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A New Bifunctional Chitosanase Enzyme from *Streptomyces* sp. and Its Application in Production of Antioxidant Chitooligosaccharides

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Abstract Chitosanases produced by microbes and plants are getting attention to explore vastly available marine waste. Chitooligosaccharides and glucosamine can be produced using chitosanase enzyme and have applications in food, pharma and other industries. A potential microbial chitosanase source was found after isolation and screening of chitosan degrading microbes from garden soil. An isolate, designated as C6 produced chitosanase enzyme upon induction by chitosan substrates. Production of 6 U/ml of chitosanase enzyme was achieved from this isolate on chitosan minimal salt broth medium at 32 °C after 3 days of growth. The enzyme was able to hydrolyse both chitosan and cellulosic substrates. Enzymatic production of D-glucosamine and chitooligosaccharides were studied with various chitosan substrates using crude enzyme. The yield of glucosamine was found to be 40% after 2 h of reaction at 40 °C, and chitosan oligomers were produced having two to six polymerizations at 60 °C reaction temperature. The hydrolysates showed 50% antioxidant activity as compared to ascorbic acid.

Keywords Chitosan · Chitosanase · Chitooligosaccharides · Glucosamine · Antioxidant

Introduction

Cellulose and chitin are the two most abundant biopolymers on earth which can be utilized as industrial raw material for cost-effective production of a range of bioactive compounds. Chitosan is the deacetylated form of chitin, and all these three compounds are structurally related due to the presence to β-1,4-linked glucopyranoses. The enzymatic depolymerization of this vastly available biomass is desirable over chemical methods due to its high yield and low pollution, but is limited due to its involvement of costly enzymes. Recently, conversion

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of chitosan to its oligosaccharides and glucosamine has attracted interest because not only are they water soluble but they possess versatile functional properties like antioxidative, antibacterial, anticoagulant, immunostimulative, anticancer, etc. [1]. Recently, chitooligosaccharides (COS) as natural antioxidant has gained attention since the synthetic antioxidants used are under strict regulation due to the potential health hazards caused by these compounds. COS act as potential radical scavenging agents due to their ability to extract hydrogen atom from free radicals [2, 3]. Furthermore, their ready uptake by cells in addition to low toxicity makes them promising natural antioxidants [4].

Chitosanase (EC 3.2.1.132) enzyme hydrolyses the glycosidic bond in chitosan and has received special attention as it is important in the maintenance of ecological balance, recycling of huge marine waste—which are chitinous in nature—for enzymatic preparation of chitooligosaccharides and biological control of fungal pathogens [5]. Use of chitosanase in hydrolysis is limited due to its high cost and unavailability in bulk quantity [6]. Most chitosanases are widespread in soil microbes. In higher plants, they are produced as a defense mechanism in response to VAM infection in roots which contain mainly zygomycetes having chitosan in their cell wall [5, 7].

On the other hand, utilization of lignocellulosic substrates depends on the production of a pool of hydrolytic and oxidative enzymes able to convert lignocellulosic compounds into low molecular molecules that can be converted into biological energy [8]. Cellulolytic enzymes like exo-cellobiohydrolase (EC. 3.2.1.91), endoglucanase (EC. 3.2.1.4) and cellobiase (EC. 3.2.1.21) produced can be utilized for this purpose. Cellulase constitutes a complex enzyme system responsible for the degradation of cellulosic substance, and many of these have been reported to have nonspecific hydrolysis towards chitosan substrates. *Myxobacter* AL-1 sp. producing endoglucanase have also been reported to have chitosan hydrolysing activity [9]. Cellulase from *Streptomyces* sp. [10] and *Bacillus* sp. [11] has also been shown to have chitosanase activity.

In the present work, we screened microbes for chitosan degrading activity isolated from garden soil (onion root). Production of glucosamine and COS were studied using partially purified enzyme from a number of chitosan substrates, and hydrolysing activities on other similar substrates like cellulose were checked. Antioxidant or radical scavenging activity of chitosan hydrolysates was checked using ascorbic acid as standard.

