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PRODUCTION AND CHARACTERIZATION OF CHITOSANASE ENZYME FROM *STREPTOMYCES* SP ISOLATED FROM BIOWASTE SOIL SAMPLES

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ABSTRACT

Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. Chitosanase enzyme has received increased attention due to its wide range of biotechnological applications. In the present study, actinomycetes isolates were screened for chitosanolytic activity and on the basis of chitosan hydrolysis zone 6 isolates were selected for chitosanase production in broth media. Based on enzyme production, the most potent isolate identified as *Streptomyces* sp SA4 was selected for further study. The effects on media composition and various fermentation conditions for optimization of chitosanase production were studied. The maximum chitosanase production was obtained at 28°C and pH 6.0 after 72 h of incubation. Among the substrates soluable chitosan (1.5%) was the best for both the strain. Regarding carbon sources, xylose (1%) was the best; while (1%) ammonium chloride was found as the best nitrogen source. The enzyme activity was maximum pH 7 and at 40°C. The crude chitooligosaccharides was prepared with SA4 enzyme and was checked for its antibacterial activity against pathogens.

KEY WORDS: **Microbial chitosanase; streptomyces; soluable chitosan; Production optimization.**

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INTRODUCTION

Chitosan [-(1P4)-2-amino-2-deoxy-D-glucose], a functional and basic linear polysaccharide, was organized by N-deacetylation of chitin [1]. Chitosan, second largest polymer next to cellulose, a partially deacetylated derivative of chitin, has emerged as biomaterial for food, pharmaceutical, waste water treatments, textile and other industries [2].The high molecular weight and higher viscous nature of chitosan makes the human intestine hard to absorb the chitosan [3]. The low viscosity and solubility at the neutral pH of chitooligosaccharide makes it more effective than chitosan [4] and has a promising bioactivity such as antitumor activity, radical scavenging, antimicrobial activity, wound healing etc. Chitooligomers were largely obtained by acid hydrolysis, oxidative degradation, microwave & gamma irradiation and enzyme hydrolysis [5]. Enzymatic hydrolysis could be considered as the preferred method due to its easily controllable nature, mild hydrolysis conditions and yielding of low environmental pollution [6]. The biodegradable nature and enzymatic hydrolysis of chitosan makes it potential material for drug delivery systems, applications in the biomedical agricultural and industrial applications, such as antibacterial, antifungal, antihypertensive and as a food quality enhancer. Chitooligosaccharides are produced by specific enzymes (chitinase, chitosanase, gluconase, and protease) and non specific enzymes [7]. Among all the specific enzymes chitosanase has a wide-range of applications such as preparation of pharmaceutically important chitooligosaccharides and is a prime tool for converting chitosan into chitooligosaccharides since all the other enzymes display activity towards various substrates but chitosanase hydrolyse only chitosan [8] into glucosamine oligomers [9]. Many chitosanases have been reported from microorganisms like bacteria, fungi and actinomycetes [10]. Microbial chitosanases differ in their pattern of hydrolytic activity either endo-type cleavage or exo-type cleavage [10]. The exotype chitosanase activity releases a single glucosamine residue and glucosmine oligomers. The endotype chitosanase produces functional chitooligosaccharides with a high degree of polymerization [11]. Even though microbial chitosanses produces chitooligosccharides, the purification process makes it expensive to be utilised in a large scale production. Thus exploration of industrial application of these enzymes needs screening of promising microbial isolates with high chitosanase activity and simple practical methodology for extraction and purification of the chitosanolytic enzymes [12]. Hence it was focussed to isolate promising chitosanase producing microbial isolate for the preparation of bioactive chitooligosaccharides. In our present study we reported the isolate *Streptomyces* sp A4, a promising chitosanase enzyme producer for degrading chitosan. The optimal production condition of chitosanase was analysed and characterized. The antibacterial effects of chitooligosaccharides were also checked against microbial pathogens.

MATERIALS AND METHODS

Sample collection and isolation of chitosonolytic organism

A total of 32 different poultry soil samples were aseptically collected from different regions of Tamilnadu and Kerala, India. The location of collected soil was chicken feather deposited pit soil samples varied from 1 month to 6 months. For screening of chitosanase producers, chitosan detection agar medium (CDA) as per [13] was amended with slight modification of adding 0.5% chitosan as substrate. The colonies showing clearance zones were considered as chitosanase-producing organism.

Screening of chitosanase producing organism

Screening was performed with bacterial isolates on the CDA plates incubated at 28°C. The isolates were selected on the basis of a larger hydrolysis zone after 96 h of incubation and further screened for maximum enzyme production in chitosan detection broth(CDA broth). The cultures were centrifuged at 10000 rpm for 15 min and the crude was used for chitosanase assay.

Assays of chitosanase activity

The chitosanase activity was assayed by measuring reducing sugar released from chitosan [14]. Briefly, 0.5ml of crude enzyme solution was mixed with 0.5ml of chitosan (pH 6.0) substrate and the mixture was incubated in water bath at 55^{o} C for 30 minutes. After incubation, 3ml of DNS (3, 5-Dinitrosalicylic acid) was added and the reaction was terminated by incubating the mixture in boiling water for 3 minutes. After cooling, the absorbance of the mixture was measured at 540 nm. One unit of the chitosanase activity was defined as the amount of enzyme which yields 1 µmol of reducing sugar per minute.

