



Isolation of chitinase producing *Streptomyces albus* FS12, production and optimization of extracellular chitinase

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Abstract

In the present study, a chitinolytic actinomycete strain FS12 was isolated from fish market soil in Chennai, India. The isolated chitinolytic actinomycete was identified as *Streptomyces albus* based on its morphological, physiological and biochemical characteristics. The maximum production of chitinase was found on fifth day of incubation, with pH 8 and 37°C using colloidal chitin as substrate by *S. albus*. The degradation of chitinous substrates (Shrimp shell powder, fish scale powder and chitin powder) was also examined on the basis of chitinase production. *Streptomyces albus* degraded all the three chitinous substrates in the following order: Shrimp shell powder > fish scale powder > chitin powder. The results revealed that the crude chitinous waste can be degraded more efficiently when compared to purified chitin powder which may be due to the presence of additional nutrients that supports their degradation. From the result of the present findings, the chitinase produced by *S. albus* FS12 can be used in the management of chitinous wastes.

Keywords: Chitinous substrate, colloidal chitin, shrimp shell waste, optimization

Introduction

Among the natural polymers, chitin is considered to be second most abundant in the world which is widely distributed as a structural component of insects, crustaceans, and cell wall component of most of the fungi (Lee *et al.*, 2009; Yang *et al.*, 2009). Chitin is the chemical analogue of cellulose, differs in the acetylation of the hydroxyl groups of each glucoside residues. About 70% of the total body weight of shellfishes is composed of chitinous substances and are often discarded as waste. Every year, approximately 1000 tons of chitin is accumulated as waste from aquatic food industries worldwide (Xu *et al.*, 2008; Wang *et al.*, 1997). The management of such large amount of waste is always a challenging task by the researchers. These wastes can be used as the renewable raw materials for the production of various high value products like chitin, chitosan, etc.

Chitinases hydrolyse the insoluble chitin to its oligo and monomeric components. Chitinases are present in various bacterial species like *Serratia*, *Aeromonas*, *Streptomyces*, etc. Based on their target, chitinases may be of endo- or ecto-chitinases. Chitinases find their application in various industries like, single cell protein preparation, protoplast isolation from yeast and fungi, chitinous waste management, malaria control, etc. The enzyme digested chitin oligomers are used in various agricultural, industrial and medical industries. The chito-oligomers can also be used as antifungal and antibacterial agents and also as a food quality enhancer (Kawase *et al.*, 2006; Yu *et al.*, 2008).

The increase in the accumulation of chitinous wastes demands the necessary 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). With these backdrops, the present study aimed at the isolation of most prominent chitinolytic actinomycete bacteria from soil sample and optimization of production parameters for maximum chitinase production. The study also aimed to utilize the chitinous natural substrates for the production of chitinase.

Materials and Methods

Actinomycetes Isolation

A total of 5 different soil samples from fish market and plant rhizosphere were collected in sterile polythene bags from different regions of Chennai, TN, India. For the isolation of actinomycetes, the starch casein agar (SCA) medium was used. The medium consists of (g/l): soluble starch, 10.0; casein, 0.3; KNO₃, 2.0; NaCl, 2.0; MgSO₄.7H₂O, 0.05; CaCO₃, 0.02; FeSO₄.H₂O, 0.01; K₂HPO₄, 2.0; agar, 18.0; pH, 7.0 (Poosarla *et al.*, 2013). The collected soil samples were then diluted serially and plated on the starch casein agar medium. The growth of actinomycete colonies over the SCA medium was observed for every 24 h interval up to seven days incubation. The colonies developed were sub-cultured to maintain pure culture and further screened for chitinase production.

Preparation of Colloidal Chitin

The colloidal chitin was prepared by slowly adding 40 g of chitin into 600 ml of concentrated HCl and left for 60 min at 30°C under vigorous stirring. The mixture was further added to 2 litres of distilled water at 10°C. The suspension was collected by filtration and washed with distilled water till the pH was adjusted to 3.5 (Saima *et al.*, 2013). The colloidal chitin obtained was used as a substrate in the medium for further studies.

