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Biochemical Characteristics of Immobilized Chitinase from *Alternaria infectoria*

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Authors' contributions

This work was carried out in collaboration between all the authors who read and approved the final manuscript.

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ABSTRACT

This research aimed to purify and immobilize chitinase from *Alternaria infectoria* and to study its biochemical characteristics for biotechnological applications. Chitinase (EC. 3. 2. 1. 14) was purified by ammonium sulphate precipitation, DEAE-cellulose and then Sephadex G-200 with specific activity of 51.9 U mg^{-1} protein. The pH values were 7, 8 and 9 for the free, silica gel – immobilized and entrapped enzyme. The optimal temperatures were 40, 50 and 60°C for the three forms of chitinase, respectively. The activation energy values were 43.2, 30.8 and 11.6 KJ mol^{-1}. Ca^{2+} was activator whereas Hg^{2+}, Cd^{2+} and Ni^{2+} were inhibitors. However, K^{+} and Mg^{2+} showed no remarkable effect. The immobilized chitinase retained appreciable activity after 8 cycles particularly entrapped enzyme. The entrapped chitinase expressed higher stability against desorptivity by SDS than silica gel-immobilized enzyme. Thus, the results reveal that immobilized chitinase expressed higher thermostability compared to the free enzyme and this was important for industrial applications.

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Keywords: Chitinase; purification; characterization; thermostability; reusability; desorptivity.

1. INTRODUCTION

Chitin is odorless, non-toxic, biodegradable, biocompatible with living tissues [1], moisture retaining, presenting antibacterial and healing characteristics [2].

The shellfish processing industry of crab shells or shrimp produce great amounts of waste from shells, which contain about 30% chitin [3].

Chitinase degrades chitin to chitooligomers, which are good for agricultural, industrial and medical functions including anti-tumor activity as well as elicitor action [4,5].

Fungal chitinases perform an important function in morphogenesis, development and nutrition processes [6]. Chitinases are found in fungi including *Trichoderma* [7] *Penicillium, Neurospora*, *Lecanicillium*, *Beauveria*, *Mucor, Aspergillus, Lycoperdon, Myrothecium, Agaricus* and *Stachybotrys* [5,8,9].

The stability of the chitinase and its cost were the main essential factors for chitinase applications. Therefore, it is necessary to study potential producers and inexpensive inducers [10].

Scientists paid an increased attention to chitinases due to their action in bio-controlling of harmful insects [11] and fungal phytopathogens [12]..

The chitinases have not established biotechnological uses at commercial scale because of their high cost. However, chitinases could act as biopesticides [13].

The goal of present work was to isolate and purify chitinase from *A. infectoria* and study its biochemical characteristics.

2. MATERIALS AND METHODS

2.1 The Experimental Organism

A. infectoria was provided by Salwa Abdel Magid Khalaf, Prof of Microbiology, Botany Department, Faculty of Science, Zagazig University, Egypt.

2.2 Culture Media Used

The basal medium used throughout the present work was Czapek-Dox's medium containing chitin as carbon source [14] composed of (g/L) 10.0 colloidal chitin, 0.5 $MgSO_4.7H_2O$, 1.0, 0.5 KCl, KH_2PO_4, 0.01 $FeSO_4.7H_2O$, 3 yeast extract and 1000 ml distilled water. The pH of the basal medium was 7.0.

2.3 Extraction of Extracellular Chitinase

Chitinase was isolated from *A. infectoria* by filtrating the culture fluid using a Whatman no. 1 filter paper. The obtained cell debris was discarded by centrifugation for 15 min at 10,000g and 4°C. The resulting supernatant was the crude enzyme extract for determination of chitinase activity [15].

2.4 Purification of Chitinase

Chitinase was purified using ammonium sulphate, ion exchange and Sephadex G_{200}.

2.4.1 Precipitation step

Firstly, the crude enzyme extract was precipitated at 4°C with ammonium sulphate to obtain 85% saturation. The precipitated protein was obtained by centrifugation (8,000g for 25 min), dissolved in phosphate buffer (50 mM, pH 7.0), finally dialyzed using the same buffer overnight in a refrigerator and then chitinase activity was determined.