Materials and Methods

Materials

Commercial chitosan from crab shell, average molecular weight 290 kDa, 93% *N*-deacetylated (DAC); were kind gifts from Marine Chemicals, Chennai, India. Chitosan from shrimp shell (>75% deacetylated) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were procured from Sigma-Aldrich, Germany. Glucosamine HCl and chitin were purchased from Hi-media. All other chemicals were procured from SRL, Mumbai, India and were of analytical grade. For screening purpose, soil from the onion root plant was used (obtained from Mini Campus, IIT Delhi, New Delhi, India).

Isolation and Screening

Chitosan minimal salt agar and chitosan minimal salt broth medium were used respectively for the isolation and screening of the chitosanase producer from soil. A soil sample was

weighed, suspended in sterile water and serial dilutions were prepared. Plating was done on chitosan minimal salt agar medium. The composition of the medium was as follows: 0.5% (*w*/*v*) chitosan, 0.5% yeast extract, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.07% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.05% KCl, 0.01% $CaCl_2$ and 2% Bacto Agar with a final pH of 7.2. In the chitosan broth preparation, yeast extract and Bacto Agar was not added.

Microbial colonies grown on the surface of the agar plates forming halo were transferred to the chitosan broth and were incubated for 6–7 days at 32 °C; the culture broth was centrifuged to remove microbial growth, chitosan, and a supernatant was used for the enzyme assay. Microbes showing high chitosanase (endo and exo) activities were selected and studied for further enzyme production. A constitutive production of chitosanase was also tested using glucose (1%) as carbon source.

Preparation of Partially Purified Chitosanase

Microbial cultures were stored on agar slants at 4 °C, scrapped off and washed out with sterile distilled water. One millilitre of microbial suspension was inoculated into a 250-ml flask containing 50 ml of the chitosan broth medium (pH 6.8) and was incubated on a rotary shaker at 200 rpm for 3–4 days at 32 °C. The microbes were removed from the culture broth medium by centrifugation at 7,500 rpm for 15 min at 4 °C, and the supernatant was collected for enzyme assay. The chitosanase was further precipitated out by chilled acetone (80–90%) at 4 °C; precipitates were collected by centrifugation at 10,000 rpm for 20 min, washed repeatedly with sodium acetate buffer and were dissolved in an appropriate volume of the same buffer (100 mM, 5.5). This partially purified chitosanase enzyme was lyophilized and stored at 4 °C to be used for further studies.

Chitosanase Enzyme Assay and Protein Determination

Chitosanase activity was determined by measuring the reducing sugars produced from chitosan. Three hundred microlitres of the crude enzyme was mixed with 500 μl of chitosan (1% *w*/*v*) and 700 μl of acetate buffer (pH 5.5). A reaction mixture was incubated for 30 min at 37 °C. In order to stop the reaction, the enzyme was deactivated by heating the reaction mixture at 100 °C for 2 min. The reducing sugars in the supernatant were measured by using a modification of the Schales method [12] using glucosamine HCl as the calibration standard. One unit of chitosanase was defined as the amount of enzyme that liberated 1 μmol of D-glucosamine per minute under the conditions described above. The yield of glucosamine was calculated by the following formula:

$$\text{Yield (percent)} = [\text{glucosamine produced(millimole)}/\text{chitosan substrate(millimole)}] \times 100$$

Glucosamine yield was checked at 2 h after hydrolysis of 100 mg of chitosan (>90% DAC) in acetate buffer, when incubated with 3 ml of partially purified enzyme; the total reaction volume was maintained at 100 ml, and the reaction temperature was 40 °C.

Cellulase Assay

A culture broth was centrifuged at 8,000 rpm for 10 min; a supernatant was filtered and was used to determine various cellulase activities. Cellulase activity was measured using micro-crystalline cellulose or avicel as substrate [13]. Endo β-1,4 glucanase activity was checked

at 45 °C using carboxymethyl cellulose (CMC) as substrate. An increase in reducing groups was measured by dinitrosalicylic acid method using glucose as standard. Cellobiase activity was determined using cellobiose as substrates. One unit of cellulase activity was defined as the amount of enzyme that releases 1 μmol of glucose equivalent per minute.