Screening of Chitinase Producing Actinomycetes

For the screening of chitinase producing actinomycete strains, colloidal chitin agar medium was used. The medium consists of (g/l): Na₂HPO₄, 6; KH₂PO₄, 3; NH₄Cl, 1; NaCl, 0.5; yeast extract, 0.05; agar, 15 and colloidal chitin 1% (w/v) (Saima *et al.*, 2013). Chitinolytic screening was performed with the actinomycete isolates on the colloidal chitin agar medium and the plates were incubated at 37°C. The actinomycete isolate FS2 shown larger hydrolysis zone after 96 h of incubation was selected for further studies.

Production of Chitinase

For the production of chitinase by *Streptomyces albus* FS2, the production medium designed by Brzezinska *et al.* (2012) was used with slight modification. The medium consists of (g/l): sodium caseinate, 2; asparagines, 0.1; K₂HPO₄, 0.5; MgSO₄.7H₂O, 0.1 and colloidal chitin, 2; pH 7.5. About 100 ml of above mentioned production medium was prepared and inoculated with 1% (v/v) of *Streptomyces albus* FS2 suspension. The production was performed at 28°C for 10 days in shaker incubator (100 rpm). After incubation, the cultures were aseptically collected and centrifuged at 10,000 rpm for 10 min. The culture supernatant acts as enzyme source for further assay procedures.

Assay of Chitinase and Protein Estimation

The chitinase activity of the culture supernatant was assayed as per the modified method of Saima *et al.* (2013). Briefly, 150 µl of the culture supernatant was added to the reaction mixture consisting of 150 µl of 0.1M phosphate buffer (pH 7.0) and 300 µl of 0.1% colloidal chitin. The reaction mixture was incubated at 55°C for 10 min followed by centrifugation at 10,000 rpm for 5 min. About 200 µl of the resulting supernatant was added to 500 ml of distilled water and 1000 ml of Schales reagent. The above mixture was boiled for 10 min and cooled immediately. The absorbance of the mixture was read spectrophotometrically at 420 nm. One unit of chitinase activity was defined as the amount of enzyme which required for the release of 1µmol of reducing sugar as N-acetyl-D-glucosamine (GlcNAc) equivalent in one minute.

Optimization of enzyme production

The effect of different operational parameters on chitinase production was optimized further. About 1% culture was inoculated into the production medium and incubated at 37°C in a rotary shaker incubator (120 rpm). For the determination of optimum incubation time for chitinase production, the cultures were harvested every day till 10 days, centrifuged at 10,000 rpm for 15 min and the supernatant was subjected to chitinase assay. For the determination of optimum temperature, the chitinase production medium was prepared in six different flasks and the chitinase production was assayed after incubating the medium at different temperatures (18, 22, 37, 40, 50 and 55 °C). The optimum pH for the chitinase production was investigated by varying the initial pH

of the culture medium from 4 to 11 and at optimized temperature and incubation period.

Assimilation of natural substrates for chitinase production

The ability of the strain FS2 in the utilization of natural substrates for chitinase production was determined. The substrates tested were shrimp shell powder, fish scale powder and chitin powder (CP). About 1% concentration of substrates were amended in optimized production media by replacing colloidal chitin and chitinase production was assayed for 10 days to determine the optimum substrate for the chitinase production.

Identification of *Streptomyces* sp. FS2

PIBwin (probabilistic identification of bacteria) software was used for the identification of the chitinase producing actinomycete strain FS2. This software served as a probabilistic identification matrix for *Streptomyces* based on 50 characteristics like spore chain morphology, pigmentation, antibiotics, antibiotic sensitivity, growth tolerances and nutritional requirements (Langham *et al.*, 1989). The *Streptomyces* strain FS2 was identified based on the morphological, physiological, biological and molecular characterization (Goodfellow and Cross, 1984).

Physiological and Biological Characteristics

The mycelia growth was observed after two weeks of incubation in the medium recommended in the International *Streptomyces* Project (ISP) (Waksman, 1961; Shirling and Gottlieb, 1966). The protocol derived by Prauser (1964) was used in the color determination. The average temperature required for the actinomycete was determined in temperature gradient incubator using inorganic salt starch agar medium (ISP 4).