Protein content

The protein content of the crude extract was checked by [15] method with Bovine serum albumin as the calibration standard.

Characterization of the isolate

Isolation of genomic DNA for 16S rRNA and PCR amplification

Total genomic DNA from chitosanase producing microbial isolate was isolated using STE buffer and chloroform extraction. The quality/quantity was determined by agarose gel electrophoresis, followed by Ethidium bromide (EtBr) staining (0.5µg/ml). 16S rDNA was amplified with isolated genomic DNA in the presence of forward primer 8F (5´-AGAGTTTGATCCTGGCTCAG-3´) and reverse primer 1942R (5´-GGTTACCTTGTTACGACTT-3´); using the cycle conditions 95^{o}C for 5 minutes followed by 35 cycles of 94^{o}C for 1 minute, 50^{o}C for 45 seconds and 72^{o}C for 1 minute, final extension was carried out at 72^{o}C for 5 minutes. The amplified product was confirmed with agarose gel electrophoresis, followed by EtBr staining. PCR product of 16SrDNA was purified using Qiaquick PCR purification kit (QIAGEN, USA). Sequencing reactions were carried out in both directions using same forward and reverse primers used for amplification of 16S rDNA region with BigDye

Version 3.1 kit (Applied Biosystems) on an ABI-PRISM 3730 DNA Sequencer (Applied Bio-systems).

Optimization of enzyme production

Effect of media and incubation time on chitosanase production

Four different broth media nutrient broth (g L_1: yeast extract, 1.5; NaCl, 5; beef extract, 1.5; with 1% chitosan), M9 medium (Na_2HPo_4-1.3g, KH_2Po_4-3g, NaCl-0.5g, NH_4Cl-1g, $MgSo_4$-0.24g, $CaCl_2$-0.01g, pH-6.5) with 1%: soluable chitosan (0.5g of Chitosan, 9ml of glacial acetic acid, 40ml of Distilled water & make upto 100ml), Minimal Salt Chitosan (MS) medium (Yeast extract-0.5g, K_2HPo_4-0.2g, KH_2Po_4-0.1g, $MgSo_4$.7H2O-0.07g, NaCl-0.05g, KCl-0.05g, $CaCl_2$-0.01g), Enrichment medium (Shrimp shell powder-15g, K_2HPO_4-1g, $MgSO_4.7H_2O$-0.3g) Starch casein broth with 1% chitosan were used to determine the growth of bacteria and chitosanase production. The culture was inoculated and incubated at 37° C for 24 h in a rotary shaker (120 rpm). After 24 h of incubation, the cultures were harvested, centrifuged at 10,000 rpm for 15 min and the supernatant used for chitosanase assay. For optimum incubation time, the culture was grown for 5 days and chitosanase production was estimated every day.

Effect of temperature and pH on chitosanase production

The effect of temperature on enzyme production was determined by incubating the culture media at different temperatures (22°C, 27°C, 30°C, 37°C, 45°C and 55°C) for an optimized period of time. The effect of the initial pH value on the chitosanase production was investigated by varying the initial pH of the culture medium from 4 to 8 and at optimized temperature and incubation period. Chitosanase assay was performed as per standard protocol.

Effect of different substrates and their concentration on chitosanase production

To find out the best substrate for enzyme production, the chitosanase production was carried out by using different substrates in medium with colloidal chitosan and soluable chitosan at previously optimized conditions. Different concentrations of substrates (0.1%,0.5%,1%,1.5%,2%) were applied in optimized media and condition to determine the best substrate concentration.

Effect of carbon and nitrogen sources on chitosanase production

The effects of various carbon and nitrogen sources (0.5%,1%,2%) were used as an additional supplement in media for maximum enzyme production. The supplemented media were inoculated with 1% inoculum and fermented at an optimized condition and the medium without any carbon and nitrogen source were used as control.

Purification of Enzyme

Ammonium sulphate fractionation

Ammonium sulphate was added in increments to a concentration of 80% of saturation while gently stirring and allowed to dissolve and equilibrate between additions. The precipitated proteins were centrifuged for 15min at 4°C. The resulted pellet was dissolved in 5ml of phosphate buffer at (pH 7.0). The left supernatant was applied again with ammonium sulphate to achieve 100% (w/v) saturation and both were dialysed.

DEAE-cellulose Ion exchange chromatography

The concentrated active enzymes were applied directly on the top of the column (2 x 18 cm) of pre-activated DEAE-cellulose equilibrated with 0.01M sodium acetate, pH 5.8. Elution was carried out using the same buffer at a flow rate of 25ml/h, with a linear gradient of NaCl (0.1-0.5M). Fractions of 10 ml were collected at the elution rate 25 ml/hr. The eluted fractions were dialyzed against water for 48 h at 4 °C and monitored at 280 nm for protein and assayed for enzyme activity and protein content. The most chitosanase active fractions were pooled and concentrated by freeze drying.

SDS-PAGE

Sodium Dodecyl Sulphate–Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was carried out and the molecular weight was determined as described by [16]. The bands obtained for the enzyme samples were compared with protein marker.

Optimization of the partially purified Enzyme Activity

The partially purified enzyme was assyed for chitosanolytic activity at pH (3-9) and for the pH stability the enzyme was incubated in buffer with above pH ranges without chitosan substrate for 90 min assayed for enzyme activity. Similarly the enzyme solution were analysed for optimum temperature 30-90°C and the thermal stability for 90 min at optimal pH [17].