Chitosan Substrate Preparation

The degree of acetylation of chitosan was assayed by the method of Dasheng et al. [14] by spectrophotometer (Optizen UV^{3220}) using dual standard (D-glucosamine and *N*-acetyl D-glucosamine). *N*-acetylation of chitosan was performed by the method of Kubota et al. [15] using acetic anhydride. Colloidal chitin and chitosan were prepared by the method of Monreal and Reese [16] and Yabuki et al. [17], respectively.

High-Performance Thin Layer Chromatography

The endo activity of chitosanase was analysed by thin layer chromatography (TLC) and high-performance thin layer chromatography (HPTLC). The hydrolysate mixture was spotted onto the TLC plate and was developed in n-propanol/water/ammonia (30% solution) (7:3:1 *v*/*v*/*v*). COS standards (glucosamine HCl, chitobiose, chitotriose, chitotetrose and chitopentaose) were run in parallel to the enzyme hydrolysate mixture. After solvent development, the plate was dried by hot air and was immersed in a spray solution containing 10% H_2S0_4 in absolute ethanol. The plate was then charred at 100 °C for 10 min to develop the stain. The plates were scanned by HPTLC (CAMAG) at 366 nm.

Antioxidant Activity

The antioxidant activity was determined using scavenging ability on DPPH as a free radical. A decrease in absorbance was measured at 517 nm at regular intervals until the reaction reached a plateau. The residual radicals in the samples were calculated according to the following equation:

$$\text{Residual DPPH radicals} = 100 - [(\text{DPPH blank} + \text{control sample}) \\ - \text{DPPH sample}/(\text{DPPH blank})] \times 100$$

where DPPH blank was the value for 0.1 ml of water/1 ml of methanol including 80 μM DPPH; the DPPH sample was the value of 0.1 ml of COS mixture/1 ml of methanol including 80 μM DPPH, and the control sample value was taken as 4 ml of COS mixture/ 1 ml of methanol. The assay was standardized or compared using L-ascorbic acid (0.001 – 0.1 M and 0.1 ml) as a standard antioxidant.

Results and Discussion

Isolation and Screening

During our screening experiments, we observed various kinds of microbial colonies (total number, 65) that formed halos on the chitosan minimal salt agar medium. Several microbial cultures (33) in which colonies formed large, clear halos were screened for their ability to produce extracellular chitosanase in chitosan minimal salt broth medium.

All 33 types were able to utilize chitosan as sole carbon source. Chitosanase enzyme production in the presence of other carbon sources like glucose was also checked. Only one of these isolates was able to produce chitosan degrading enzyme constitutively, but activity was very low (0.1–0.6 U/ml), hence was not selected for the study. Twenty isolated microbes having enzymes able to produce D-glucosamine from chitosan (>90% DAC) were further screened for their endo activity by TLC to produce chitooligomers. Only two of these isolates showed endo activity and were able to produce dimer to pentamers. Isolate C6 was selected for the study as it showed a maximum activity of 6 U/ml, a specific activity of 113 per milligramme of protein and was able to produce chitooligomers. *Streptomyces* sp. (no. 6) reported to have 105 U/mg of specific activity while the other two actinobacterial sp. *Streptomyces griseus* and N-174 have been reported to have specific activities of 2.7 and 59.8 U/mg, respectively [18]. The selected isolate was found to be a gram-positive flagellated actinobacteria (Fig. 1). According to the result of the 16SrDNA partial base sequence, C6 was found to be closely related to the *Streptomyces zaomyceticus* sp., and similarity of base sequence was more than 99%. The sequence has been submitted to GenBank and has been assigned the accession number JN613285. A fourfold purification and 80% yield was achieved by treating crude enzyme with chilled acetone for 2 h. This partially purified enzyme (10 U/ml) was used for further studies.