Molecular Identification of *Streptomyces* sp. FS2

The chitinolytic *Streptomyces* strain FS2 was identified using 16S rRNA sequence analysis. For this

purpose, the total genomic DNA was extracted from the cells by using the phenol–chloroform method and from the isolated DNA, 16S rRNA region was amplified with universal forward and reverse bacterial primers using PCR. The PCR amplified product was further processed for sequencing using the same set of primers in both the directions to check the validity of the sequence. The sequence was later subjected to sequence similarity search using BLAST tool for the identification of the strain *Streptomyces* sp. FS2.

Results and Discussion

A total of 18 morphologically different actinomycetes were isolated from five soil samples collected from different locations of Chennai, India. Based on the colloidal chitin degradation on starch casein agar plates, three colonies were found to be possessing chitinolytic activity. Among them, the strain FS2 possessing maximum chitinase production based on zone of clearance was selected for further studies. Colloidal chitin served as a better chitin source used for the screening of chitinolytic organisms. Karunya and co-workers (2011) also observed that the presence of colloidal chitin in screening and production medium supports chitinase production from bacteria.

While assaying the effect of incubation time on chitinase production, the actinomycete strain *Streptomyces albus* showed maximum chitinase production after fifth day and remains constant up to sixth day incubation (U/ml). After sixth day, the enzyme production gradually decreased which might be due to the lack of nutrients or production of secondary metabolites in the medium which may affect the production of enzyme (Fig. 1).

Nawani *et al.* (2002) and Faramarzi *et al.* (2009) have also reported similar results where they observed the maximum chitinase production during fourth day of incubation. In few other reports, maximum chitinase production was observed on the fifth day of incubation when inoculated with *Penicillium aculeatum* (Binod *et al.*, 2005) and *Trichoderma harzianum* (Sandhya *et al.*, 2005).

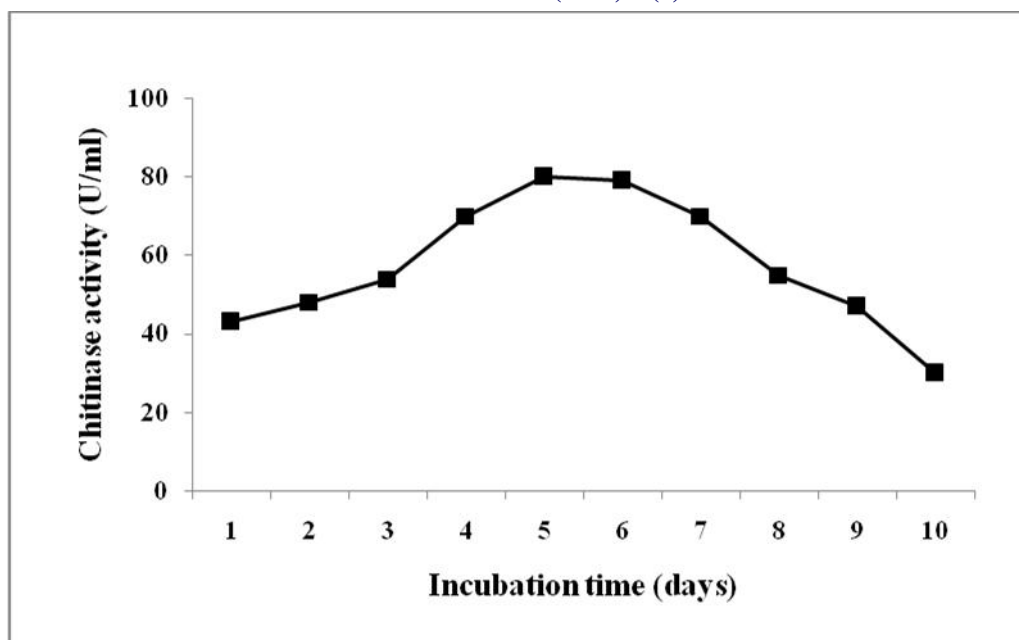


Fig. 1: Effect of incubation time of chitinase production

Temperature determines the fate of various biological processes. The growth of the organism and chitinase production are also depended on the variation in incubation temperature. Hence, the optimum growth temperature for chitinase production by *Streptomyces*

albus strain FS2 was evaluated by growing in different temperatures. From the observations, maximum chitinase production was observed in the medium incubated at 37°C (Fig. 2). The further increase in temperature decreased the chitinase production.

