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RESEARCH ARTICLE

IMMOBILIZATION AND SOME BIOTECHNOLOGICAL APPLICATIONS OF CHITOSANASE FROM *MONASCUS PURPUREUS*

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Abstract

Polyamines induced chitosanase (Chsn, EC: 3.2.1.132) production by *M. purpureus* with increasing their concentrations. Polyamines could be arranged according to Chsn activity as agmatine < putrescine < spermine < spermidine < cadaverine. Chsn was purified from *M. purpureus* with specific activity of 35 units mg^{-1}protein and 9.02-fold of purification. Chitosan 100 % of degree of acetylation (DA) as a substrate achieved maximum activity. The molecular weight of purified Chsn was 66 KDa. K_m and V_{max} for free Chsn were 1.4 mg/ml and 200 units mg^{-1} protein, respectively. However, K_m and V_{max} of immobilized Chsn on Ca-alginate were 2.5 mg/ml and 500 units mg^{-1} protein, respectively. The optimum pH values of free and immobilized enzymes on Ca-alginate were 6 and 7, respectively. The optimal temperatures were 40 °C and 50 °C for the two forms of the enzyme in the same order. Immobilized Chsn expressed appreciable heat stability at 60, 70 and 80°C. K_d values for immobilized Chsn were 10 x 10^{-2}, 12.6 x 10^{-2} and 13.7 x 10^{-2}. $T_{0.5}$ values were 6.93, 5.50 and 5.01 min at 60 °C, 70 °C and 80 °C, respectively. It was observed that the inhibitory effect of chelating agents such as ethylenediaminetetraacetic acid (EDTA), *o*-phenanthroline, 8-Quinolinol, 8-Hydroxy-5-quinoline sulfonic acid (8-H5QS) and α,α`-dipyridyl was less pronounced in the case of the immobilized enzyme and this give immobilized Chsn a great advantage. Chsn reaction product chitooligosacharides (CHOS) have antioxidant activity and can eliminate undesirable effects of reactive oxygen species in foods and scavenge some free radicals such as hydrogen peroxide (H_2O_2). Immobilized *M. purpureus* Chsn acts as strong inhibitor for chitinase and this plays an important role in malaria and asthma therapy.

INTRODUCTION

The study of enzymes is an important area since it exists just on the borderline where the biological and physical sciences meet (Yogananth et al., 2012). Chsn enzyme catalyzes the hydrolysis of the β-1,4 glycosidic bond of chitosan, a partially deacetylated derivative of chitin, which comprises N-acetyl-D-glucosamine and D-glucosamine residues (Zhang and Zhang, 2013).

Structure of Chsn was found to consists of two globular domains, with a high α-helical content. In between the two lobes a large substrate binding and catalytical cleft is formed, which consist of two α-helixes and a three-stranded β-sheet (Marcotte et al., 1996). Chsn was found to hydrolyze the substrates according to an endo mode of action. In addition, degradation products were found to be in the α-anomer form, meaning that Chsn use the inverting mechanism (Fukamizo et al., 1995).

Fungal Chsn has been produced and characterized from different fungi such as *Aspergillus* (Chen et al., 2008), *Chaetomium globosum* (Provost et al., 2013) and *Trichoderma* (Ike et al., 2006).

CHOS that produced by hydrolosis of chitosan by Chsn have anti-tumor effects (Muzarelli, 1977), and there is also evidence for positive effects of CHOS in reducing metastasis from tumors (Shen et al., 2009). It was first suggested that the anti-tumor activity was due to the cationic properties of CHOS (Chen et al., 2005).

The present work aimed to investigate the activity level, purification, immobilization and some applications of extracellular *M. purpureus* Chsn. Also, it aimed to compare the characteristics of the free and immobilized Chsn.

Materials and Methods

Fungal source

Sugarcane trash is used as source for *M. purpureus* producing extracellular Chsn.

Inoculum preparation

The inoculum was prepared by growing the organism on potato dextrose agar (PDA**)** (Vanderzant and Splittstoesser, 1992) plates for 5 days at 30 ºC. The conidia were harvested by covering the plates with isotonic sodium chloride solution and brushing gently with an inoculum loop. The spore suspension was filtered through double cheese cloth (mira cloth filter) to remove hyphae and conidiophores. The spores were diluted with the same solution, vertex and then counted in a haemocytometer. The spore suspension was added to the flasks at a final concentration of 10^7 spores/ml.

Mode of fermentation

Cultivation of *M. purpureus* was carried out by submerged fermentation (SmF) in 250 ml Erlenmeyer flasks using the modified Czapek's dox liquid medium (Eaton et al., 1998) with 1 % colloidal chitosan as source of carbon, autoclaved at 121°C; 15 1bs pressure for 20 min and then inoculated with prepared inoculum a final concentration of 10^7 spores/ml and incubated in the dark at 30 ºC for 7 days. Samples were withdrawn at regular intervals of 24 hours and observed for Chsn activity.

Extraction of extracellular Chsn from M. purpureus

Extraction of extracellular Chsn was carried out according to the method described by Chen et al. (2008) and El-Shora et al. (2014).

Assay of Chsn

The assay of Chsn was carried out according to the method of Pechsrichuang et al. (2013).

Determination of protein content

Soluble protein content was estimated as described by Bradford (1976). The concentration of protein was determined from standard curve using bovine serum albumin (BSA) as standards.