Preparation of Chitooligosaccharides

Chitooligosaccharides are prepared by the hydrolysis of chitosan by partially purified chitosanase enzyme. 1ml of the enzyme was added to 1ml of 1% chitosan and incubated at 55°C for 30 minutes. The reaction was stopped by heating at 100°C for 5minutes and after cooling the mixture was mixed with 0.25M of sodium hydroxide and centrifuged for 2 minutes at 1,000 X g. The chitooligosaccharides present in the supernatant was separated and stored for further studies [18].

Antimicrobial activity of Chitooligosaccharides

The multi drug resistant pathogens *Escherichia coli*, *Staphylococcus aureus* was procured from KMCH Laboratories, KMCH hospitals, Coimbatore and Christian Medical College, Vellore, Tamilnadu. The optimal 24 hours broth cultures of *Escherichia coli* and *Staphylococcus aureus* was used for antimicrobial activity [19]. The enzyme solution, chitosan and chitooligosaccharides at various concentrations (10-100µl) was added into the well and incubated at 37°C for 24 hours. The Chitooligosaccharide samples that found effective, as antimicrobial agent during qualitative test were tested to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for each strain. MIC was determined by broth dilution method. The chitosanoligosaccharides samples were diluted to final

concentrations of 0.25, 0.5,1,5,10,15, 25,50 ,75,100… mg/ml. About 100 µl of 10^5 CFU/ml of the test culture was inoculated in tubes with equal volume of nutrient broth and chitosanoligosaccharides samples. The tubes were incubated aerobically at 37°C for 24h. Two control tubes were maintained for each strain (media control and organism control). The dilutions that showed no turbidity were incubated further for 24h at 37°C. The lowest concentration that produced no visible turbidity after a total incubation period of 48h was regarded as final MIC. MBC value was determined by sub culturing the test dilution [which showed no visible turbidity] on to freshly prepared nutrient agar media. The plates were incubated further for 24-48h at 37°C. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as MBC.

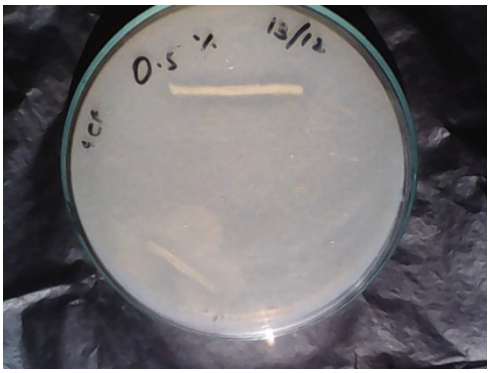


Figure 1
Growth of chitosanase producing organism on CDA plate

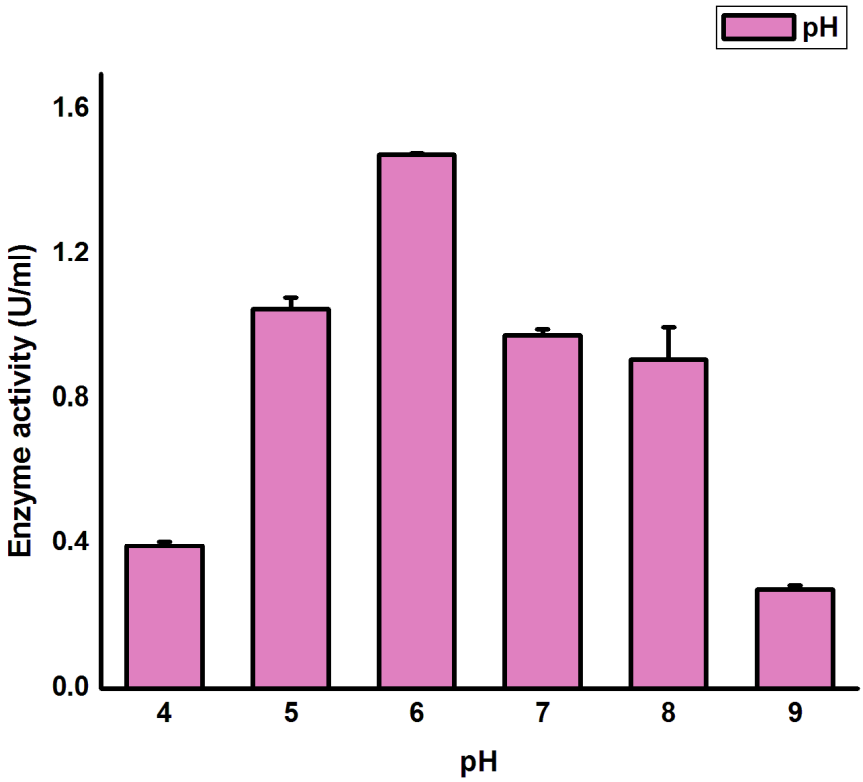


Figure 2
Effect of pH on chitosanase production by SA4.