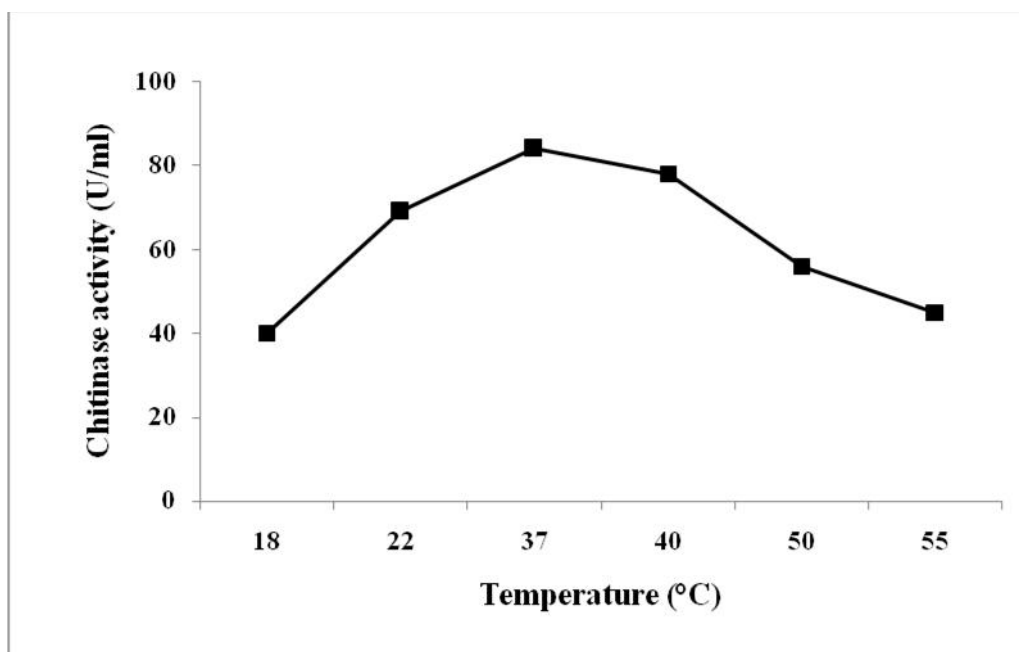


Fig. 2: Effect of temperature on chitinase production

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).

Further to the incubation time and temperature, the pH of the production medium was optimized by growing the bacterial cultures at different pH. Among them, the medium with initial pH 8 served as optimum for chitinase production by *Streptomyces albus* strain FS2

(Fig. 3). The turbidity of the medium also increased in medium with pH 8. From the observations, it can be concluded that pH of media assists in both the chitinase production as well as in the cellular growth.