Purification of Chsn

The purification of Chsn from *M. purpureus* was carried out through three steps using ammonium sulfate precipitation, ion exchange and gel filtration chromatography. Solid ammonium sulfate was added to the crude filtrate until it reached 65 to 85 % saturation. Precipitation of the protein will then occur due to the aggregation of proteins via the exposed hydrophobic portions.

A column of DEAE-(diethylaminoethyl) cellulose was prepared and the desalted enzyme sample was poured on the surface of column (3 x 50 cm). The elution of sample was carried out with 0.1 M phosphate buffer (pH 6.0). A column of Sephadex G-100 (3 x 50 cm) was prepared and the eluent from the previous step was added. Elution was then carried out by 0.1 M phosphate buffer (pH 6.0). A column of Sephadex G-200 (3 x 50 cm) was prepared and the eluent from Sephadex G-100 was applied and eluted with the same phosphate buffer at a constant drop rate. A total of 20-fractions of three-ml each were collected. The enzyme activity was assayed and the protein estimation was carried out. Fractions containing enzyme activity were pooled and lyophilized (Jakoby, 1971). The purity of the enzyme was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

SDS-PAGE and staining of purified Chsn from M. purpureus

Purity of Chsn from *M. purpureus* at each step was checked by SDS-PAGE (Laemmli, 1970). The molecular weight of the Chsn from *M. purpureus* was estimated using the protein standards: Ferritin (440 KDa), Catalase (232

KDa), myosin (136.0 KDa), bovine albumin (66.0 KDa), ovalbumin (45.0 KDa), carbonic anhydrase (29.0 KDa) and lactalbumin (14.2 KDa) (Laemmli, 1970).

Immobilization of Chsn on Ca-alginate bead

Immobilization of Chsn on Ca-alginate was carried out according to the method described by Maheswari and Priyadharshini (2014) with some modification.

Determination of the optimal pH for Chsn activity

The optimal pH for enzyme activity was determined over a range from 3-10. Sodium acetate/acetic (pH 3.0 - 4.0 - 5.0), potassium phosphate buffer (6.0 - 7.0), Tris-HCl buffer (8.0 - 9.0) and sodium bicarbonate buffer (10.0). From the results obtained a graph of enzyme activity vs. pH was plotted, and the optimum pH for Chsn was subsequently determined.

Effect of temperature on activity of Chsn

Reaction mixture was incubated at different temperatures 10, 20, 30, 40, 50, 60, 70 and 80 ºC. The optimum temperature was determined from the graph of enzyme activity against
temperature. The enzyme activity was estimated by the method described above.

Hydrogen peroxide scavenging activity by immobilized Chsn

The capability of Chsn to scavenge hydrogen peroxide was determined. Chitosan 100 % DA (0.5 ml of 0.8 mg/ml) was added to 1.5 ml 50 mM phosphate buffer (pH 7) and immobilize Chsn was added at three concentrations 50, 100 and 150 µg/ml and each mixture was incubated at various time intervals (30, 60, 90, 120, 150 and 180 min) at 50 °C. After that 0.5 ml of 50 mM of H_2O_2 was added to each treatment and absorbance was determined at 230 nm after 20 min. Blank was prepared using phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 was calculated using the following equation:

Antioxidant activity = $(A_{control} - A_{test\ sample}) / A_{control} \times 100$.

Assay of chitinase

The assay of chitin was carried out according to the method described by Chen et al. (2013).

Experimental Results

Induction of Chsn from M. purpureus by polyamines

In this experiment the effect of some polyamines namely: cadaverine, spermidine, spermine, putrescine and agmatine were tested at various concentrations (50, 100, 150, 200 and 250 µM) in the growth medium. The results in Fig. **1** show that the five polyamines induced Chsn production by *M. purpureus* with increasing their concentrations. Polyamines arranged according to Chsn activity as agmatine < putrescine < spermine < spermidine < cadaverine. The activity with cadaverine was 97.1 U/ml at 250 µM and with spermidine was 87.2 U/ml at 250 µM.

Purification steps of Chsn from M. purpureus

In this experiment Chsn from *M. purpureus* was purified by ammonium sulfate (65 -85 %), DEAE-Cellulose**,** Sephadex G-100 and Sephadex G-200. The obtained results in Table 1 indicate that specific activity was 35 units mg^{-1} protein with purification-fold of 9.02. The Chsn purification was supported by single band at 66 KDa obtained by SDS-PAGE (Fig. 2).

Substrate specificity for free Chsn purified from M. purpureus

This experiment aimed to study substrate specificity for Chsn activity from *M. purpureus*. Various substrates such as chitosan 100 % DA, chitosan 80 % DA, chitosan 60 % DA, chitosan 50 % DA, crystalline chitosan, colloidal chitosan, carboxymethyl chitosan (CM-chitosan), shrimp shell powder (SSP) and glycol chitosan were tested at concentration of 0.4 mg/ml with maintaining pH, temperature and Chsn concentration constants. The results were shown in Fig. 3. These results indicate that the activity of Chsn from *M. purpureus* varied according to the substrates used and the various substrates could be arranged according to their specificity as chitosan 100 % DA > chitosan 80 % DA > colloidal chitosan > chitosan 60 % DA > chitosan 50 % DA > CM-chitosan > crystalline chitosan > SSP > glycol chitosan.

