0.3%, the substrate concentration reduced the enzyme activity. Nevertheless, many researchers claim that colloidal chitin is the best inducer of chitinases (Kim *et al.*, 2007; Zarei *et al.*, 2010).

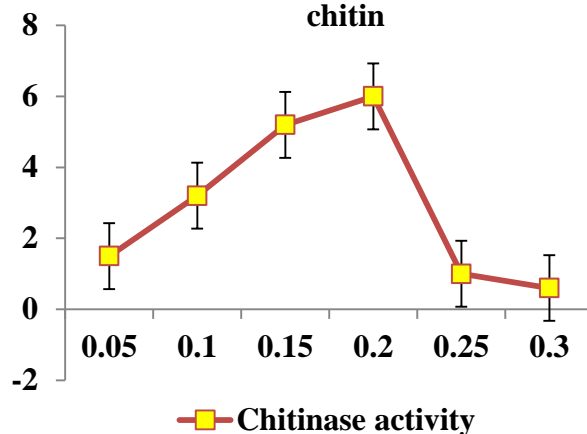
The pH activity profile showed an optimal pH of 7.0 and retained more than 80% activity in a wide pH range of 7.0 to 10 with maximum stability in the pH range of 6.0 to 8.0. Interestingly, there was no chitinase production at pH values below 6.0. Previous reports available also suggested that slightly alkaline pH favoured the chitinase production by various bacteria including *Aeromonas* sp. (Ahmadi *et al.*, 2008), *Micrococcus* sp. (Annamalai *et al.*, 2010) and *Serratia marcescens* (Xia *et al.*, 2011). A finding by Hiraga *et al.* (1997) supported the present findings by reporting the optimum pH for chitinase production by bacteria lies between 5 and 8.

The optimum temperature range was determined to be 35°C. At temperatures above and below this optimal value, the chitinase activity was decreased sharply. Similar reports were also available which reported 35°C served as optimum temperature for chitinase production by *Streptomyces* sp. (Narayana and Vijayalakshmi, 2009) and *T. harzianum* (Sudhakar and Nagarajan, 2011). In general, most of the soil borne *Streptomyces* sp. showed the optimum temperature for maximum chitinase production lies between 30 to 40°C (Gomes *et al.*, 2001; Shanmugaiah *et al.*, 2008; Subramaniam *et al.*, 2012).

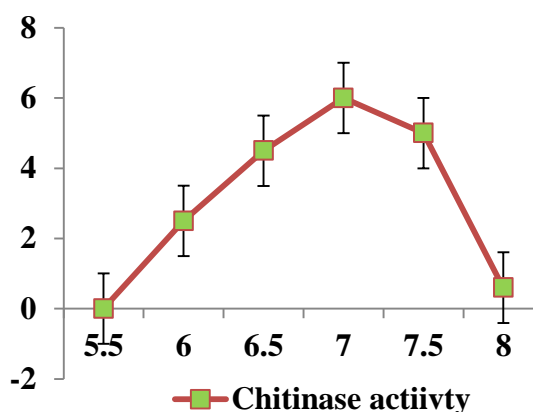
It was observed that the strain SETB16, showed maximum chitinase activity (6.24 units/ml) at the 14<sup>th</sup> day of incubation. As per previous works, 5<sup>th</sup> day seemed to be the optimum day for the maximum chitinase activity in the case of *Streptomyces* species

and common other bacterial strains other than actinobacteria. The actinomycete strain *Streptomyces albus*. With the increase in the number of days of incubation, there was a drastic decline in the production of chitinase.

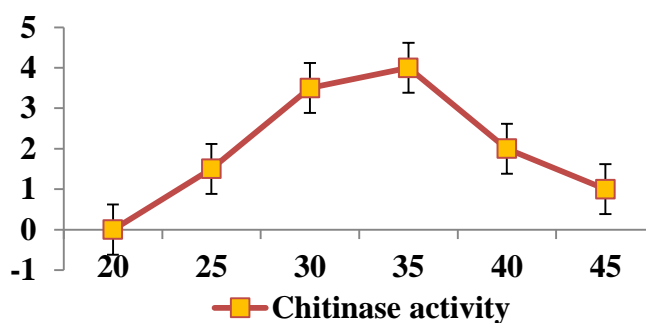
Graph 1 Concentration of colloidal chitin



Graph 2 pH



Graph 3 Temperature



#### IV. CONCLUSION

The present investigation appears to be the first record of chitinase activity by a non-streptomycete rare actinomycete belonging to the genus *Streptosporangium*. In the present study, a rare marine actinomycete strain *Streptosporangium sp.* was isolated from the marine sediments and screened for its ability to degrade chitin. The effects of media composition and various fermentation conditions were optimized. Maximum chitinase activity was observed in the CCMB medium with 0.1% of colloidal chitin. The optimum pH value was found to be 7 and the temperature range was 35<sup>o</sup>C. Highest chitinase activity was observed on the 14<sup>th</sup> day of incubation, where the growth of the organism was found to be maximum.

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