2.4.2 Ion-exchange chromatography on DEAE –Cellulose

A column (2.5 × 30.0 cm) was packed with a slurry of the diethylaminoethyl-cellulose (DEAE-Cellulose) and equilibrated with phosphate buffer (50 mM, pH 7.0). The dialyzed enzyme from the first step was applied to DEAE-Cellulose column. Elution was performed with 50 mM NaCl in phosphate buffer (50 mM , pH 7.0).

2.4.3 Gel filtration chromatography on Sephadex G-200

The concentrated protein from DEAE-Cellulose column was applied to a column of Sephadex G-200 (2.5 × 45.0 cm), pre-equilibrated with 50 mM phosphate buffer (pH 7). The enzyme was eluted with 50 mM NaCl in phosphate buffer (50 mM, pH 7.0) with flow rate of 2 ml h^{-1} followed by determination of the enzyme activity.

2.5 Estimation of Enzyme Molecular Weight

The molecular weight of the resulting protein band was calculated from the authentic protein markers according to Laemmli [16]. The markers consist of myosin (200 KDa), phosphorylase (97 KDa), BSA (66 KDa) and ovalbumin (45 KDa).

2.6 Determination of Protein Content

The protein content was estimated by the method of Bradford [17] using Coomassie Brilliant Blue (CBB) G-250 and bovine serum albumin (BSA) as a standard.

2.7 Immobilization of Chitinase on Different Beads

2.7.1 Immobilization of chitinase on Ca-alginate

Sodium alginate solution (5% w/v) was prepared in sodium phosphate buffer (50 mM, pH 7.0) by warming at 50°C, cooled to room temperature and 2 ml of chitinase preparation was mixed with 8 ml of sodium alginate preparation.

The mixture was placed in a syringe and dropped into 1 M calcium chloride with continuous stirring for 2 h at 4°C. The formed bead was dried by filter paper then exposed for 1 h to the open air before being used [18]. The filtered calcium chloride solution was collected for determination of chitinase activity.

2.7.2 Immobilization of chitinase on agar-agar

The purified chitinase was immobilized in agar-agar. The agar was prepared by warming at 50°C in 20 mM potassium phosphate buffer (pH 7.0), then cooled to room temperature. One ml of the enzyme preparation (0.4 mg protein/ml) was mixed with 9.0 ml of the matrix solution (the total volume was 10 ml) and immediately casted on pre-assembled glass plates. After solidification of the mixture, the agar-agar gel was cut into small chips (5 × 5 mm) and washed several times for removing any enzyme attached to the gel surface. The immobilized chips were kept in extraction buffer and stored in refrigerator for determination of the enzyme activity as described before.

2.7.3 Immobilization of chitinase on silica gel

One gram of silica gel was mixed with prepared chitinase solution and stored at 4°C overnight. The unbounded chitinase was removed from the silica gel through washing four times with 3.0 ml of phosphate buffer (10 m, pH 7.0).

2.8 Characterization of Free and Immobilized Chitinase

2.8.1 Optimal pH of free, entrapped and silica gel-immobilized chitinase

The activity of both the free and immobilized chitinase was estimated. Chitinase was determined at pH range 3.0 – 10.0 using 0.05 M acetate buffer (3.0 – 5.0), 0.05 M phosphate buffer (5.0 –8.0) and 0.05M sodium carbonate buffer (8.0 – 10.0). The chitinase activity was then estimated at the various pH values.

2.8.2 Optimal temperature of free and immobilized chitinase

The enzyme activity was measured at various temperatures (20 to 80°C). The other factors affecting the enzyme activity were kept constant.