Lineweaver–Burk plot for free and immobilized Chsn from M. purpureus

The activities of Chsn against various chitosan 100 % DA concentrations (w/v) were studied. Plotting the relationship between 1/V against 1/S for Chsn gave a straight line. These results are shown in Fig. 4. From this figure the values of K_m and V_{max} were calculated and were 1.4 mg/ml and 200 units mg^{-1} protein, respectively. The V_{max} value defines as the maximum velocity when all of the enzyme molecules are saturated with substrate whereas K_m is the substrate concentration at which an enzyme reaches 1/2 V_{max}. The calculated results for immobilized Chsn are illustrated in Fig. 5. From this figure K_m and V_{max} were calculated and the values were 2.5 mg/ml and 500 units mg^{-1} protein, respectively.

Effect of pH on activity of free and immobilized Chsn from M. purpureus

The enzyme activity of any enzyme is dependent on pH value. Therefore, it was thought of interest to study the pH profile of the purified Chsn from *M. purpureus*. In fact, for such sort of study the other factors, which may affect the enzyme activity including substrate concentration and the time of incubation were fixed. So, Chsn activity from *M. purpureus* was measured at a range of pH value between 3 and 10. The results in Fig. 6 indicate that Chsn activity increased gradually up to optimal pH 6.0 for free Chsn and pH 7.0 for immobilized one after which a gradual decrease of the activity was observed.

Effect of temperature on activity of free and immobilized Chsn from M. purpureus

The effect of temperature on free and immobilized Chsn from *M. purpureus* was studied. It was observed that increasing of temperature from 10 ºC concomitantly increased the activity of both free and Ca-alginate-immobilized Chsn up to 40 ºC and 50 ºC, respectively (Fig. 7). Therefore, these two temperatures are the optimum for free and immobilized Chsn, respectively. Beyond 40 ºC and 50 ºC, the free and immobilized Chsn displayed lower activities. Indeed the immobilized enzyme retained higher activity at 60 ºC, 70 ºC and 80 ºC compared to the free enzyme. As a general observation the immobilized enzyme expressed higher activity than the free one.

Heat stability of immobilized Chsn from M. purpureus

This experiment aimed to study the thermal stability of Chsn from *M. purpureus*. This was carried out at temperatures 60, 70 and 80 °C throughout time intervals (30, 60, 90, 120, 150 and 180 min). The results are shown in Fig. 8. The results indicate that Chsn from *M. purpureus* was stable at 60 ºC and the stability was reduced with rising of the temperature to 70 and 80 ºC.

K_d and $t_{0.5}$ of immobilized Chsn from M. purpureus

The K_d and $t_{0.5}$ of the immobilized immobilized Chsn purified from *M. purpureus* was determined and the results are shown in Fig. 9. The K_d increased with increasing temperature from 60 ºC to 80 ºC, whereas the $t_{0.5}$ value decreased with increasing the temperature and reached down 5.1 min at 80 ºC.

Effect of chelating agents on activity of free and immobilized Chsn from M. purpureus

This experiment aimed to investigate the effect of various chelating compounds namely: EDTA, O-phenanthroline, 8-Quinolinol, 8-H5QS and α,α`- dipyridyl at concentration of 5 mM on free and immobilized Chsn activity from *M. purpureus*. The results are shown in Fig. 10. These results indicate that all tested compounds inhibited the activity of free and immobilized Chsn and suggest that the enzyme is metalloenzyme. It was observed that the inhibitory effect of these reagents was less pronounced in the case of the immobilized enzyme and this give immobilized Chsn a great advantage.

Hydrogen peroxide radical scavenging immobilized Chsn from M. purpureus

The effect of Chsn reaction product (CHOS) on hydrogen peroxide scavenging activity was investigated. Chsn was tested at 50, 100 and 150 µg/ml and each mixture was incubated at various time intervals (30, 60, 90, 120, 150 and 180 min) at 50 °C. The results in Fig. 11 indicate that the percentage of hydrogen peroxide scavenging activity of Chsn reaction product (CHOS) exhibited appreciable effects. The scavenging activity was dependent on Chsn concentration and the incubation time of the reaction mixture.

Effect of immobilized Chsn from M. purpureus on chitinase activity

This experiment was designed for studying the effect of different concentrations of 5, 10 and 15 units of immobilized Chsn from *M. purpureus* on activity of chitinase. The experiment was carried out for 7 days and the chitinase activities were measured every day. The results in Fig. 12 indicate that increasing the concentration of

Chsn resulted in inhibition of chitinase activity which decreased down to 0.12 units mg^{-1} protein at 15 units of Chsn at th 7^{th} day. This inhibition by *M. purpureus* Chsn may suggest important role of Chsn in malaria and asthma therapy.

Discussion

The present results indicate that treatment of *M. purpureus* growth media with polyamines namely: agmatine, putrescine, spermine, spermidine and cadaverine resulted in the induction of Chsn activity. In fact, these results are in agreement with those reported by Tanemura and Yoshino (2006). The increase in the enzyme activity could be due to activation of enzymes involved in biosynthesis of RNA and enzyme protein (Abraham and Pihl, 1981; El-Shora, and Metwally, 2008). Spermidine could prevent superoxide-generating NADPH oxidase activation and thereby prevent regeneration of free radicals which attack enzyme protein (Shen et al., 2000). Both free and conjugated polyamines have the potential to act as free-radical scavengers (Belle et al., 2013).