Chitosanases occur widely in soil microorganisms and are considered important for extensive carbon and nitrogen recycling [18]. Ubiquity of chitosan degrading organism in soil may be due to common soil microbes like *Zygomycetes*, which contain chitosan in their cell wall [2]. A large majority of higher plants are colonized by VAM fungi, which contains *Zygomycetes*, which has been shown to trigger host defense mechanism inside the plants, in response of which they release certain enzymes like chitinases, chitosanases, peroxidases, etc. [19]. Some workers have reported chitin/chitosan degrading microbes as biocontrol agents against plant pathogens as they have the ability to degrade fungal cell wall and can be a good non-toxic alternative to chemical fungicides [20]. Some other reports where actinobacteria have been isolated from soil are *Streptomyces* N174 [21], exochitosanases producing *Amycolatopsis orientalis* subsp. *orientalis* [22], etc.

Glucosamine Production by Chitosan Hydrolysis

Different chitosan substrates (30-90% DAC), chitosan from shrimp shell (>75% DAC), colloidal chitosan, glycol chitosan, chitin and colloidal chitin were used as substrates for

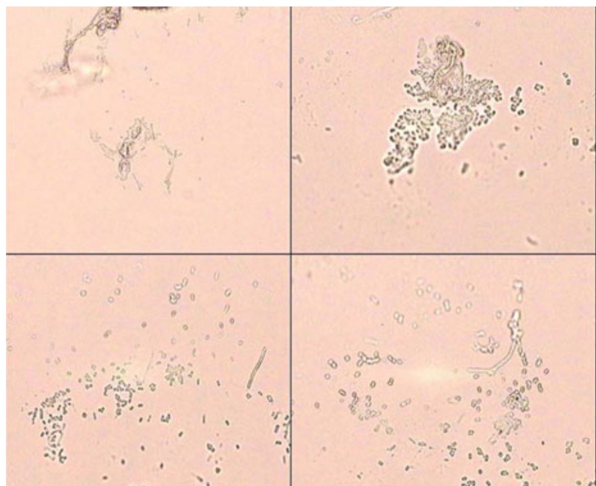

Fig. 1 Photomicrograph of cells and mycelia of *S. zaomyceticus* C6 (100×)

glucosamine production. Crude enzyme showed maximum activity on 90% DAC chitosan, and it decreased with the increasing degree of acetylation (Table 1). The yield of glucosamine was 55% with shrimp shell (>75% DAC) chitosan as compared to crab shell (65%) with the same degree of acetylation. The enzyme was not able to hydrolyse glycol chitosan, chitin and colloidal chitosan; however, glucosamine yield was 12% with colloidal chitin. Solubility of chitosan increases with deacetylation of chitosan, and thus soluble chitosan are hydrolysed more rapidly than powdered chitosan, the same trend of hydrolysis was observed in the case of *Aspergillus fumigatus* hydrolysing chitosan [23].

Shrimp shell waste is an important source of bioactive material, and major components are protein, chitin and mineral [24]. Chitin is further deacetylated to chitosan to be used for different purposes. A lower yield can be explained by the fact that crude chitosanase enzyme can be divided into three classes based on their hydrolytic patterns [25] which is mainly based on the presence of glucosamine and *N*-acetylglucosamine present on bond to be hydrolysed. In the deacetylation process and in the process of making chitosan colloidal, the removal of the acetyl group is not specific and thus glucosamine yield may differ from other sources of chitosan like crab shell and squid pen, etc. A good yield of glucosamine (25 mg) was achieved after almost 2 h at 40 °C from 100 mg of chitosan (Fig. 2) using 30 U of enzyme. In the first 60–70 min, it increased linearly and a maximum hydrolysis was achieved after 130 min. The TLC of the hydrolysates was also done to confirm glucosamine production (Fig. 3). Glucosamine is reported to have a therapeutic activity in osteoarthritis and has been evaluated as a food supplement recently [26].