2.8.3 Thermostability of free and immobilized chitinase at 60°C and 70°C.

Chitinase denaturation was studied at 60°C and 70°C for 120 min and the enzyme activity was measured every 20 min. The residual activity of chitinases from the three forms of the enzymes was expressed as percentage of remaining activity using the following equation:

$$\%\text{Remaining activity} = \frac{(\text{Enzyme activity at time } (t))}{(\text{Enzyme activity at time } (t_o))} \times 100$$

2.8.4 Effect of metal ions on free and immobilized chitinase

The activity of free, entrapped and silica gel-immobilized chitinase was measured using standard assay method in the presence of K^{+}, $Mg^{+2,}$ Co^{+2},Ca^{+2}, Hg^{+2}, Cd^{+2} and Ni^{+2}. These cations were tested as metal chloride at 5 mM.

2.8.5 Reusability of entrapped and silica gel-immobilized chitinase

The activity of immobilized enzyme (entrapped and silica gel-immobilized chitinase) was tested throughout 8 cycles followed by measuring the enzyme activity. The relation between cycle number and residual activity was plotted.

2.8.6 Desorptivity of entrapped and silica gel-immobilized chitinase

SDS was used at different concentrations (0.4, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8 w/v%) followed by determination the activity of entrapped and silica gel immobilized enzyme.

3. RESULTS AND DISCUSSION

3.1 Purification of Chitinase from *A. infectoria*

Chitinase was purified by ammonium sulphate (85%), DEAE-Cellulose and then Sephadex G-200. The final specific activity was 51.9 U mg^{-1} protein and 1730-fold. The obtained results were in Table 1. The elution profile (Fig. 1) indicated that the highest chitinase activity was recorded in fraction number 10. The purity of the enzyme was confirmed by SDS-PAGE (Fig. 2).

Table 1. Purification of chitinase from *A. infectoria*

Fraction	Total protein (mg)	Total activity (U)	Specific activity (Umg^{-1})	Yield (%)	Fold of Purification
Crude extract	271.4	820	3.0	100	100
Ammonium Sulphate precipitation (85%)	50.2	620	12.4	75.6	413
DEAE- Cellulose	12.4	350	28.2	42.7	940
Sephadex G-200	5.2	270	51.9	32.9	1730

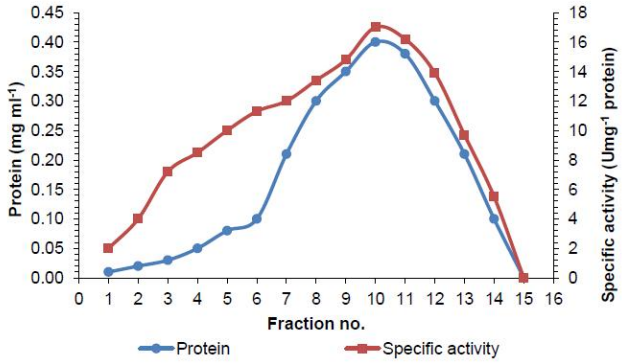


Fig. 1. Profile of purified chitinase from *A. infectoria*

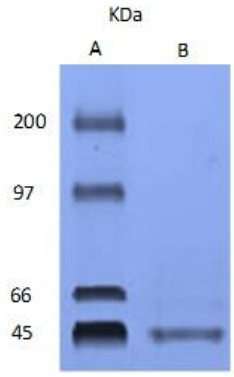


Fig. 2. SDS-PAGE for purified chitinase enzyme. A: markers; B: Sample.

3.2 Immobilization of Chitinase on Different Beads

Immobilization of an enzyme aimed to increase its stability to various deactivating agents due to restricted conformational mobility of the molecules. The immobilized enzyme could act under harsh conditions and losing little activity as compared to its free form [19,20].

Chitinase was immobilized on calcium alginate, agar-agar and silica gel. The immobilization efficiency (%) was calculated for the enzyme from each bead. The results in Fig. 3 indicated that Ca-alginate was the best bead compared to silica gel and agar-agar, where the immobilization efficiency was 88%.

