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Purification and characterization of chitosanase from *Paecilomyces lilacinus*

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Abstract

Chitosanase, a useful enzyme, degrades chitosan and produces chitooligosaccharides. The aim of this study was to purify the chitosanase enzyme from soil fungus, *Paecilomyces lilacinus* and applications on its substrate in various fields. The enzyme was released extracellularly from the *P. lilacinus* fungus in M 9-chitosan agar medium and the crude protein in the culture filtrate was precipitated by 80% ammonium sulfate. The protein was dissolved in 20 mM acetate buffer (pH 5.6), dialyzed against the same buffer and injected into Resource Q anion-exchange column in FPLC system. The active fractions were estimated for chitosanase activity. The specific activity of the purified enzyme was 1050 U/mg with a purification fold of 95. The molecular mass of the purified enzyme was estimated to be 40 kDa by SDS-PAGE and its absolute mass was 39.6 kDa by ESI-MS-Q-TOF analysis. Chitosanase showed highest activity at pH 6 at 50°C. It showed antimicrobial effect, being maximum in *Staphylococcus aureus* followed by *Bacillus subtilis* and *Pseudomonas aeruginosa* and least in *Escherichia coli*. Chitooligosaccharides isolated from chitosan showed maximum antimicrobial effect in *S. aureus* in comparison to *P. aeruginosa.* Chitooligomeric mixtures separated by HPLC produced high amount of *N*-acetyl glucosamine (monomer) in different time intervals with the production of low amount of dimmer in 7h.

Keywords. *Paecilomyces lilacinus;* Chitosanase; Chitin; Chitosan; Chitooligosaccharides; Glucosamine

Introduction

Cellulose, chitin and chitosan are polysaccharides consisting of β-1,4- linked glucopyranose units differing only in functional groups at the C-2 positions of their constituent sugars, i.e , the hydroxyl, acetamido, and amino groups respectively. Chitosan is a polysaccharide of β-1,4- linked glucosamine(GlcN) residues, derived by deacetylation of chitin. Chitin is widely distributed in exoskeleton of insects, crustaceans as well as molluscs. Chitosan like chitin is distributed in the cell wall of fungi particularly zygomycetes. Chitosanases are group of enzymes that degrade chitosan. They have been purified from a variety of microorganisms including bacteria, fungi, actinomycetes, plants etc[1-12]. Purified chitosanase have been characterized mostly as endotype enzymes which cleave chitosans at random, their reaction rate being highly dependent on the degree of acetylation of chitosan. An exo-type chitosanase called exo-β-*D-glucosaminidase* has been purified from the actinomycete *Nocardia orientalis*[13] and fungus *Trichoderma reesei* PC-3-7. Three main classes of chitosanase are classified according to their substrate specificities. First type is the chitosanase that degrades chitosan upon recognizing a GlcNAc-GlcN bond, second is that recognizes both the GlcNAc-GlcN and GlcN-GlcN, and the third is that specific for GlcN bond only. Recently, chitosanase has drawn attention for its potential application in medicines, industry and agriculture [14-16]. In plants chitosanases are believed to play role in self-defense against pathogenic fungi. When plants attacked by pathogenic fungi or bacteria, they exploit some hydrolytic action of cleaving the β-1-4- linkages between *N*-acetyl-D-glucosamine and D-glucosamine residues in a partially acetylated chitosan in fungal cell wall as a component of a larger post-attack defense response. However, these enzymes may also function in pathogenesis-related (PR) signal transduction. Glucosamine oligomers, released from fungal cell wall after hydrolysis with chitinase or chitosanase, are elicitors of plant defense responses such as stomal closure[17] and cell wall lignification. The responses elicited by these molecules depend on the length and degree of acetylation of the oligomers released. More specifically, long oligomers or intact fungal cell walls will cause little or no reaction. Some chitosanases have hydrolytic activity on substrate other than chitosan, like chitin[18] and cellulose[19].Chitosanases have been found and cloned from several organisms such as viruses[20] bacteria[21] fungi[22-25] and plants[26].

Chitosanases are produced by a large number of microorganism including bacteria and fungi[27]. In most cases bacterial chitosanases are inducible by the substrate chitosan and play role in the degradation. In

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contrast there have been few reports for fungal chitosanase[27]. Their physiological role remained unclear, since exogenously added chitosan could not be used efficiently as a carbon source by fungi in most cases. Information on chitosanase is relatively limited.

The present communication describes the isolation and characterization of a new exo-type enzyme from *Paecilomyces lilacinus* fungus. The purified chitosanase was used for the preparation of chitooligosaccharides. We also have described here in the antimicrobial effect of chitooligosaccharides and chitosan against gram positive and gram negative bacteria.

Materials and methods

Fungal strain and culture medium

The strain *Paecilomyces lilacinus* used in this study was obtained from "The Advanced Mycology Centre, Post Graduate Institute of Medical Education & Research, Chandigarh, India and maintained on M 9 chitosan agar medium (0.13 g Na_2HPO_4, 0.30 g KH_2PO_4, 0.05 g NaCl, 0.10 g NH_4Cl, 0.024 g $MgSO_4$, 0.001 g $CaCl_2$, 0.15 g dextrose, 0.5 % chitosan and 2 gm agar in 100 ml). In the medium chitosan acts as the sole carbon source. The pH was adjusted to 5.6. After formation of a single colony it was repeatedly sub-cultured in fresh M 9 chitosan broth to obtain maximum growth adaptability of this fungus.