The Chsn was purified from *M. purpureus* using ammonium sulfate, DEAE-Cellulose, Sephadex G-100 and Sephadex G-200 with specific activity of 35.0 units mg^{-1} protein and 9.02-fold of purification. However, the purified Chsn from *Trichoderma reesei* had a specific activity of 27.3 units mg^{-1} protein (Nogawa et al., 1998). The specific activity of Chsn from Bacillus cereus was 347 units mg^{-1} protein which is higher than that observed in the present investigation (Gao et al., 2008). The specific activity of Chsn from *Aeromonas sp*. was 8.5 units mg^{-1} protein with purification fold of 21.25 (Sun et al., 2009). The specific activity Chsn from *Capsicum annuum* leaves was 57.8 units mg^{-1} protein (El-Sayed et al., 2012).

The purification of *M. purpureus* Chsn in the present investigation was supported with the single band at 66 KDa obtained by SDS-PAGE. The molecular weight of Chsn from *Aspergillus sp*. were 25, 23.38 and 111.23 KDa (Eom and Lee, 2003; Li et al., 2013). The molecular weight of Chsn from *Bacillus circulans* MH-K1 and *Gongronella sp*. were 30 and 90 KDa, respectively (Yabuki et al., 1988; Wang et al., 2008).

The substrate specificity for Chsn of *M. purpureus* could be arranged as chitosan 100 % DA > chitosan 80 % DA > colloidal chitosan > chitosan 60 % DA > chitosan 50 % DA > CM-chitosan > crystalline chitosan > SSP > glycol chitosan.

It is suggested that the presence of N-acetylglucosamine residues in the molecule of chitosan plays an important role in the recognition of the substrate by the enzyme. Chsn showed an endo-splitting type of activity, and the end product of chitosan degradation contained a mixture of the dimer and trimer of glucosamine. The smallest of the substrates was a tetramer of glucosamine (Yabuki et al., 1988).

The obtained relationship between the velocity (V) and the concentration (S) of chitosan is in accordance with the typical enzyme reaction (Michaelis and Menten, 1913). Also, it was apparent that the typical substrate concentration achieved the maximum velocity of enzyme reaction as shown at 0.7 mg/ml of chitosan 100 % DA for free enzyme and 0.8 mg/ml chitosan 100 % DA for immobilized enzyme. This may be attributed to the fact that higher substrate concentration may cause an inhibition of the enzyme reaction (Palmer et al., 1995).

Plotting V^{-1} versus S^{-1} gave a linear relationship from which K_m value of Chsn from *M. purpureus* was calculated. V_{max} and K_m values were 200 units mg^{-1} protein and 1.4 mg/ml, respectively. K_m for Chsn from *Gongronella* sp. was 4.5 mg/ml (Wang et al., 2008). The K_m and V_{max} for Trichoderma reesei were 0.06 mg/ml and 27.3 units mg^{-1} protein, respectively (Nogawa et al., 1998). K_m of *Penicillium* immobilized Chsn on chitin was 1.064 mg/ml (Wang et al., 2012).

The immobilized enzyme expressed structural changes of the enzyme which are introduced by the applied immobilization procedure and/or to the lower accessibility of the substrate to the catalytic site of the immobilized enzyme. Generally, several reasons can account for the variations of the K_m and V_{max} values of the enzyme upon immobilization (Yildiz, 2005; El-Shora and Youssef, 2008; El-Shora et al., 2015a).

The variations of the K_m and V_{max} values are attributed to several factors such as the interactions of the immobilized enzyme molecules with bead surface might have induced an inactive conformation to the enzyme molecules. It should be noted that immobilization process does not also control the proper orientation of the immobilized enzyme on the support (Vinod et al., 2006; El-Shora et al., 2009). This improper fixation and/or the change in the property of the active sites might hinder the active site for binding of the substrate to the immobilized enzyme molecules (El-Shora et al., 2014).

The optimal pH value for the free Chsn from *M. purpureus* was 6.0. However, optimal pH of *Gongronella* sp. Chsn was 5.6 (Wang et al., 2008). The pH optimum of the immobilized Chsn in the present work was 7.0. However, optimum pH was 6.0 for immobilized Chsn by Abdel-Aziz et al. (2014). Also, optimal pH for free and immobilized *Aspergillus* Chsn were 4 and 6.5, respectively (Sinha et al., 2011).

Furthermore, the optimal pH for the *Penicillium* immobilized Chsn was 7.0 (Wang et al., 2012). These authors reported that immobilized Chsn was more stable at this pH than its native counterpart suggesting that the attached

polymer chain can protect the active enzyme conformation at alkaline pH and avoids protein unfolding. A shift of pH optimum of Chsn on immobilization is well known properties of many immobilized enzymes (Rogaiski et al., 1999, El-Shora et al., 2015 a).

The fixed position of immobilized enzyme on the surface of the carrier is thought to prevent extensive distortion of the native conformation of the enzyme protein caused by external pH (El-Shora et al., 2014, El-Shora et al., 2015b).

The optimal temperatures of free and immobilized Chsn were 40 and 50 °C, respectively. The optimal temperature for free Chsn from *Fusarium solani* was 50 ^{o}C (Liu and Bao, 2009) which is more or less comparable to our results. Abdel-Aziz et al. (2014) reported optimal temperature of 30 ^{o}C for free Chsn from *Mucor rouxii*.