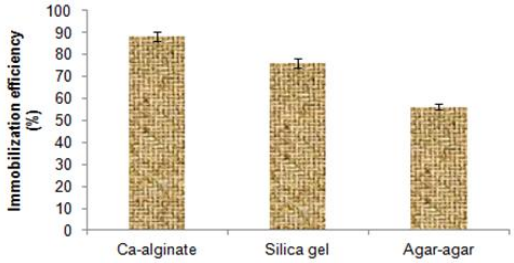


Fig. 3. Immobilization of chitinase on different beads

3.3 Saturation Curves of free and Immobilized Chitinase Activity from *A. infectoria*

The rate of chitinase reaction and the chitin concentration were consistent with the typical enzyme reaction [21]. The results were shown in Fig. 4 and indicated continuous increase in chitinase activity with increasing chitin concentration. The free enzyme expressed the higher activity compared to the immobilized form. The entrapped chitinase was higher than silica gel-immobilized enzyme.

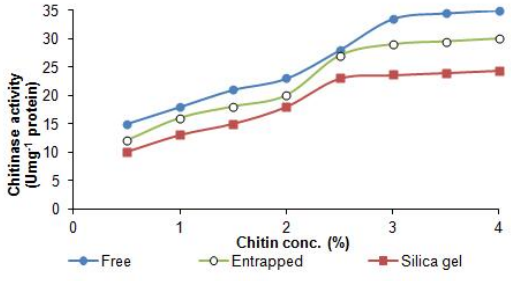


Fig. 4. Relationship between chitin concentration and the activity of free, entrapped and silica gel-immobilized chitinase from *A. infectoria*

3.4 Effect of pH on free and Immobilized Chitinase Activity from *A. infectoria*

Generally, changes in pH affect the enzyme activity. The pH can influence the enzyme activity in a number of ways: 1) it changes the ionization of enzyme substrate complex; 2) it changes the ionization of different groups in the enzyme which may affect its affinity to the substrate; 3) it changes the ionization of the substrate that may affect the binding process with the substrate and 4) it changes the protein structure [22]. At extreme pH, the tertiary structure of enzyme may be affected and possibly the protein is denatured [23].

The results in Fig. 5 indicated continuous increase in the chitinase activity with increasing the pH up to 7, 8 and 9 for the free, silica gel-immobilized and entrapped enzyme, respectively. After the optimal pH there was continuous reduction in the enzyme activity for three forms of chitinase. Other studies showed that the optimal pH values for free chitinase from *Aspergillus terreus* were 5 and 6 [5].

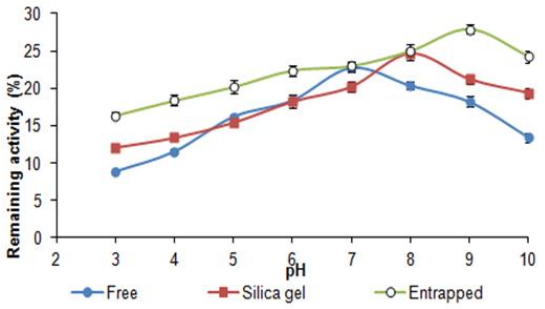


Fig. 5. pH optima of free, entrapped and silica gel-immobilized chitinase from *A. infectoria*

3.5 Effect of Incubation Temperature on Free and Immobilized Chitinase Activity from *A. infectoria*

The results in Fig. 6 indicated continuous increase chitinase activity with increasing the incubation temperature up to 40, 50 and 60°C which were the optimal temperatures for the free, silica gel-immobilized and entrapped enzyme, respectively. After the optimal temperature for each form of enzyme, there was reduction in the enzyme activity of the free and immobilized enzyme. Other studies showed that the optimal temperature for free chitinase from *Aspergillus terreus* was 50°C [5].