Enzyme production

Fungal culture was inoculated on to a chitosanase detection agar (CDA) plate which was prepared by mixing of chitosan (0.5 g) pre-dissolved in 100 ml of 0.5% acetic acid with agar (2 g). The pH of the culture medium was adjusted to 5.6. Extracellular chitosanase was detected by visualizing the clear zone around the growth of fungal colony. Fungus culture was transferred from CDA plate to 100 ml of the culture medium (M 9) and the flask was incubated at 30^{0}C with 175 rev / min on a shaker for 20-30 days.

Purification of enzyme

Ammonium sulfate precipitation

After cultivation, the fungal biomass was removed from the culture broth by filtration. The culture filtrate was concentrated by ultrafiltration using YM-10 membrane. To this filtrate ammonium sulfate was added up to 80 % saturation and kept at 4 ^{0}C for overnight in stirring condition. The pellet was collected by centrifugation at 10,000 rpm for 30 min and dissolved in an appropriate amount of 20 mM acetate buffer (pH 5.6). The dissolved protein was dialyzed against the same buffer. The dialyzate was centrifuged to remove the insoluble materials and was used as semi purified chitosanolytic enzyme fraction.

Anion exchange chromatography

The semi purified enzyme fraction was loaded into Resource Q anion-exchange column in a Akta Prime system. The column was equilibrated with 20 mM acetate buffer (pH 5.5). The fractions were eluted with the same buffer containing 0 to 0.5 (M) NaCl at a flow rate of 0.5 ml/min. The fractions showing chitosanase activity were pooled, concentrated and dialyzed against the same buffer.

Enzyme assay

The activity of the enzyme was assayed by estimating *N*-acetylglucosamine released from the reducing end of hydrolyzed chitosan. The assay was performed by mixing 0.2 ml chitosanase fractions containing 10 µg protein with 0.2 ml chitosan (0.2%) as a substrate at a pH 5.6. After incubation for 30 min at 37°C, hydrolysis reaction was terminated by adding 0.6 ml of dinitrosalicylic acid reagent (DNSA) [DNSA (0.5 g), sodium sulfite (0.025 g), sodium potassium tartarate (20 g), NaOH (1.07 g), phenol (0.1 ml)] dissolved in 50 ml H_2O. After boiling for 15 min it was cooled and the absorbance was measured spectrophotometrically at 575 nm.

Protein assay and gel electrophoresis

The protein concentration of different chitosanolytic enzyme fractions were determined by Bradford method[28] using bovine serum albumin as the standard. Molecular weight of the above fractions were estimated by 10 % SDS-PAGE by the method of Laemmli[29] under denaturing condition. The proteins in the gel was stained with 0.2% Coomasie Brilliant Blue G 250 in 10% acetic acid and destained with 10 % acetic acid containing 30% methanol.

Electron spray ionization-mass spectroscopy

The molecular mass of chitosanase was also determined by ESI-MS-Q-TOF mass spectrometry by dissolving the enzyme in CH_3CN:H_2O (1:1) mixture.

Enzyme characterization

Thermal stability

To determine the thermal stability purified chitosanase was incubated separately in water bath at different temperatures ranging from 10^0 to 80^{0}C for 30 min. After cooling its activity was measured at room temperature.

pH stability

The pH stability of purified chitosanase was determined by dialyzing its aliquots against buffers of different pH ranging from pH 2 to 10 for 6 h at 4^0C, *viz*, 20 mM glycine-HCl buffer (pH 2), 20 mM citrate phosphate buffer (pH 4), 20 mM sodium citrate buffer (pH 6), 20 mM TBS buffer (pH 8), 20 mM glycine-NaOH buffer (pH 10).

Effect of divalent metal ions

The effect of different divalent metal ions on the activity of purified chitosanase was tested. Chitosanolytic activity was assayed after addition of different metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}) to the reaction mixture at a final concentration of 1 mM**.** The enzymatic activity was assayed after incubation at 37^0 C for 30 min.

Preparation of chitooligosaccharides

Chitooligosaccharides were prepared by hydrolysis of chitosan by chitosanase. Chitosanase solution (4 U/ml) dissolved in 1 ml of 0.02 M acetate buffer (pH 5.6) was added to 3 ml of 1% (w/v) chitosan and the mixture was incubated for different time intervals, *viz*., 1, 3, 5 and 7 h at 37^0C. The reaction was terminated by heating at 100^0 C for 5 min. The pH of the solution was adjusted to 7 by adding 1M NaOH solution. The mixture was centrifuged at 1000 x g for 20 min. The precipitated chitosan was discarded and the supernatant containing chitooligosacharides was concentrated by freeze drying and was ready for analysis by HPLC (UFLC, Shimadzu) using μBondapak NH_2 column (125Å, 10 μm, 3.9 X 300 mm; Waters). The mobile phase was linear gradient of acetonitrile: water (65:35). The run was taken for 20 min at a flow rate of 0.6 ml / min.