Cellulase Activity

A maximum cellulase activity of 0.6 U/ml was observed on crystalline cellulose after 3 days of growth at 32 °C but was unable to hydrolyse the soluble form of cellulose, i.e. CMC. No cellobiohydrolase activity was observed with the crude enzyme. The trend of chitosanase and cellulase enzyme activity with time was the same as both activities reached a maximum at 72 h with the growth and has been shown in Fig. 4. Some microbes have been reported to produce enzymes with the ability to degrade polysaccharides linked by β-1,4-glycosidic linkages like chitin, cellulose and chitosan. This exocellular microbial β-1,4-glucanases may

Table 1 Relative yield of glucosamine from different chitosan substrates using crude chitosanase enzyme (reaction mixture contained 1% substrates, 1 ml of crude enzyme (10 U/ml) and 200 mM acetate buffer, pH 5.5)

Substrates	Relative yield of glucosamine (%)
Chitosan (>90% DAC)	100[a]
Chitosan (>80% DAC)	78
Chitosan (>70% DAC)	65
Chitosan (>60% DAC)	44
Chitosan (>50% DAC)	32
Chitosan (>40% DAC)	21
Chitosan (>30% DAC)	22
Shrimp shell chitosan (>75% DAC)	55
Colloidal chitosan	8
Glycol chitosan	0
Chitin	5
Colloidal chitin	12

[a] Highest activity with chitosan (>90% DAC) was taken as 100%

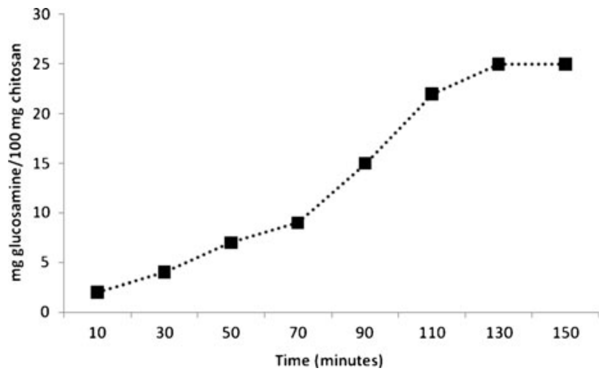

Fig. 2 Time course of glucosamine production per 100 mg of chitosan by chitosanase enzyme: chitosan (>90% DAC), 1% (*w*/*v*); enzyme, 6 U/ml; sodium acetate buffer, 250 mM

be of great use in the brewing industry, in bioconversion of agricultural wastes and the biological control of fungal pathogens [27]. A bifunctional enzyme with chitosanase and CMCase activity was purified from commercial cellulase produced by *Trichoderma viride* [28]. Since both enzymes are secreted maximally at the same time during growth phase, same proteins having two catalytic activities cannot be ruled out and need further study.

COS production

Chitosan hydrolysis for 24 h at 60 °C led to the production of COS (dimer to pentames), which was confirmed by TLC (Fig. 5). From HPTLC (Fig. 6), it was found that till the third hour, only trimer and pentamers were formed, after that all COS (dimer to pentamer) were formed. After 24 h of reaction, the mixtures of the dimer, tri and pentamers were observed. Glucosamine was not present in the final reaction mixture at 60 °C. It was concluded that the enzyme was also having endo activity. Since it is showing exo and endo activity at different temperatures, it was assumed that crude enzyme might be containing more than one enzyme having different optimum temperatures. Wang et al. [29] showed similar results of a study on fungal chitosanase where *Gongronella* sp. have been shown to produce two chitosanase isoenzymes.

Antioxidant Activity

The residual DPPH at 200 s for the hydrolysate mixture was 54% (Fig. 7) as compared to that of ascorbic acid (80 μg/ml). Ascorbic acid is known to have strong antioxidant properties and was used as reference compound for checking radical scavenging activity.

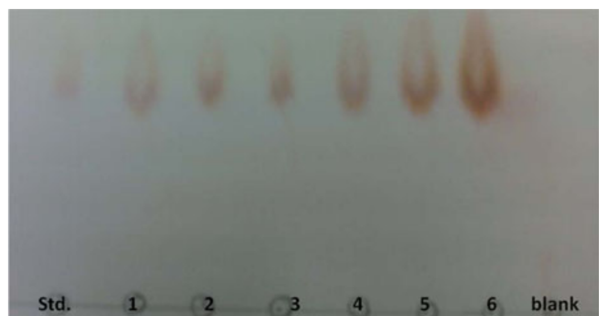

Fig. 3 Thin layer chromatography of glucosamine produced by chitosanase enzyme: *Std.*, glucosamine HCl (2 mg/ml); *1*, *2*, *3*, *4*, *5* and *6* are samples withdrawn from 10 to 60 min, respectively; *blank* was without enzyme