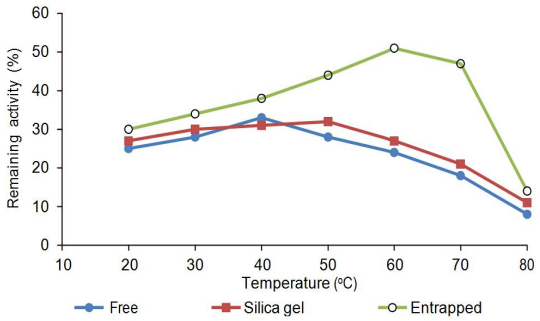


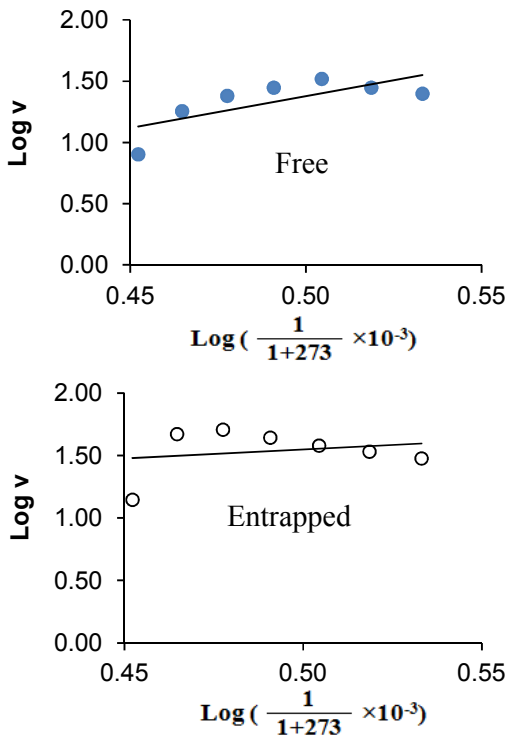
Fig. 6. Optimal temperatures of free, entrapped and silica gel-immobilized chitinase from *A. infectoria*

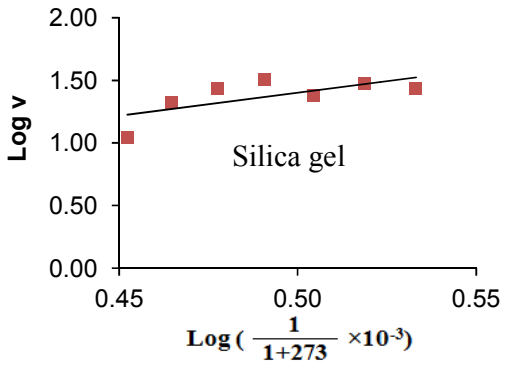
3.6 Arrhenius Plot of Free and Immobilized Chitinase from *A. infectoria*

Plotting log v against log [1/ (t+273) × 10^{-3}] represented Arrhenius plot. Therefore, the relation between log v against Log [1/ (t+273) × 10^{-3}] for free, entrapped and silica gel-immobilized enzyme was investigated. The results in Fig. 7 showed straight lines with slopes of 5.2, 3.7 and 1.4 for the three forms of the chitinase mentioned above in the same order. The calculated activation energy values were 43.2, 30.8 and 11.6 KJ mol^{-1} for the free, silica gel-immobilized and entrapped enzyme, respectively.

3.7 Thermostability of Free and Immobilized Chitinase from *A. infectoria* at 60°C and 70°C

The inactivation of chitinase at higher temperature over the optimal could be attributed to hydrolysis of the peptide chain, aggregation, incorrect confirmation or destruction of amino acids [24]. At high temperature, it was possible that enzymes could be inactivated by aggregation at hydrophobic sites [25]. Unfolding of protein at high temperature occurred due to breaking of the hydrogen bonds as well as the other interactions which maintain the tertiary structure. The orientation of the residual groups in the protein was lost, and interaction between the water molecules and exposed regions may take place causing denaturation of the enzyme protein [26].





	y=mx+b	slope	Ea
Free	y=5.2x-1.22	5.2	43.2
Silica gel	y= 3.7x-0.45	3.7	30.8
Entrapped	y= 1.4x+0.84	1.4	11.6

Fig. 7. Arrhenius plot for free, entrapped and silica gel-immobilized chitinase from *A. infectoria*

In this experiment, the thermostability of free chitinase was studied at 60°C and 70°C and the residual activity was calculated. It is apparent from the results in Figs. 8 and 9 that thermostability at 60°C was higher than that at 70°C. Also, it was observed that entrapped enzyme was the most thermostable throughout the experimental period compared to silica gel-immobilized enzyme and the free one. On the other hand, the chitinase produced from *Penicillium oxalicum* was fairly stable below 45°C [27].