Antimicrobial assay

Antimicrobial activities of chitosan were determined against four bacterial strains *viz., Pseudomonas aeruginosa* (ATCC 49189), *Staphylococcus aureus* (ATCC 6538P)*, Bacillus subtilis* (KCTC 1028) and *Escherichia coli* (ATCC 1150) and in case of chitooligosaccharides two bacteria *viz., Pseudomonas aeruginosa* and *Staphylococcus aureus* were used. One ml of each bacterial culture (1 X 10^6 cells / ml) was transferred to 4 ml LB (Luria Bertani) broth. Different concentrations (0.1 - 0.6%) of chitosan (0.5 ml) was added to the bacterial culture of LB broth and was incubated for 5 h at 37^0C with shaking condition. Chitooligosaccharides produced after hydrolyzing chitosan by chitosanase with different time intervals as described above was added to bacterial cultures at different concentrations (0.1, 0.3 and 0.5%). Bacterial cultures without chitosan and chitooligosaccharide were considered as control. The inhibitory effect was assayed periodically by measuring the turbidity of the culture medium at 660 nm.

Results

The fungus isolated from CDA plate showed highest chitosanase activity after 7 days' incubation at 30°C as detected by the clear zone of growth and identified as *Paecilomyces lilacinus* by The Advanced Mycology Centre, Postgraduate Institute of Medical Education and Research, Chandigarh, which produced a buff-colored, granular colony. Repeated subcultures with extended incubation period of 2 weeks yielded mauve-colored colonies consistent with those typically seen with *P. lilacinus* in (Figure 1). The microscopic structure is shown in (Figure 2).

Chitosanase was purified to homogeneity successively by ammonium sulfate precipitation and Resource Q anion-exchanger column in Akta Prime system. Figure 3 represents the fractions eluted from the column as well as their chitosanase activity. The fractions under peak No. I and II showed 28 U/mg and 12.7 U/mg chitosanase activity respectively. The active fractions under peak I showing maximum chitosanase activity were pooled, mixed and concentrated. The homogeneity of the purified enzyme was judged by 10 % SDS-PAGE.

Table 1 shows the purification scheme of chitosanase. Chitosanse activity was expressed in unit; one unit

Table.1 Purification scheme of Chitosanase

Step	Total volume (ml)	Concentration of protein (mg / ml)	Total Protein (mg)	Total activity (U)	Specific activity (U / mg)	Purification fold	Yield (%)
Culture filtrate	100	0.08	8	87	11	1	100
0-80 % $(NH4)_2SO_4$ precipitation	6	0.6	3.6	127	35.2	3.2	45
Purified enzyme	0.5	0.4	0.2	210	1050	95.45	2.5

would liberate 1 µmol of glucosamine (GlcN) from chitosan per min at pH 5.5 at 37^{0}C. Chitosanase activity was found to be present in the culture filtrate as well as in 80% ammonium sulfate. The chitosanase was purified 95 fold with a specific activity of 1050 U/mg of protein. It exhibited a single band by 10% SDS-PAGE under denaturing condition (Figure 4). The molecular mass of the enzyme as observed by SDS-PAGE was 40 kDa. The absolute mass of chitosanase was 39.6 kDa as obtained by ESI-MS-Q-TOF analysis (Figure 5).

The activity of chitosanase was dependent on temperature. The activity of chitosanase was gradually increased with the increase in temperature being maximum at 50^{0}C and then gradually decreased with increase in temperature and completely abolished at 80^{0}C (Figure 6). The activity of chitosanase was pH dependent. The activity of chitosanase was gradually increased with the increase in pH being maximum at 6 and then gradually decreased with increase in pH (Figure 7). Chitosanase activity was not affected by Ca^{2+}, Zn^{2+} or Mg^{2+} ions but activated slightly by Fe^{2+} and Mn^{2+} (Figure 8).

HPLC profiles of the chitooligosaccharides mixtures are presented in Figure 9. High amount of *N*-acetylglucosamine (monomer) was produced in different time intervals except in1 h whereas very low

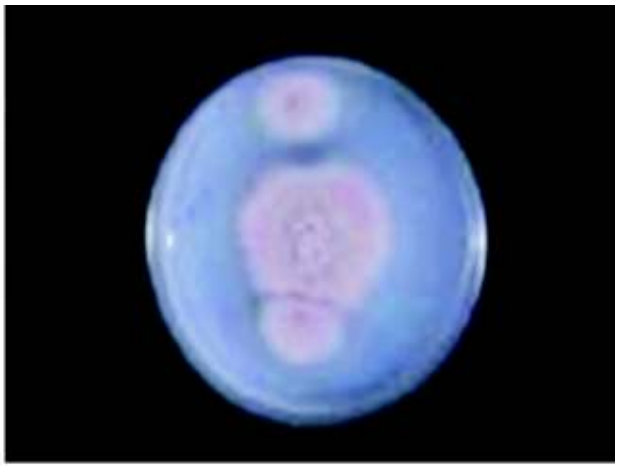

Figure 1. Fungal colony on chitosan agar medium after 1 week of incubation at 30°C.