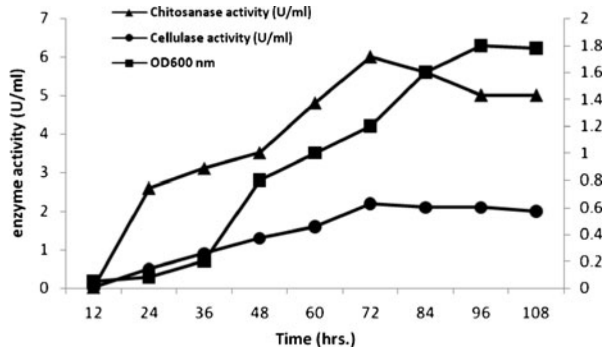

Fig. 4 Cell growth and enzyme (chitosanase and/or cellulase) activity of *S. zaomyceticus* C6 grown in chitosan minimal salt broth medium at 32 °C. At appropriate time intervals, cell density was measured at 600 nm, and chitosanase activity was measured using >90% D.A. chitosan as substrate. Cellulase activity was measured using Avicel or crystalline cellulose as substrate

The same study has been conducted on the culture supernatant of *Serratia marcescens* TKU 011 [30] grown on 2% shrimp shell waste, and 22% DPPH scavenging ability was observed per millilitre. However, no other actinobacterial sp. enzymes or COS produced by them have been studied for radical scavenging activity or antioxidant activity. An interest in identifying new natural antioxidants is growing to overcome the damaging effect of freer radicals on biological systems. Many carbohydrates, peptides and phenolic compounds have been shown to have antioxidant properties. The radical scavenging ability of chitosan and COS is dependent on the degree of deacetylation and molecular weights. COS with lower molecular weight have been shown to have higher potential to scavenge different radicals [31]. The precise mechanism of radical scavenging activity is not clear and is attributed to amino and hydroxyl group attached. Recently, it has been revealed that metal ion uptake ability of COS has greater influence on hydroxyl radical scavenging ability and is partly due to the chelating ability of transition Fe^{2+}, molecular charge properties and proton donation via hydroxyl and amino group [3].

Conclusion

From this work, it was concluded that crude enzyme shows wide substrate specificity and is advantageous, since purification of enzyme involves a lot of cost. Rhizosphere soil harbors a

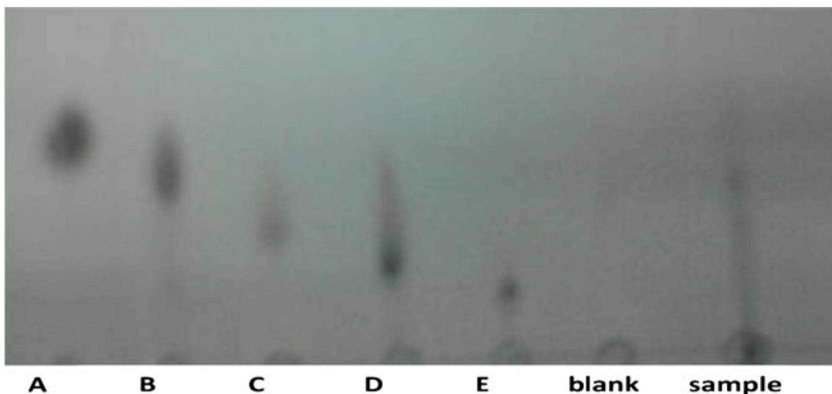

Fig. 5 Thin layer chromatography of chitooligosaccharide produced at 60 °C for 2 h reaction time. *Lane A*, *B*, *C*, *D*, *E* denoted COS standards of GlcN, $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$, respectively. *Blank* is reaction mixture without enzyme and *sample* is hydrolysate mixture