The increase in optimum temperature of immobilized Chsn in the present work may be caused by the changing enzyme conformational structure upon immobilization (Deere et al., 2002). Optimal temperature for free and immobilized *Aspergillus* Chsn was 30 °C (Sinha et al., 2011). However, optimal temperature for the *Penicillium* immobilized Chsn on chitin was 60 ^{o}C (Wang et al., 2012).

The immobilized Chsn in present investigation expressed appreciable stability at 60, 70 and 80 ^{o}C. The higher heat resistance of immobilized Chsn could be explained on the basis that the support of the immobilization generally has a protecting effect at high temperatures at which deactivation occurs (El-Shora et al., 2014).

The conformational flexibility of the enzyme is affected by immobilization. The immobilization of enzyme causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denaturation by raising the temperature (Chang and Juang, 2004; El-Shora et al., 2014, El-Shora et al., 2015). Immobilization of the enzyme on Ca-alginate is supposed to preserve tertiary structure of enzyme from conformational changes causing effects of the environment.

$T_{0.5}$ values of immobilized *M. purpureus* Chsn decreased with increasing the temperature and reached down 5.1 min at 80 ºC. Enzymes are usually quite stable at the temperature ambient for the organism from which they are obtained and lose their activity when the temperature is increased to a significantly higher level. There is still much uncertainty about the mechanism of thermal inactivation of enzymes. The first step in enzyme thermoinactivation is partial unfolding of the protein molecule (El-Shora et al., 2012; El-Shora et al., 2014).

The chelating agents namely: EDTA, *o*-phenanthroline, 8-Quinolinol, 8-H5QS and α,α`-dipyridyl inhibited the free and immobilized Chsn from *M. purpureus*. This is harmony with the results of El-Sayed et al. (2012). These compounds inhibited other enzymes such as glucose oxidase (El-Shora et al., 2014). These results reveal that the chelating agent react with the metal ion leading to the formation of stable complex and suggest that Chsn is a metalloenzyme.

On the other hand Chsn purified from *Streptomyces aureocirculatus* was stimulated by EDTA (El-Shirbiny et al., 2007). However, it was observed that the inhibitory effect of these reagents was less pronounced in the case of the immobilized enzyme. This may be due to the fact that the enzyme has a fixed structure and that the carrier provides a protective role to the immobilized enzyme. Similar results were previously reported for other immobilized enzymes (Guerfali et al., 2009; El-Shora et al., 2014).

In conclusion, our results indicate that M. *purpureus Chsn* reaction product (CHOS) expressed appreciable antioxidant activity for reactive oxygen species such as hydrogen peroxide. Therefore, the enzyme could be used to eliminate the undesirable effects of reactive oxygen species in foods. Also, the immobilized Chsn from M. *purpureus* acts as inhibitor for chitinase and this may play an important role in malaria and asthma therapy.

Table 1: Purification steps of Chsn from *M. purpureus*.

Purification step	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U mg^{-1} protein)	Yield (%)	Fold of purification
Crude enzyme	27.8 ± 1.10	108.1 ± 2.1	3.88 ± 0.7	100	1
Ammonium sulfate ppt 65 - 85 %	15.61 ± 1.20	99.30 ± 1.1	6.36 ± 0.6	91.8	1.64
DEAE-Cellulose	5.23 ± 0.40	76.50 ± 1.3	14.6 ± 0.8	70.8	3.76
Sephadex G-100	1.58 ± 0.05	40.21 ± 1.2	25.5 ± 0.7	37.2	6.57
Sephadex G-200	0.30 ± 0.01	10.62 ± 1.0	35.0 ± 0.5	9.82	9.02

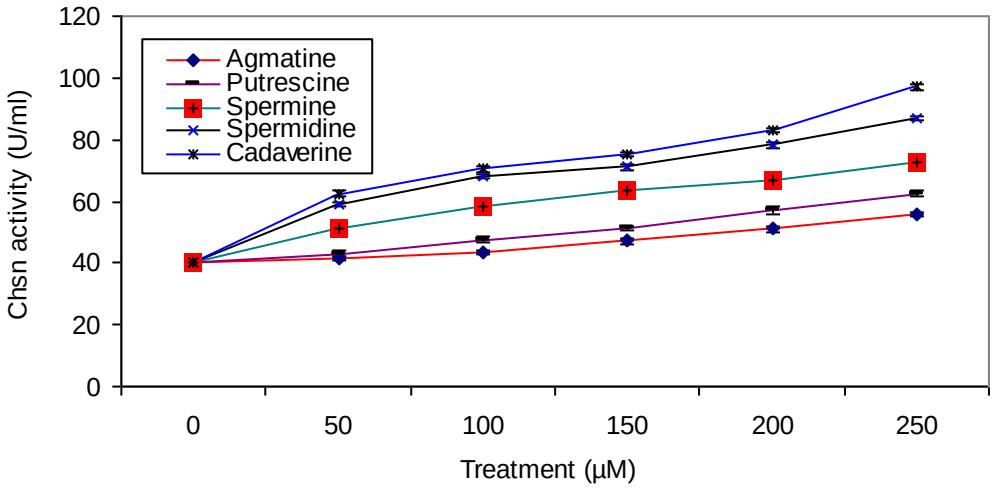

Fig.1: Induction of Chsn from *M. purpureus* by polyamines.