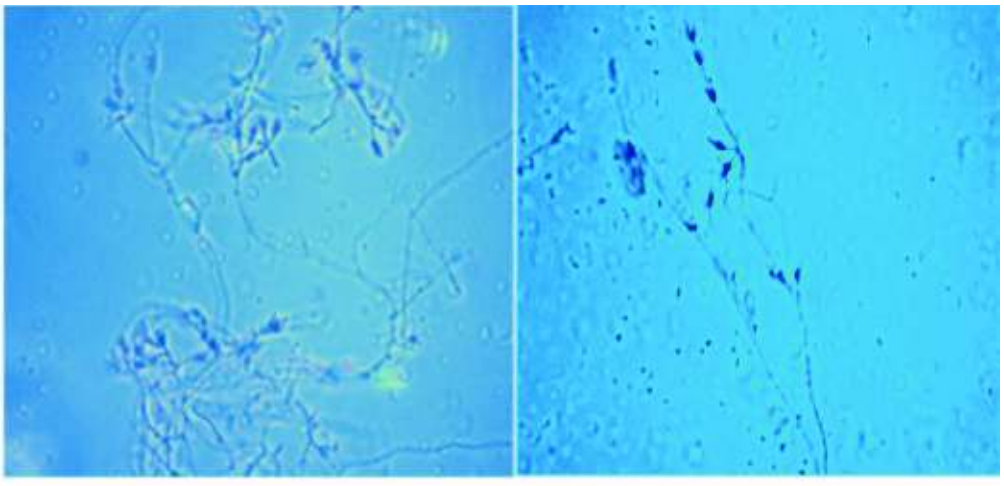

Figure 2. Microscopic structure of *P. lilacinus.*

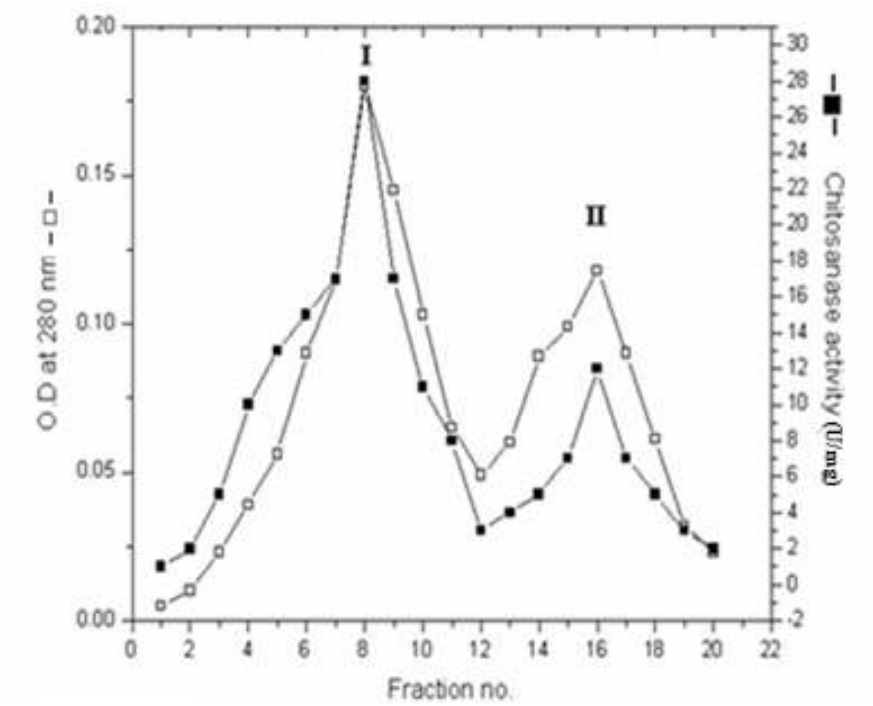

Figure 3. Purification profile of chitosanase from *P. lilacinus* on Resource Q anion- exchange column

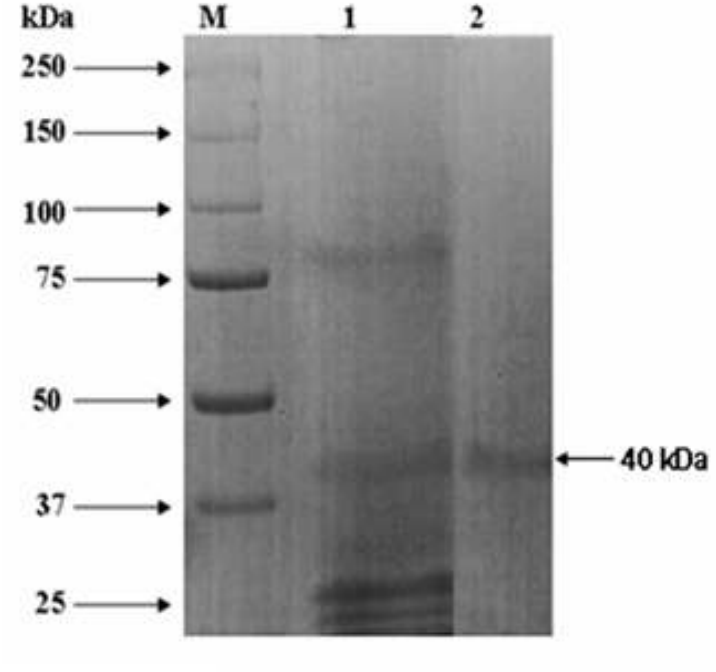

Figure 4. Homogeneity of the purified chitosanase. Proteins in chitosanase fractions of each purification step were subjected to 10 % SDS-PAGE. Lane1, M, Precision Plus Protein™ standard from Bio-Rad; lane 2, Ammonium sulphate precipitated fraction (10 µg), lane 3, Purified enzyme (5 µg of protein).