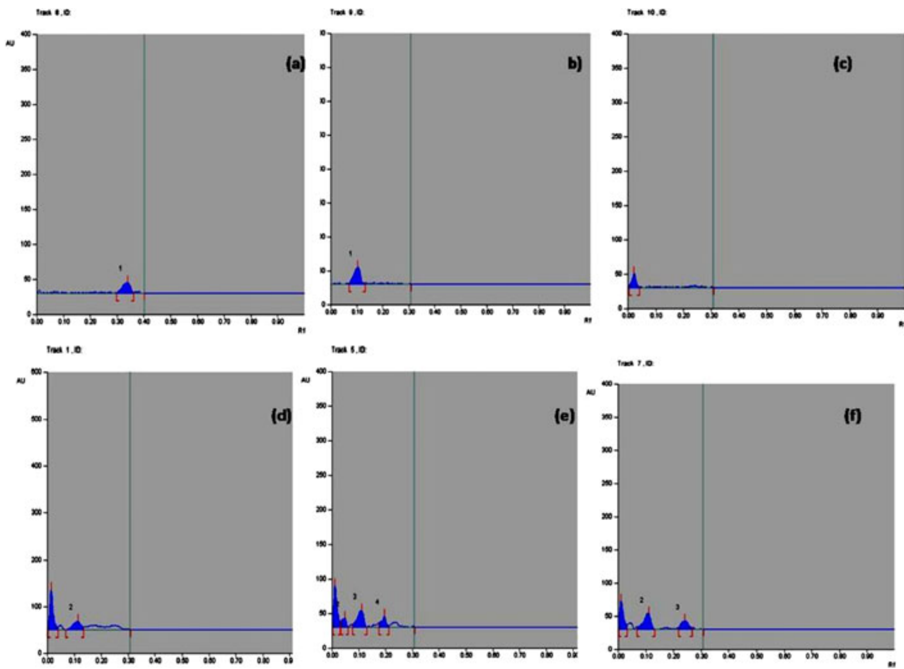

Fig. 6 HPTLC of COS. **a–c** Standards of glucosamine (R_f value, 0.35), chitotriose (R_f value, 0.15) and chitopentaose (R_f value, 0.5), respectively. **d–f** are results of reaction mixture taken after 3, 4 and 24 h, respectively

number of industrially important microbes. Isolated actinobacteria from soil produced bifunctional chitosanase, which was able to degrade chitosan as well as cellulosic substrates. This enzyme was able to produce biofunctional oligosaccharides from different chitosan substrates and could be a suitable candidate for large-scale industrial production of chitooligosaccharides using marine waste. Although radical scavenging activity has been reported

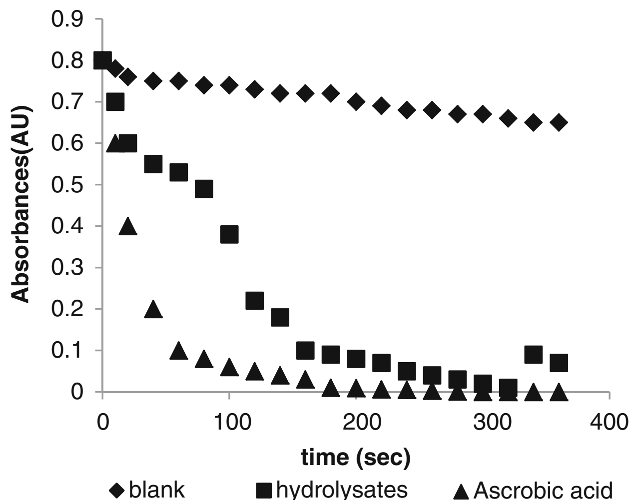

Fig. 7 Antioxidant activity of chitosan hydrolysates using ascorbic acid as standard. *Blank* was taken as methanol DPPH mixture with water; *ascorbic acid* (80 μM) was added in the mixture which served as standard; 4 ml of *hydrolysates* was added in the methanol DPPH mixture to check sample antioxidant activity

in bacterial cultures producing chitosanase enzyme, to the best of our knowledge, this is the first report of actinobacterial chitosanase having antioxidant properties, which can be used in the health and pharma industries and need to be scaled up and purified further. With optimization of media and cultural conditions, production of both chitosanase enzyme and biofunctional oligosaccharides can be enhanced and could be used in areas like agriculture, waste management, industries and others.

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