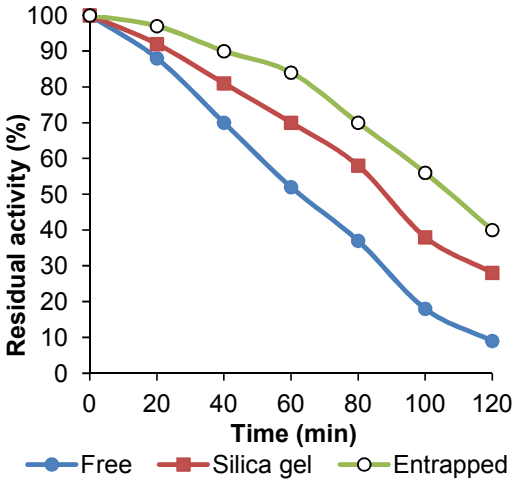


Fig. 8. Thermostability of free and immobilized chitinase from *A. infectoria* at 60°C.

The higher thermostability of immobilized chitinase could be explained on the basis that the carrier protects the enzyme at higher temperature at which deactivation occurred. The immobilization of an enzyme increase its rigidity, which was reflected by increasing its stability against thermal denaturation [28,29]. Immobilization of chitinase on alginate and silica gel was supposed to retain the tertiary structure from conformational changes under harsh conditions.

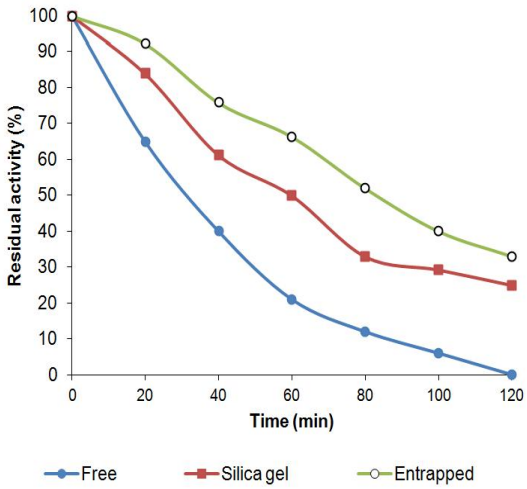


Fig. 9. Thermostability of free and immobilized chitinase from *A. infectoria* at 70°C

3.8 Effect of Metal Ions on Free and Immobilized Chitinase Activity from *A. infectoria*

In the present investigation Ca^{+2} activated chitinase activity whereas K^{+} and Mg^{+2} did not show any remarkable effect. However, Co^{+2}, Ni^{+2}, Cd^{+2}, and Hg^{+2} inhibited chitinase activity (Fig. 10). The inhibition of chitinase by Hg^{+2} indicated

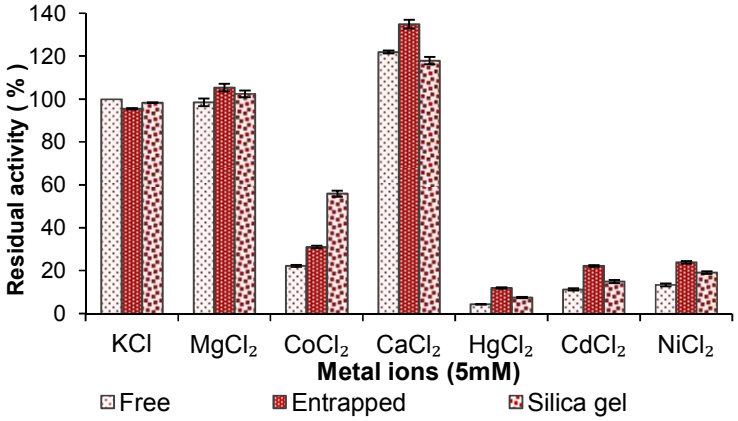


Fig. 10. Effect of metal ions on free and immobilized chitinase from *A. infectoria*

the essentiality of –SH group for enzyme catalysis [30]. Chitinase from *Enterobacter* spp. was activated by Ca^{+2}, K^{+} and Mg^{+2}, while it was strongly inhibited by Hg^{+2} and Co^{+2} [31]. Bhushan et al. [32] reported that Ca^{+2} activated the enzyme up to 20%.