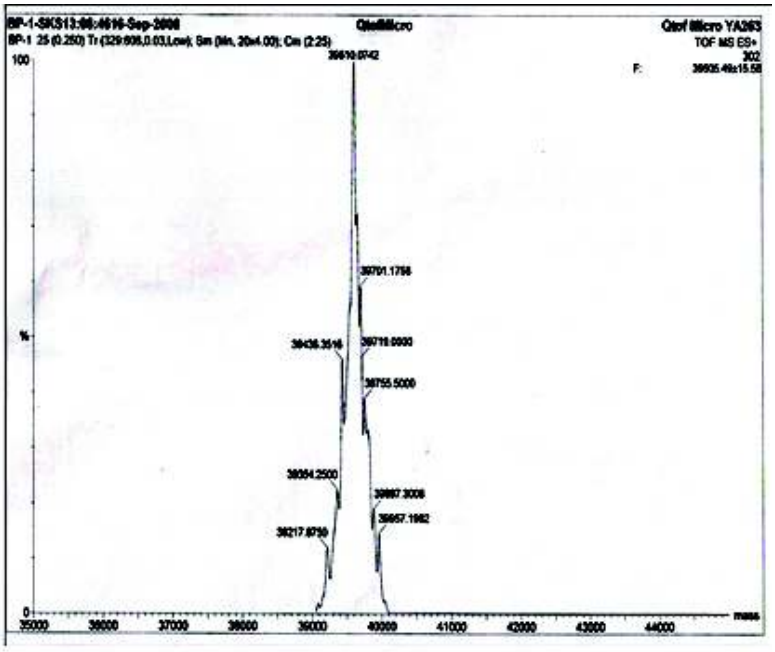

Figure 5. ESI-MS-Q-ToF mass spectrum of chitosanase.

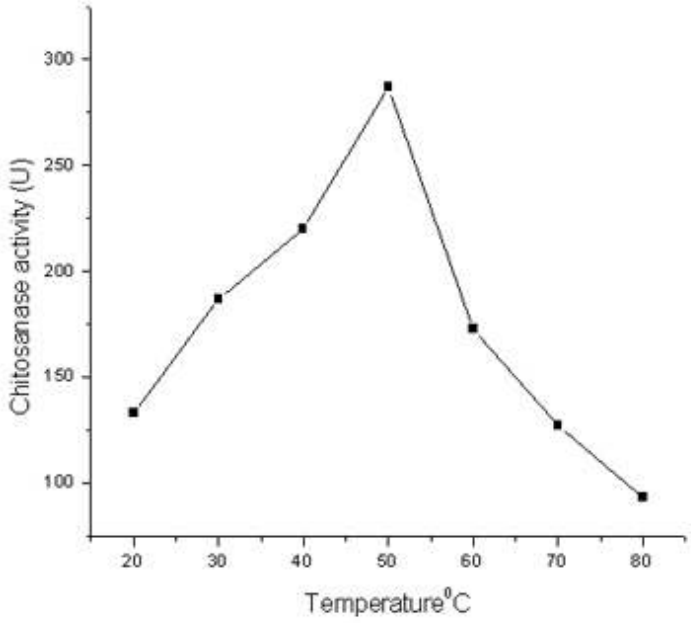

Figure 6. Effect of temperature on chitosanase activity.

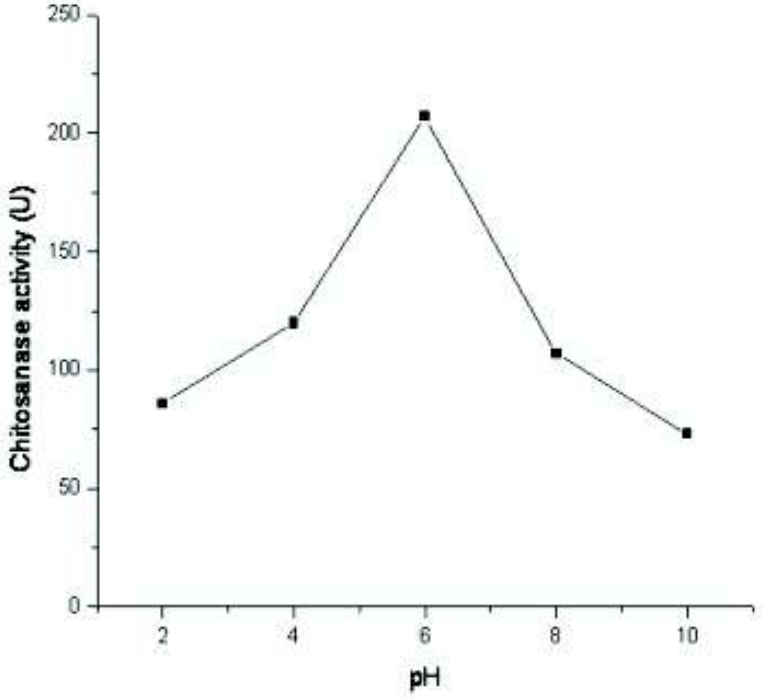

Figure 7. Effect of pH on chitosanase activity.