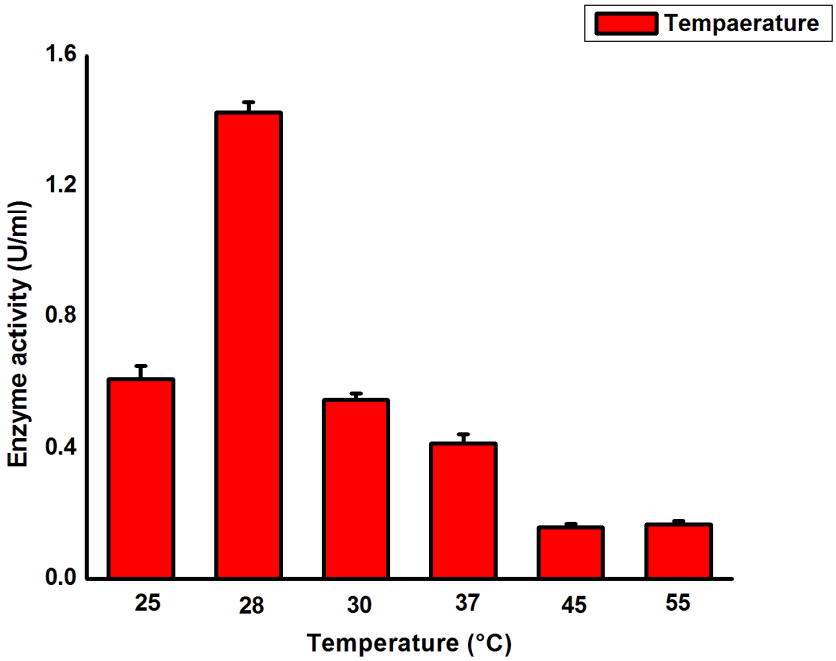


Figure 3
Effect of temperature on chitosanase production by SA4

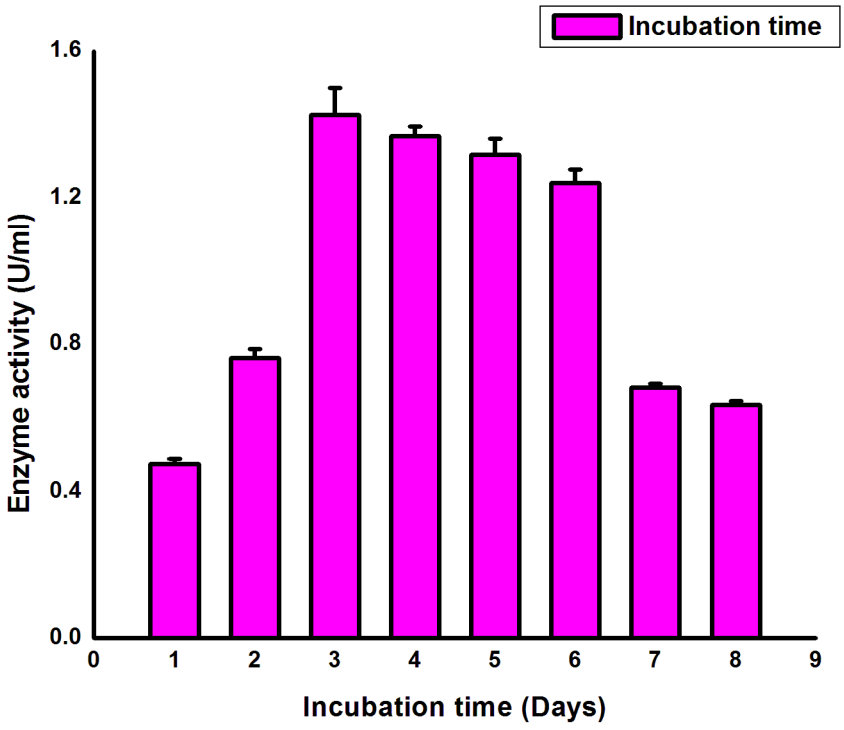


Figure 4
Effect of incubation period on chitosanase production by SA4

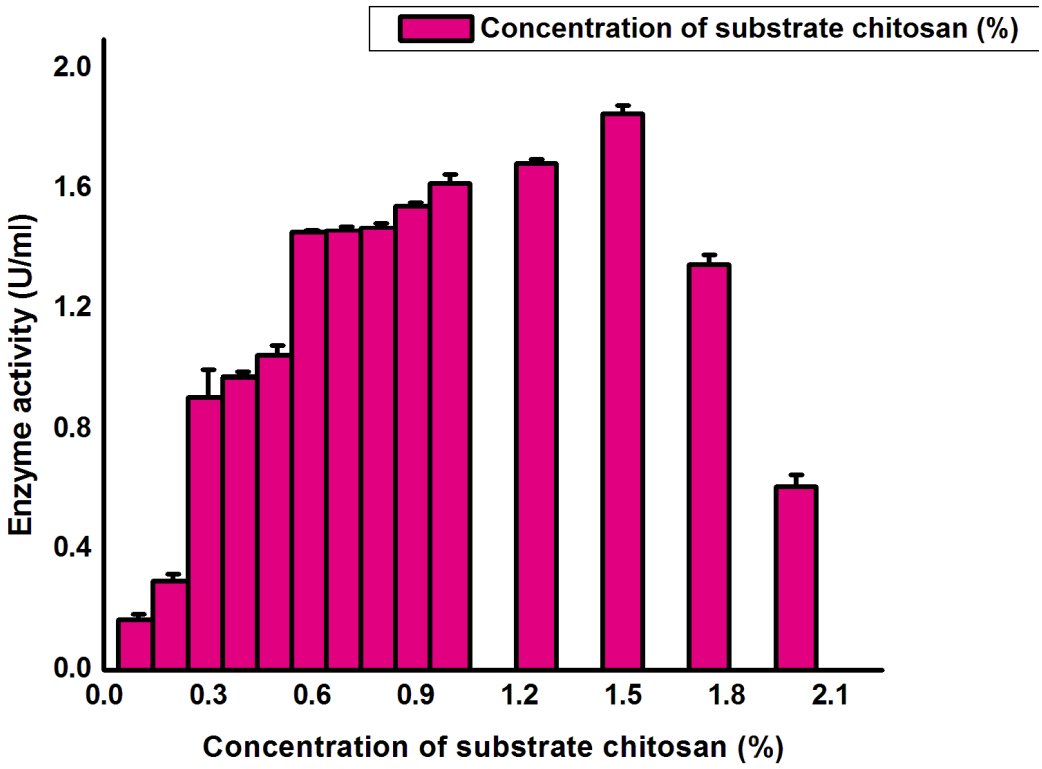