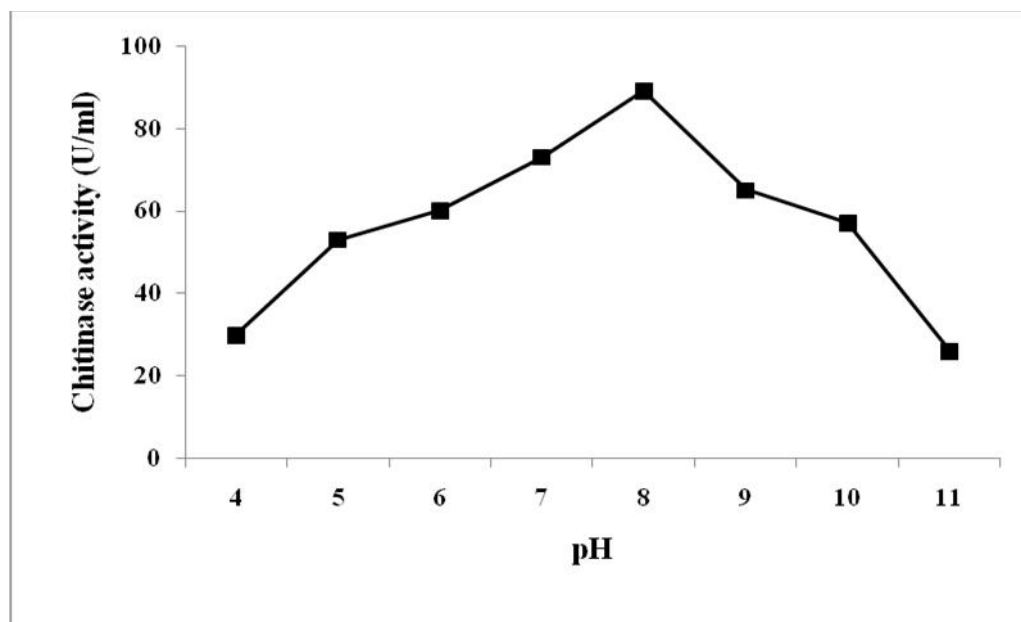


Fig. 3: Effect of pH on chitinase production

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 ability of the actinomycete in the assimilation of chitinous substrates for the chitinase production was evaluated. Among the substrates tested, shrimp shell powder was found to be the best substrate for chitinase production by *Streptomyces albus* followed by fish scale powder and chitin powder (Fig. 4). The present finding was evidenced by the previous reports with *Streptomyces lydicus* (Monreal and Reese, 1969), *Streptomyces viridificans* (Gupta *et al.*, 1995) and *Streptomyces rimosus* (Brzezinska *et al.*, 2012). In addition, the crude chitinous wastes were found to favour chitinase production when compared to purified chitin. This might be due to the presence of additional ingredients that favour the chitin degradation. The colloidal chitin with sucrose acted as sole carbon and nitrogen source for chitinase production by *Streptomyces* sp. (Subramaniam *et al.*, 2012).

The spore pattern of the strain FS2 was spiral with smooth surface, the spore mass was dirty- white to grey in colour. The aerial mycelium was gray white with the substrate mycelium was yellow brown to olive green. The growth of strain was observed well between pH 6 to 10 with incubation temperature of 35 - 40° C and NaCl tolerance of 10% in the medium. The potential strain was Gram positive, non motile, absence of any diffusible pigment and melanin production and also shows positive for oxidase, catalase and did not reduce nitrate. Several other researchers have also studied the morphological, physiological and biochemical parameters for the identification of *Streptomyces* spp. (Nayaka and Babu, 2014; Sankary *et al.*, 2015). The isolate FS2 was able to hydrolyse chitin, gelatin, casein, starch, cellulose but not utilized pectin and urea. The isolates were also able to utilize sugar such as, raffinose, sucrose, xylose, mannitol, galactose and fructose. The biochemical and cultural characteristics of the strain FS2 were shown in table 1. The results showed that the strain showed resistance to carbenicillin, amoxicillin, penicillin, rifampicin and sensitive to gentamicin, tetracycline, kenamycin, streptomycin and erythromycin. The morphological, physiological and biochemical characteristics based on ISP, Bergey's manual of Systematic Bacteriology and PIBW in ID scores, the strain FS2 was found to be *Streptomyces albus* (Shirling and Gottlieb, 1966; Holt, 1994).