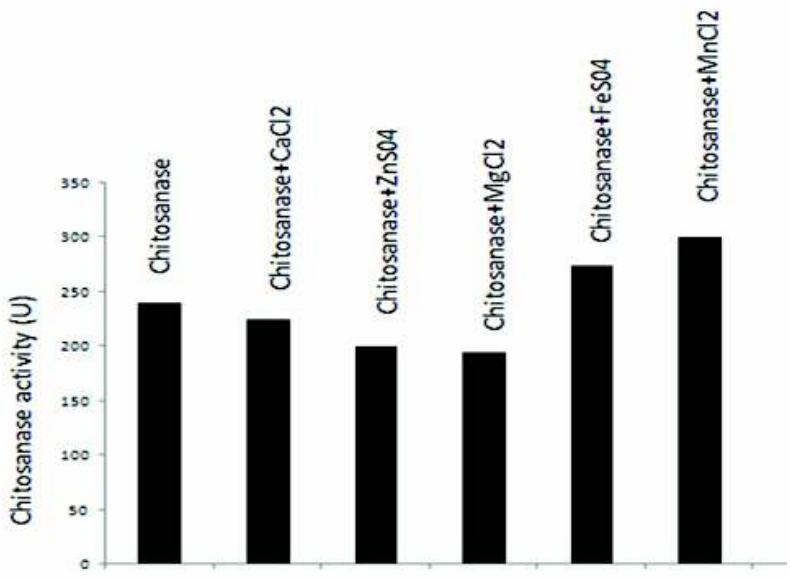

Figure 8. Effect of metal ions on chitosanase activity.

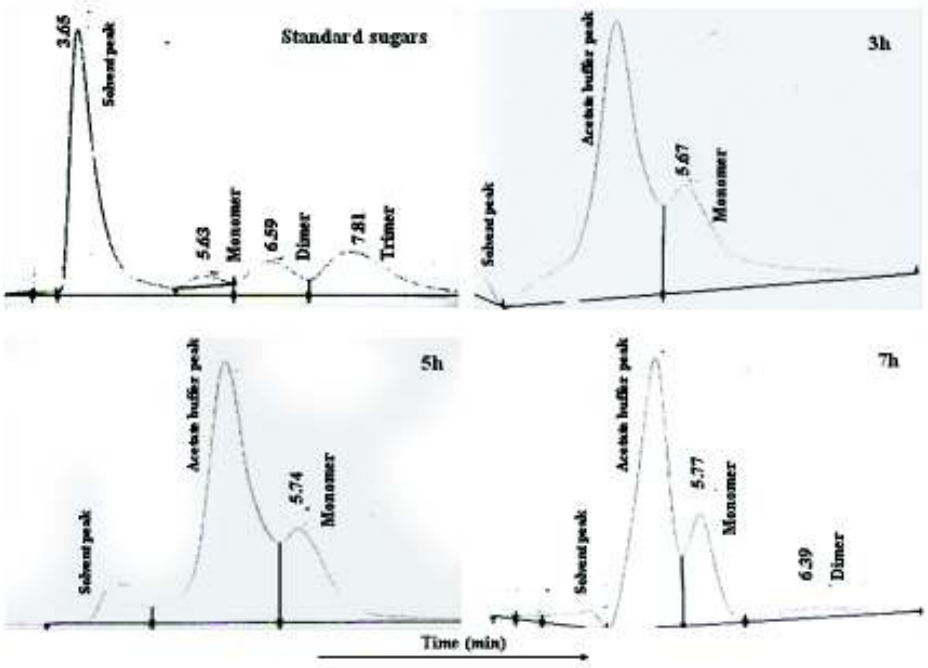

Figure 9. HPLC profile of chitooligosaccharides released from chitosan after treatment with chitosanase.