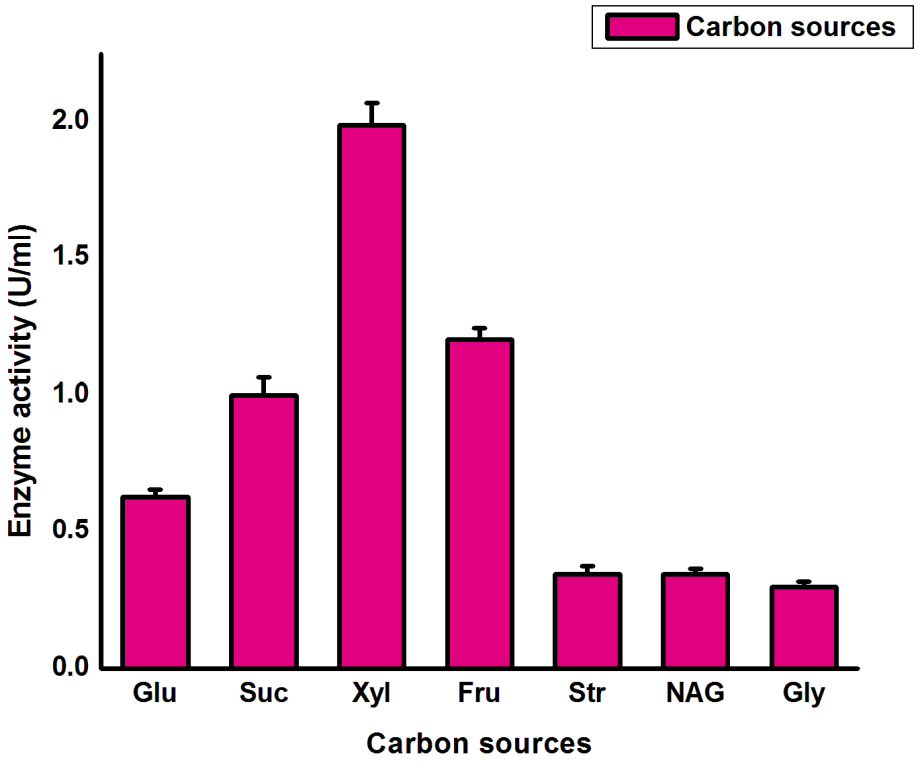


Figure 5
Effects of different substrate concentration on chitosanase production by SA4



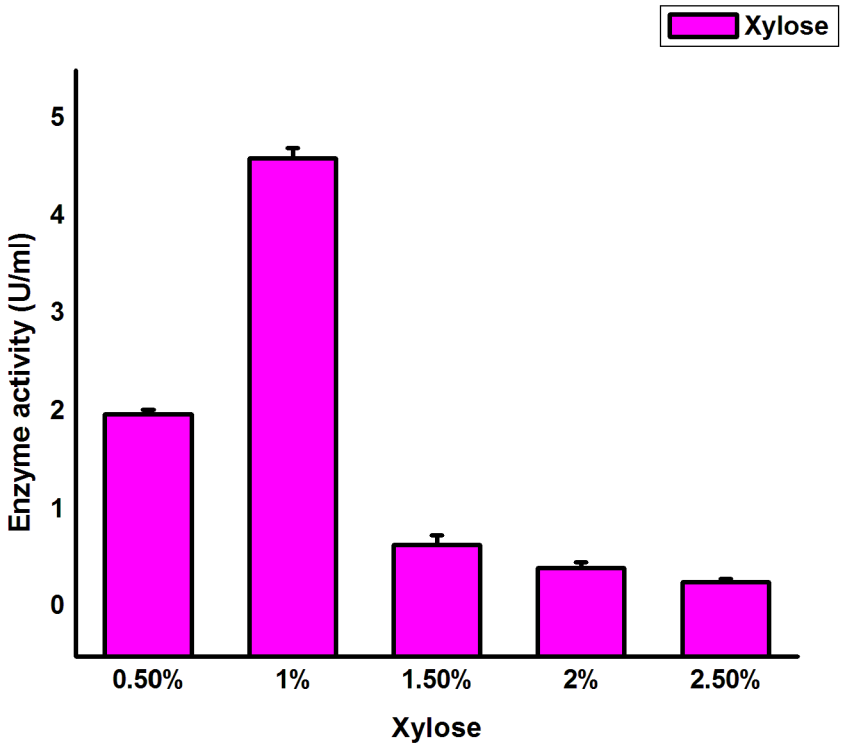


Figure 7
Effect of different concentration of xylose on chitosanase production by SA4