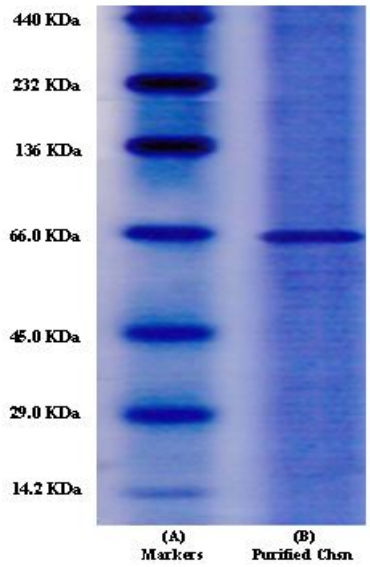

Fig. 2: SDS-PAGE of Chsn from *M. purpureus*.

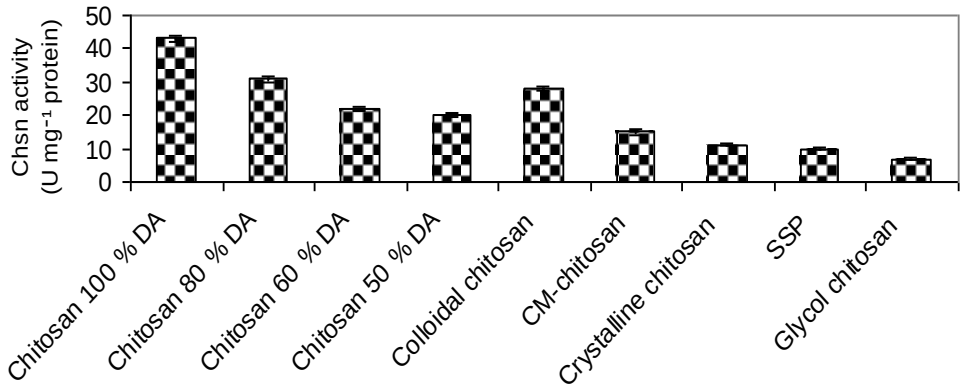

Fig. 3: Substrate specificity for free Chsn purified from *M. purpureus*.

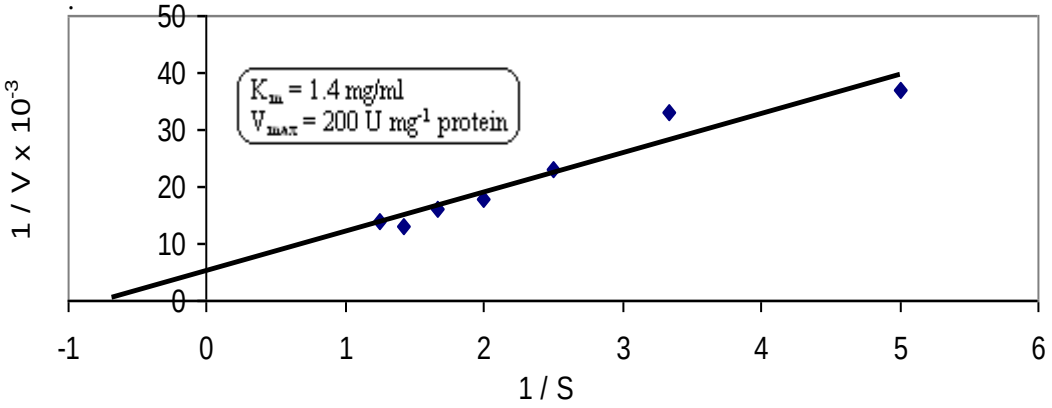

Fig. 4: Lineweaver–Burk plot for free Chsn from *M. purpureus*

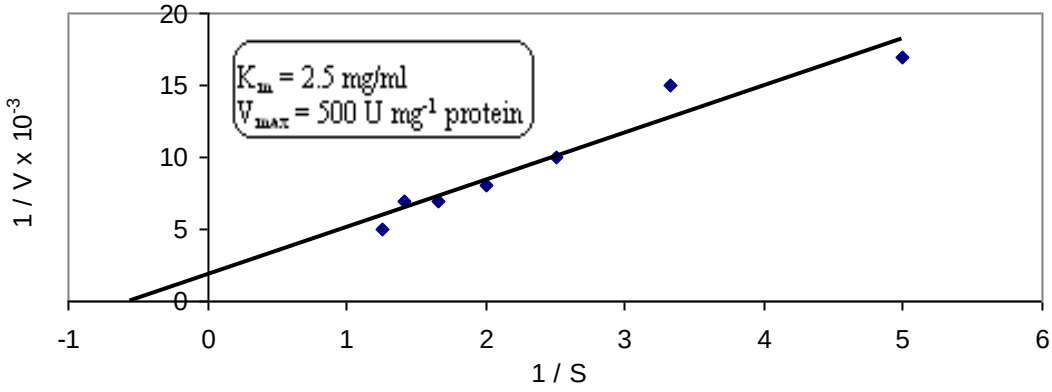

Fig. 5: Lineweaver–Burk plot for immobilized Chsn from *M. purpureus*.