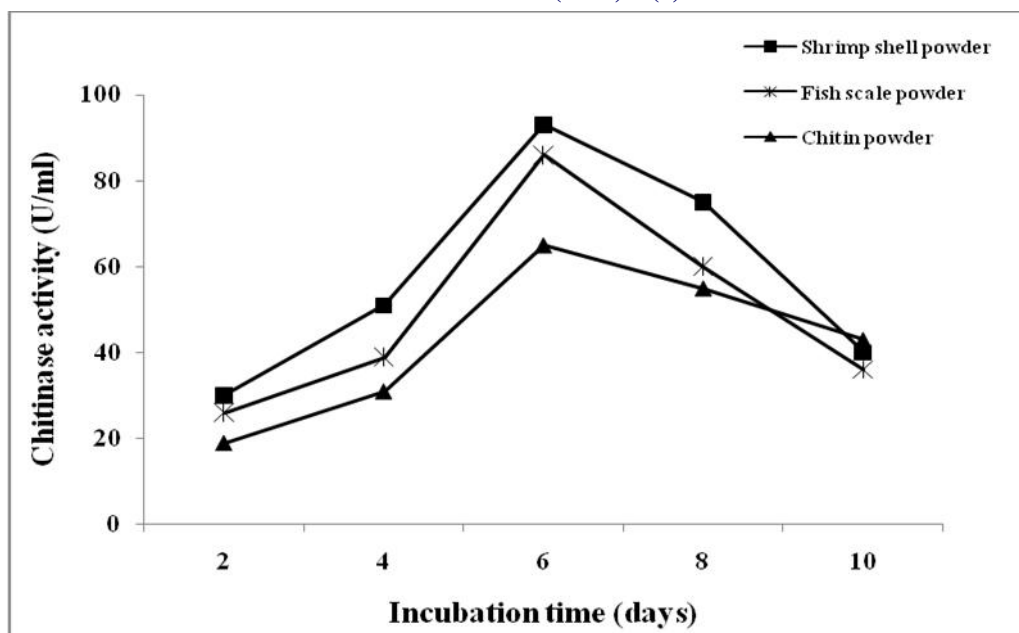


Fig. 4: Assimilation of natural substrates on chitinase production

Table 1: Morphological, physiological, biochemical characteristics and antibiotic susceptibility patter of the isolate *Streptomyces albus* FS2

Test Particulars	Observations
Gram's staining	Gram positive
Motility	-
Spore chain	Spiral
Spore surface	Smooth
Spore mass	Dirty white – gray
Aerial mycelium	Gray – white
Substrate mycelium	Yellow brown - olive green - brown
Diffusible pigment	-
Melanin production	-
Oxidase	+
Catalase	+
Nitrate reduction	-
pH	6-10
NaCl Tolerance	0 -10%
Growth in lysozyme	+
Caesin	+
Starch	+
Gelatin hydrolysis	+
Cellulose hydrolysis	+
Pectin hydrolysis	-
Urea	-
Sugar utilization	+
Raffinose	+
Sucrose	+
Xylose	+
Mannitol	+
Galactose	+

Galactose	+
Fructose	+
Carbenicilin	Resistant
Gentamicin	Sensitive
Amoxycilin	Resistant
Penicillin	Resistant
Tetracycline	Sensitive
Rifampicin	Resistant
Kenamycin	Sensitive
Streptomycin	Sensitive
Erythromycin	Sensitive

The homology of the partial 16S rRNA gene sequence of the isolate FS2 was analyzed using the BLAST algorithm in GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The highest-scored BLAST result was considered for phylotype identification of the isolate. The BLAST showed that the partial 16S rRNA gene sequence of the isolate FS2 has maximum homology (99%) with *Streptomyces albus*. Hence, the identity of the isolate was confirmed as *Streptomyces albus* with the help of 16S rRNA analysis and the sequence was submitted to Genbank.

Conclusion

Several streptomycetes were isolated from different environmental sources which are able to produce various beneficial enzymes. The present findings concluded that *Streptomyces albus* strain FS2, an efficient chitinolytic actinomycete isolated from fish market soil showed a maximum production of chitinase on fifth day of incubation at temperature 37°C with the initial pH of 8. Since the optimum temperature of *S. albus* is the field temperature for the cultivation of most of the crops in India, it can be applied to fields as antifungal agent. These streptomycetes can also be exploited in the production of enzyme which can be used in the management of sea food waste industries containing huge amount of chitinous wastes. However, further understanding of enzyme kinetics and economical factors which need to be studied before applying for any waste management of chitinous wastes.

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