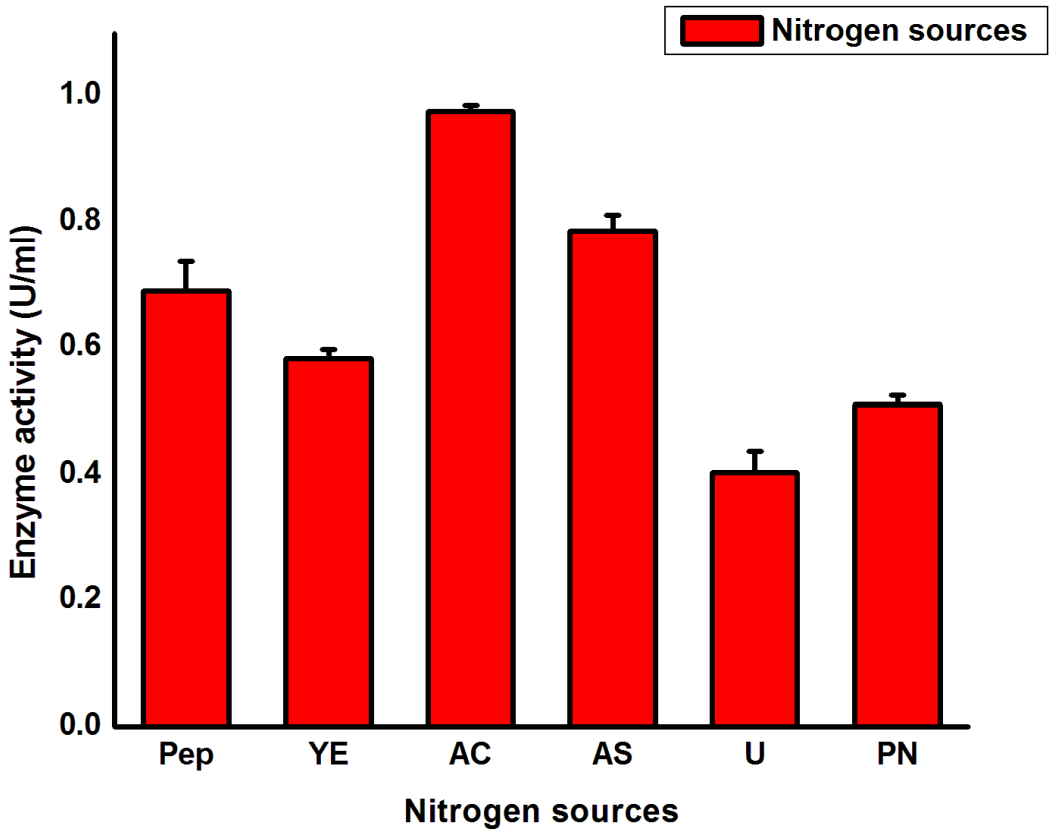


Figure 8
Effect of nitrogen sources on chitosanase production by SA4

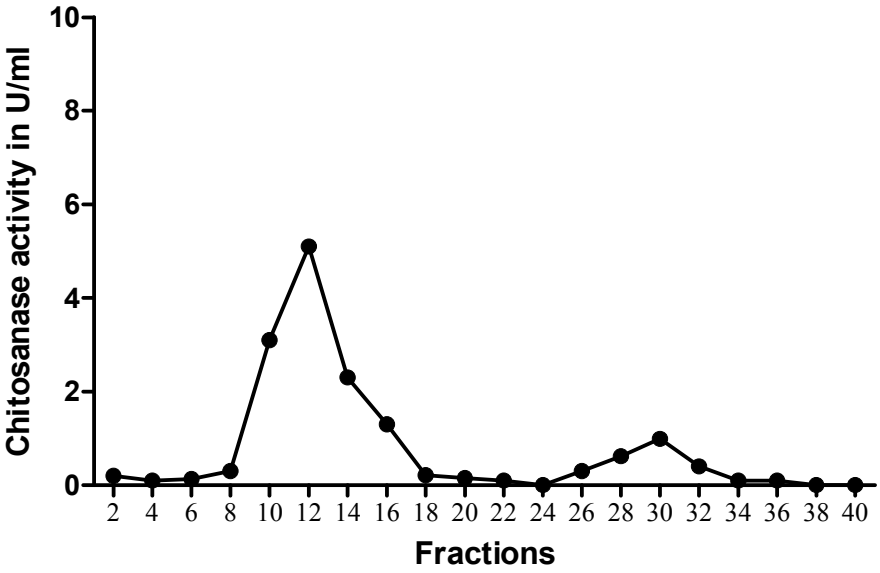


Figure 9
Enzyme activity of the fractions of DEAE cellulose chromatography purification steps