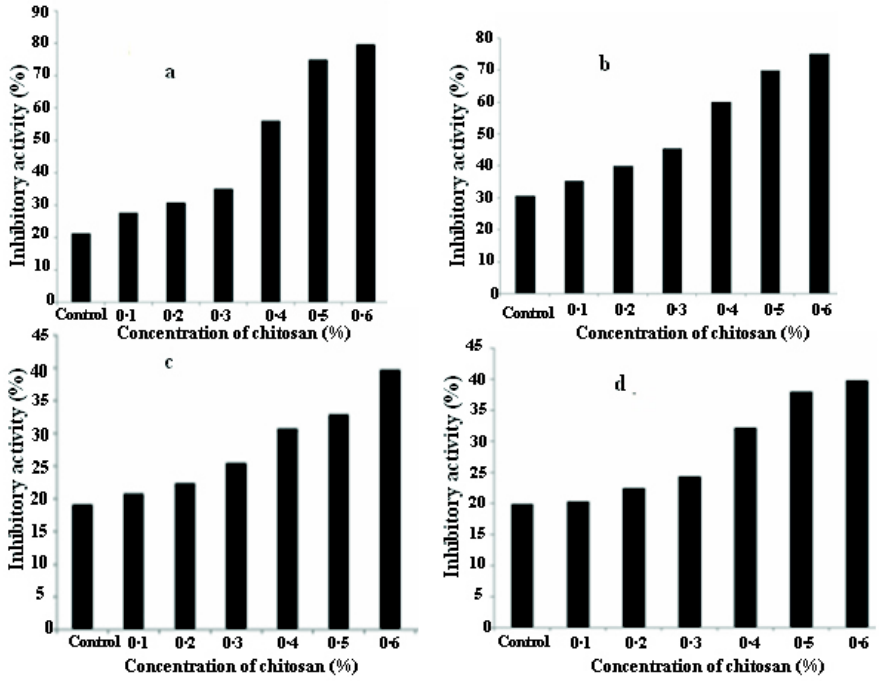

Figure 10. Antimicrobial activity of chitosan at various concentrations on the growth of (a) *Staphylococcus aureus;* (b) *Bacillus subtilis;* (c) *Pseudomonas aeruginosa;* and (d) *Escherichia coli.* Control (bacterial culture without chitosan); chitosan concentration (0.1% - 0.6%).

amount of dimmer was released at 7 h.

The inhibitory effect of the chitosan on bacterial growth as investigated at different concentrations ranging from 0.1 to 0.6 % (Figure 10 a, b, c, d). The inhibitory effect was observed with increased chitosan concentration. The O.D value of bacterial growth containing chitosan at 660 nm was very less (not shown) with respect to that of control (without chitosan). This shows that the chitosan has good antimicrobial effect on gram positive bacteria like *S. aureus* (79.8 %) followed by *B. subtilis* (75.28%). However, less antimicrobial effect was observed on gram negative bacteria like *P. aeruginosa* (39.8 %) and *E. coli* (38.81 %). On the other hand chitooligosaccharides showed maximum antimicrobial effect on *S. aureus* (100 %) in comparison to *P. aeruginosa* (86 %), a gram negative bacteria. Antimicrobial property of chitooligosaccharides gradually increased as the time of preparation of chitooligosaccharides increased and inhibition was observed (Figure 11 a, b).