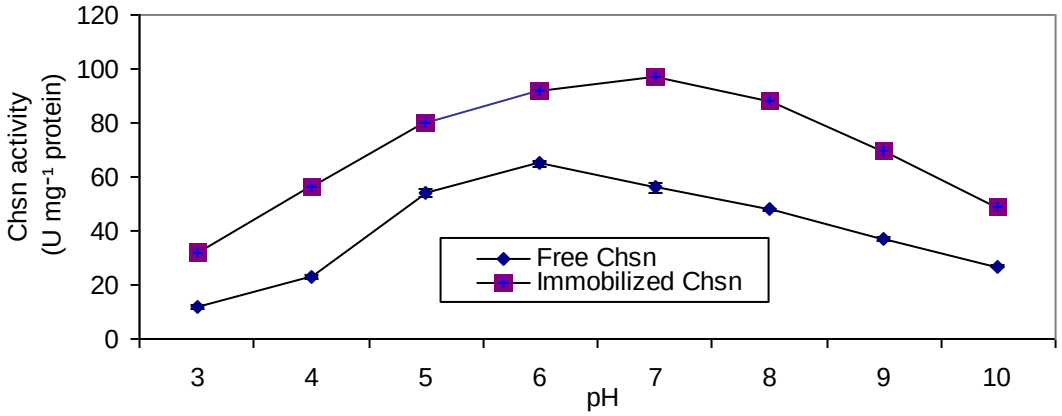

Fig. 6: Effect of pH on activity of free and immobilized Chsn from *M. purpureus*.

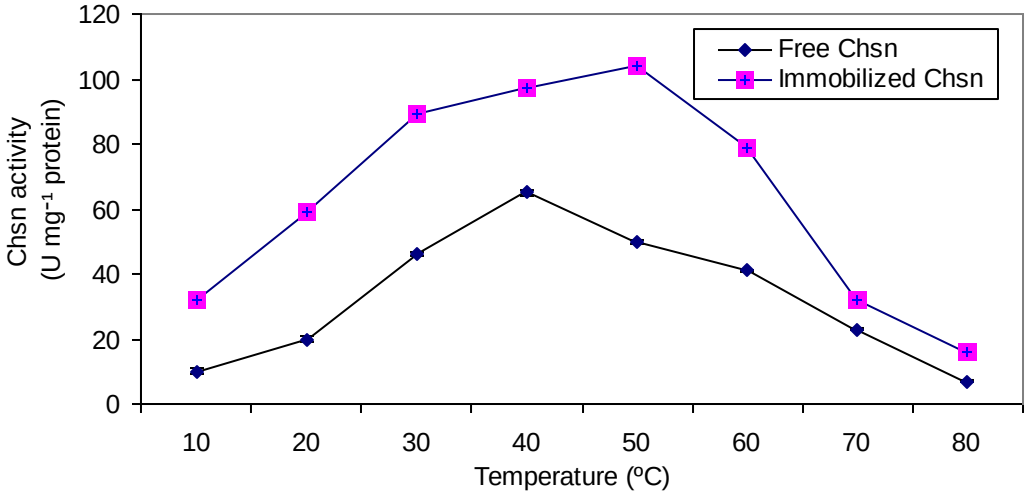

Fig. 7: Effect of temperature on activity of free and immobilized Chsn from *M. purpureus*.

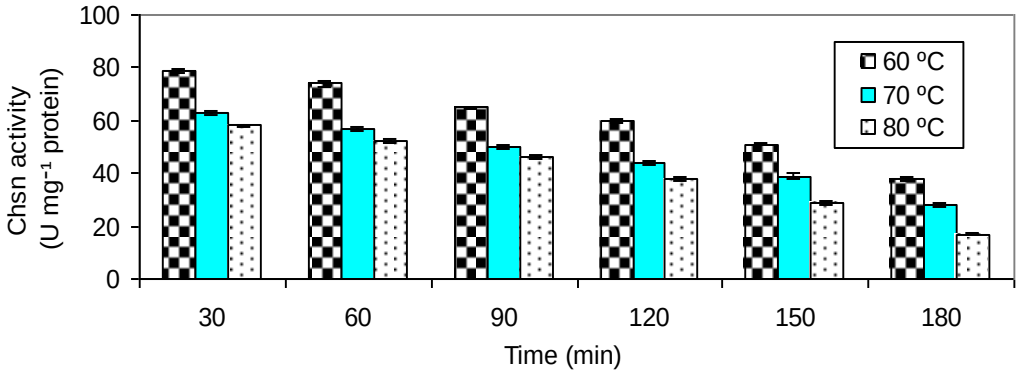

Fig. 8: Heat stability of immobilized Chsn from *M. purpureus*.