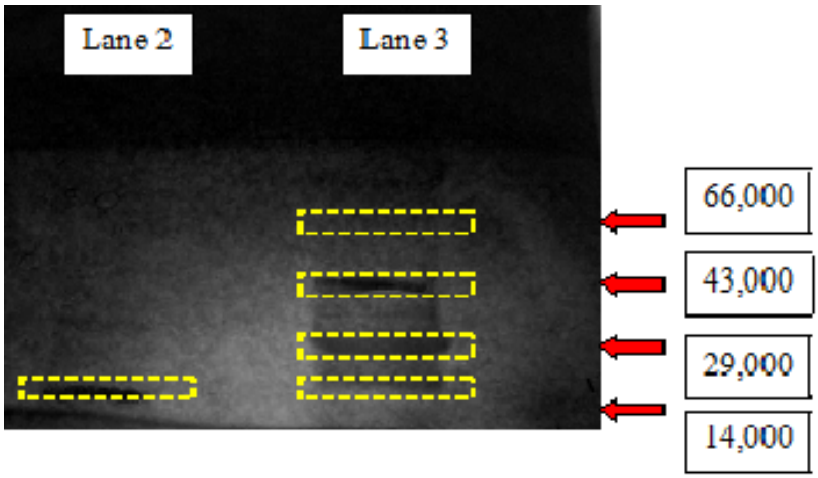


Figure 10
SDS PAGE analysis of partially purified chitosanase of Streptomyces sp A4.a Lane 2-Streptomyces A4.a and Lane 3- Molecular marker protein

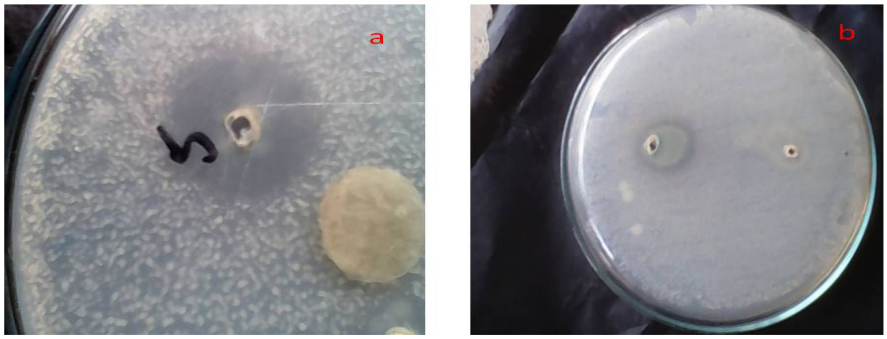


Figure 11
Antibacterial effect of chitooligosaccharides against 1)Staphylococcus aureus 2) E.coli

Table 1
SA4 Enzyme purification steps

Purification steps	Total enzyme activity	Total protein in	Specific activity in **U/ml**	Purification fold	Yield %
Fungal crude	215.5	2132.5	0.1009	1	100
Ammonium sulphate purification	162.8	1249.03	0.130	1.3	75.54
09-19 fractions in DEAE-Cellulose	124	51.5	2.41	23.88	57.54

Antimicrobial effects of chitooligosaccharides

Table 2
Antimicrobial effects of chitooligosaccharides against bacteria
Minimum inhibitory concentrations of chitooligosaccharides

Groups	Test Organisms	Inhibitory Zone (mm)
Bacteria	*E.coli*	25 + 0.1
	S.aureus	26 + 0.1

Table 3
Minimum inhibitory concentrations of chitooligosaccharides

Groups	Test Organisms	MIC %
Bacteria	*E.coli*	0.12 + 0.01
	S.aureus	0.08 + 0.1

RESULTS AND DISCUSSION

A total of 77 morphologically different chitosanolytic actinomycetes were isolated from 32 soil samples collected from different poultry farms of Tamilnadu and Kerala, India. On the basis of chitosan degradation (Fig. 1) and zone of clearance (>0.2 cm) on CDA plate, 15 colonies were selected for secondary screening in broth media and tested for enzyme activity. Based on maximum chitosanase production a potential isolate SA4 isolated from the soil of chicken feather decomposed pit sample of 6 months was selected for further study.

Characterization of bacterial isolates
Analysis of DNA sequences
The homology of the partial 16SrRNA gene sequence of the isolates was analyzed using the BLAST algorithm in GenBank. Ambiguous sequences from the base sequences were corrected with Chromas (Version 2.01) and the sequences were condign assembled with BioEdit (Version 7.0.9.0). All the processed sequences were submitted to NCBI databank using Bankit tool. Phylogenetic analyses were conducted using a multiple sequence alignment tool (Clustral W). Only the highest-scored BLAST result was considered for phenotype identification. BLAST showed that the isolate SA4 linear DNA has maximum homology (99%).

Optimization of enzyme production - Effect of media and incubation period
Among all the tested media, M9 broth with chitosan was more productive in SA4 (1.406U/mg) (Fig. 2). The effects of incubation time on chitosanase production are shown in Fig. 5. SA4 produced highest chitosanase after 72 h (1.566 U/ml) .Enzyme production was gradually decreased after 72 h. One of the reasons for decreased production may be the lack of nutrients or production of toxic chemicals in the medium resulting in the inactivation of the enzyme production .

Effect of pH on chitosanase production
The effect of pH of media on the chitosanase production was evaluated by bacterial cultures grown at different pH (4– 9). Among the tested pH, pH 6 supported the maximum chitosanase production (1.484 U/ml) (Fig. 3).

Effect of temperature for chitosanase production
Temperature not only affects various biological processes the enzyme productions are also affected with the change in incubation temperature. To evaluate the optimum growth temperature for chitosanase production, the culture was grown at 25–55°C. Chitosanase production was maximum at 28°C with 1.4806 U/ml (Fig. 4). The enzyme production was decreased above 40°C and 50°C.