Chitinase activation by Ca^{+2} could be attributed to strengthening of instruction within the molecules and binding to autolysis site [33,34]. Hg^{+2} inhibited the activity up to 50%. Also, Co^{+2} was an inhibitor for chitinase activity as reported by Dahiya et al. [31].

3.9 Reusability of Entrapped and Silica Gel-immobilized Chitinase from *A. infectoria*

The results in Fig. 11 indicated that the immobilized enzyme could be reused particularly the entrapped enzyme which expressed higher activity than silica gel-immobilized enzyme. This was important for application in the industry for reducing the cost.

3.10 Desorptivity of Entrapped and Silica Gel-immobilized Chitinase from *A. infectoria*

The results in Fig. 12 revealed that the immobilized enzyme on silica gel was desorbed easily than the entrapped chitinase.

SDS is well known as denaturant of proteins and could unfold most proteins through the interaction between the charged head groups of SDS and amino acid chains of proteins with positive charges, in addition to the interaction between alkyl chains of SDS and non-polar parts on the surface [34]. The reduction in the activity could be attributed to conformational changes in the enzyme molecule which rendered the enzyme not active [35]. Treatment of the enzyme with higher concentration of SDS resulted in formation of small charged domains in the protein and repelled each other and this caused denaturation of the enzyme protein [36].

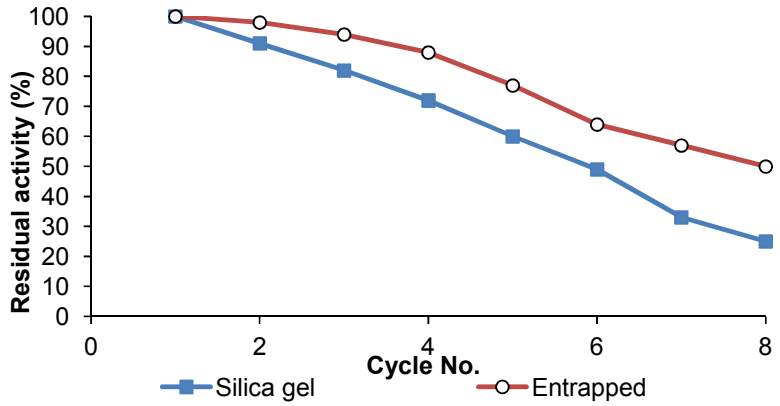


Fig. 11. Reusability of entrapped and silica gel-immobilized chitinase from *A. infectoria*

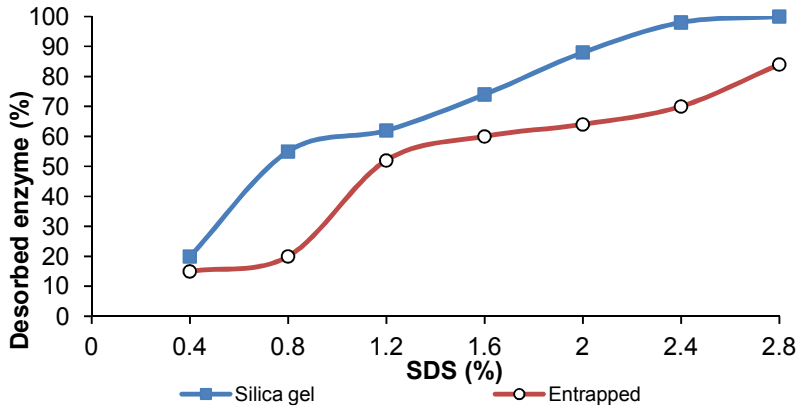


Fig. 12. Desorptivity of entrapped and silica gel-immobilized chitinase from *A. infectoria*

4. CONCLUSION

The results of our work showed successful method for chitinase isolation as well as methods of immobilization. The immobilized chitinase expressed a higher thermostability compared to the free one at 60°C and 70°C. This thermostability is important for industrial's application.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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