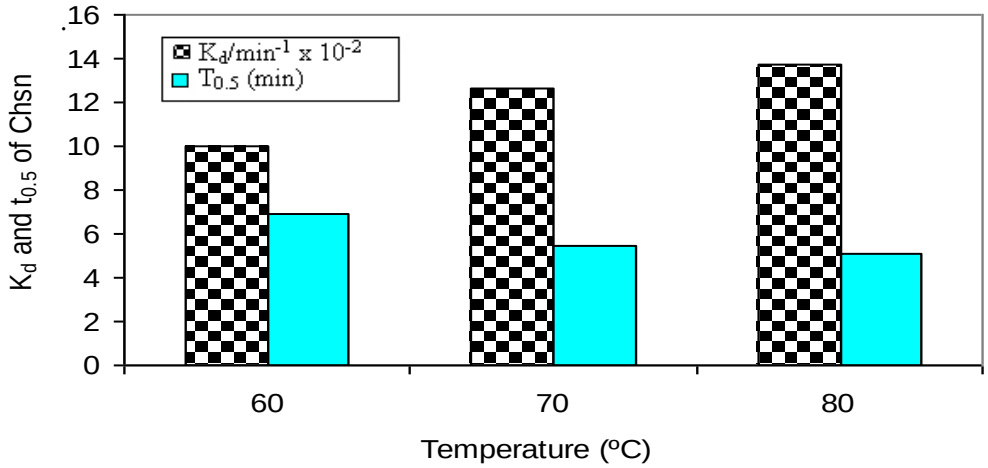

Fig. 9: K_d and $t_{0.5}$ of immobilized Chsn from *M. purpureus*

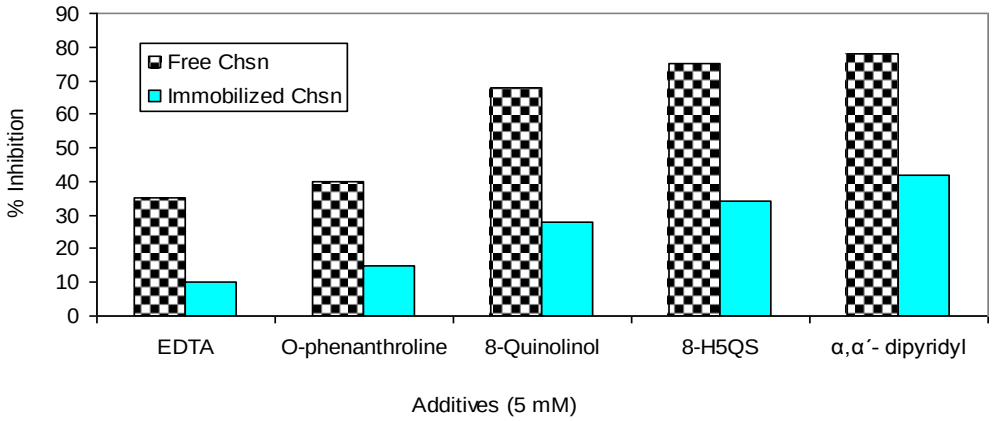

Fig. 10: Effect of chelating agents on activity of free and immobilized Chsn from *M. purpureus*.

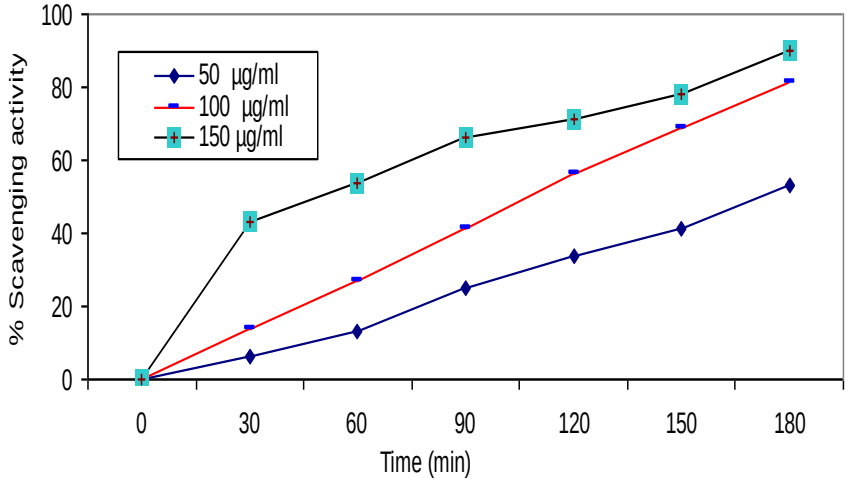

Fig. 11: Hydrogen peroxide radical scavenging activity of immobilized Chsn from *M. purpureus.*

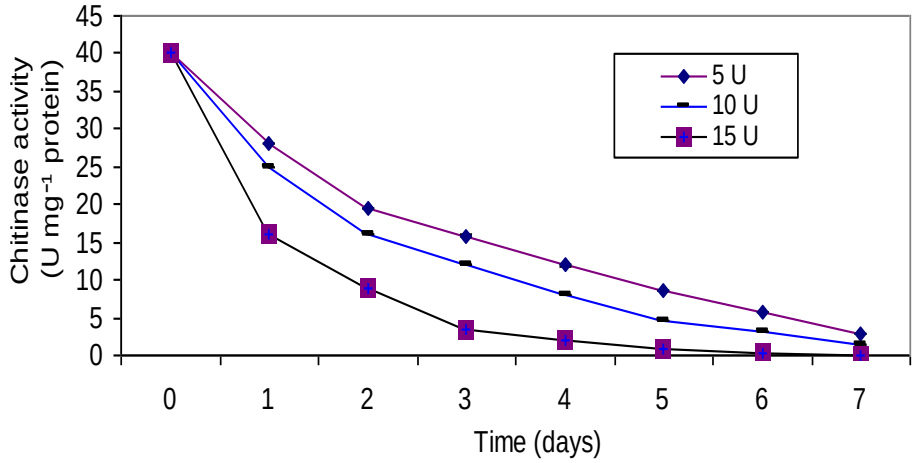

Fig. 12: Effect of immobilized Chsn from *M. purpureus* on chitinase activity.

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