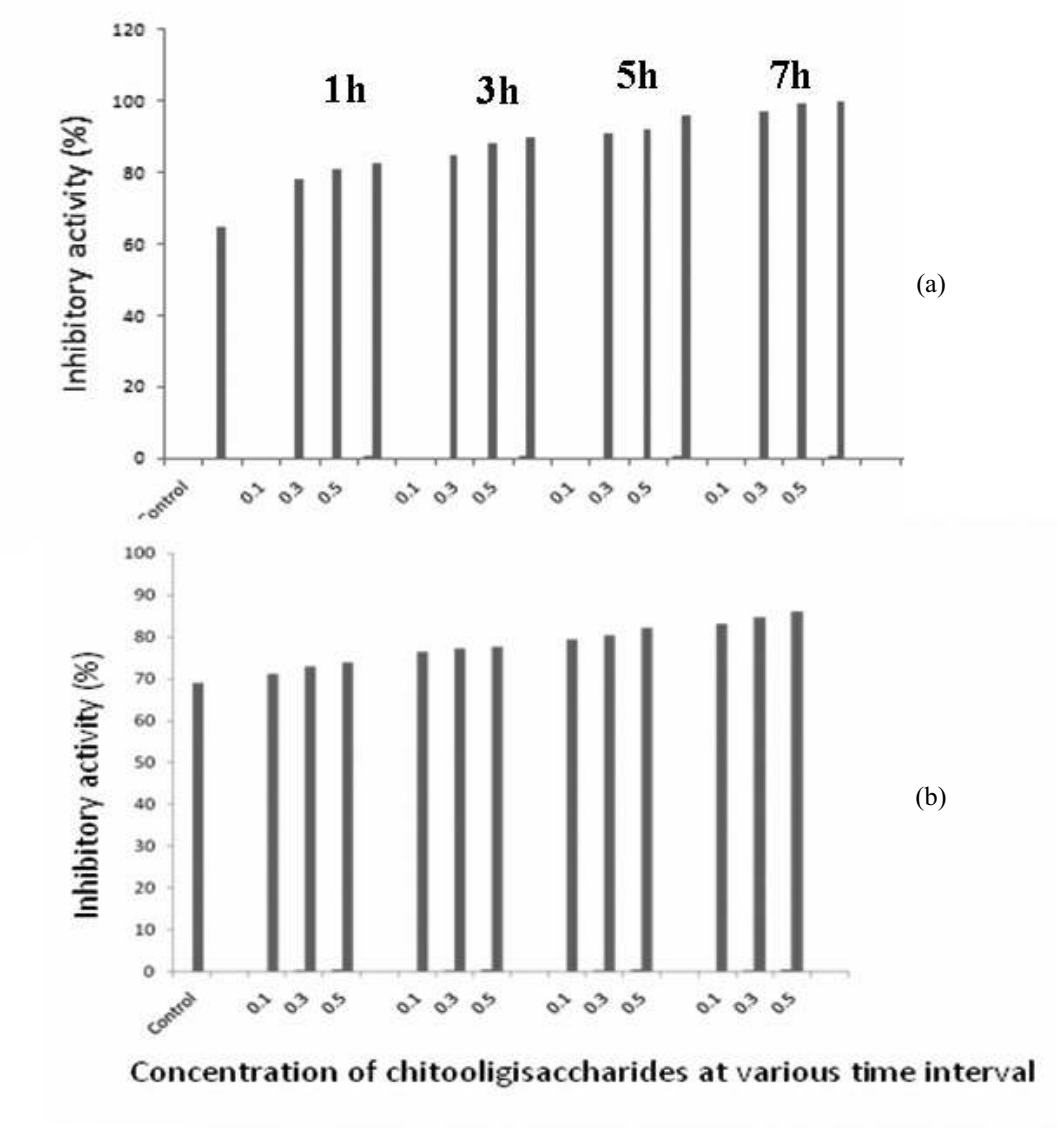

Figure11. Antimicrobial activity of chitooligosaccharides at various time intervals. (a) *Staphylococcus aureus* and (b) *Pseudomonas aeruginosa.* Control (bacterial culture without chitooligosaccharides), chitooligosaccharide concentration (0.1%, 0.3% and 0.5%).

Discussion

Fungal chitosanolytic enzymes have been purified and characterized from *Penicillium islanicum, Mucor rouxii, Fusarium solani, Trichoderma reesei* and *Aspergillus fumigates.* Most of these enzymes have been characterized as endotype chitosanases but exotype chitosanase was reported in limited number. Chitosanases from individual organisms differ in their hydrolytic action. Some chitosanases have hydrolytic activity on substrate other than chitosan[21]. The rate of reaction of chitosanase is highly dependent on the degree of acetylation of the substrate chitosan. There is a structural similarity among chitosan, chitin, and cellulose. For many years, chitosanases have remained poorly defined since besides chitosanase, other enzymes such as lysozyme, chitinase and exo-β-D-*N*-acetylglucosaminidase can hydrolyze partially acetylated chitosan more or less efficiently. A classification of glycosyl hydrolases based on hydrophobic cluster analysis for deduced amino acid sequences has been proposed. According to this classification, bacterial chitosanases belong to 46 families and are clearly distinguished from the families of chitinases or other related enzymes. It was previously reported that first deduced amino acid sequence of fungal chitosanase from plant pathogenic fungus, *Fusarium solani* had no homology with bacterial chitosanases of 46 families. This indicates that chitosanases, like cellulases and chitinases can be classified into several families. Therefore cloning and sequencing of the chitosanase gene was done.

Several chitosanolytic enzymes have been identified in filamentous fungi. An exo-β-D-glucosaminidase does not hydrolyze chitin, cellulose, carboxymethyl cellulose and glycol chitosan showing that they have strict substrate specificity. Furthermore, these exo-β-D-glucosaminidases released only GlcN residues from the nonreducing end of the chitosan polymer and cleaved GlcN-β(1-4)-GlcN and GlcN-β (1-4)-GlcNAc bonds but not the GlcNAc-β(1-4)-X bond. It was proposed that the classification of chitosanases into three classes according to their substrate specificities[30]. Class I chitosanases, represented by the chitosanases from *B. pumilis* BN262, *Streptomyces sp.* N174 and *Penicillium islandicum*, split the GlcNAc-GlcN linkage in chitosan. Class II chitosanases represented by the chitosanase from *Bacillus sp*. No. 7-M are exclusively specific towards the GlcN-GlcN linkage. Class III chitosanases represented by chitosanases from *Streptomyces griseus* HUT6037, *B. circulans* MH-K1[31], *Nocardia orientalis*, and *B. circulans* WL-12, can split both GlcN-GlcN and GlcN-GlcNAc linkages.

In the present work we found that specific activity of purified *P. lilacinus* chitosanase (40 kDa) was 1050 U/mg and the purification fold was 95 and the recovery of the enzyme was obtained 2.5%. The activity of enzyme was not affected by Ca^{2+}, Zn^{2+} or Mg^{2+} ions and was maximum at pH 6 and dependent on temperature. Antimicrobial properties of chitosan have been attributed to its cationic nature due to free amino groups present in chitosan. The molecular weight and the molecular fraction of glucosamine units in the polymer chain (usually referred as the degree of chitosan N-deacetylation), pH of chitosan solution and the nature of the target microorganism also responsible for this[32-35]. Chitosan is a kind of higher molecular polymer with positive charge. The net positive charge binds the negatively charged bacterial walls and breaks it, which leads to the release of bacterial contents into the environment. The antimicrobial effect of chitosan is maximum in *S. aureus* and minimum in *E. coli.* Thus we can say that chitosan was more effective against gram positive bacteria than gram negative bacteria tested. This difference is due to the cell wall structures of bacteria so that gram positive bacteria are more sensitive to such agents. In gram positive bacteria the major constituent of cell wall is peptidoglycan and very little protein. The cell wall of gram positive bacteria is made up of 30-40 layers of peptidoglycans, which contain GlcNAc, *N*- acetylmuramic acid as well as D- and L- amino acids including isoglutamate and teichoic acid to which the positively charged amino groups of chitooligomers/ GlcN can bind resulting in cell wall distortion-disruption, exposure of cell membrane to osmotic shock and exudation of the cytoplasmic contents. On the other hand the cell wall of gram negative bacteria is thinner but more complex and contains various polysaccharides, proteins and lipids beside peptidoglycan. The cell wall of gram negative bacteria also has outer membrane, which constitute the outer surface of the wall. Therefore, outer membrane core LPS appears to be the basis for the resistance of gram negative bacteria.

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