Effect of different substrates on chitosanase production
Among the various substrates like colloidal chitosan (CC), soluable chitosan (SC) soluble chitosan was found to be a best substrate for chitosanase production for the strain (1.484 U/ml).

Effect of substrate concentration on chitosanase production
Different concentrations of soluble chitosan were used to elucidate the best concentration for maximum chitosanase production. The strain produced 1.88 U/ml of enzyme maximally at 1.5% (Fig. 5) produced. The substrate concentration from 0.5% influenced the

production of the enzyme and above 1.75% decreased the production.

Effect of carbon sources on chitosanase production

A total of seven different carbon sources (1%) namely glucose, fructose, sucrose, starch, glycerol, n-acetyl glucosamine and xylose were tested for maximum chitosanase production. Among the carbon sources, 0.5% xylose supported maximum chitosanase production (2.0816 U/ml) followed by fructose (1.2742 U/ml) and sucrose (1.1303 U/ ml) (Fig. 6). The enzyme production reached the maximum (4.8103 U/ml) by the addition of 1% xylose to M9 medium with 1.5% chitosan substrate at pH6 incubated with 28°C (Fig 7).

Effect of nitrogen source for chitosanase production

As per the results, only ammonium chloride had a significant effect on chitosanase production among the various tested nitrogen sources(yeast extract, peptone, ammonium chloride, ammonium sulphate, urea and potassium nitrate) (Fig. 8).

Partial purification of chitosanase

The partially purified chitosanase was precipitated with 80% ammonium sulphate and was dialysed and the dialysate was applied to DEAE cellulose chromatography. The active fraction (Fig.10) No.10 – 16 showed specific chitosanase activity. All the active fractions were collected and used for further work. The summary of purification steps were summarized in Table 1. The degree of purity was 1.3 after Ammonium sulphate precipitation, and was 23.88 fold after ion exchange chromatography with final yield of 57.54%. The molecular mass of the chitosanase revealed as 14 kDa chitosanase by SDS-PAGE (Fig-10). Earlier reports has showed chitosanase with wide range of molecular weights from *Streptomyces*.sp as 26 kDa [20], 34 kDa [21] and 46 kDa [22].The molecular weight of most of the microbial chitosanase ranged from 10-50 kDa [23] and [3] reported two chitosanase enzyme chiA and chiB was 108 and 29 kDa from *Aspergillus* CJ22-326. Similarly [24] reported 21kDa chitosanase from *Serratia marcescens* sub sp *marcescens*.

Characterization of the enzyme activity

The present study revealed that though the optimal pH for the production medium was 6.0, the optimal pH for the enzymatic activity was noticed to be 7.0. similar with results of [25,2,26]. The widest range of pH stability of the present study revealed within 5-8 [22]. Regarding the temperature as a parameter, it was observed that 50°C was the optimal temperature for maximal enzyme activity.

Preparation of chitooligosaccharides

The portion of the mixture was taken out and mixed with alcohol [27]. No precipitate indicated that the chitosan has depolymerised into small oligomers. The chitosan hydolysis was determined at 55°C. The concentration of pentamer, tetramer, trimer in the hydrolysate differs according to the hydrolysis time. The monomer concentration increase with the hydrolysis time, concentration of tetramer and pentamer decreases with the hydrolysis time but trimer remains constant [28].

Antibacterial activity of chitooligosaccharides

The chitooligosaccharides showed the higher zone of inhibition from 35 µl for both the organism. The crude enzyme showed no zone of inhibition where as the 70 µl of chitosan showed the inhibition zone lower to that of chitooligosaccharides (Fig 11,Table 2). Thus it was clear that the chitooligosaccharides has higher antimicrobial potency over the chitosan. The MIC of the chitooligosaccharides was also determined from 0.2µl to 100µl (Table 3). Though the earlier report of [27] and [29] shows that chitooligosaccharide, chitosan and chitosanase showed stronger antimicrobial activity, our study revealed the antimicrobial potency of chitooligosaccharide over chitosanase and chitosan. *E.coli* cells were greatly inhibited by the chitooligosaccharides compared with *Staphylococcus aureus* [30] and the results of the present investigation had revealed that the prepared chitooligosaccharide has strong bioactivity against *E.coli* than *Staphylococcus aureus* [31,32] revealed that chitosan exhibited stronger antibacterial activity against Gram positive than the Gram negative whereas chitooligosaccharide of the present investigation exhibited stronger activity against Gram negative organism in the present study revealing that prepared chitooligosaccharide is novel and has greater potency in biomedical research.

CONCLUSION

Chitosanase plays an important role in the degradation of chitosan and potentially in the utilization of chitosan as a renewable resource. The result concluded that SA4 are novel *Streptomyces* sp strain that have the ability to produce chitosanase for the preparation of chitooligosaccharide enzyme. The prepared chitooligosaccharide showed higher antibacterial activity against selected test pathogens. Xylose and sucrose could be a potent carbon source for maximal chitosanase production at industrial scale. The outcome from the present study it was concluded that the reported chitosanase could be utilized for enzymatic hydrolysis of chitosan and the product chitooligosaccharide could be used in treatment of diabetic foot ulcer. Thus the bio waste soil sample could be a promising source for isolating a novel chitosanase for preparing a novel bioactive chitooligosaccharide.

Conflict of interest

All the authors has no conflict of interest

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