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Covalent Immobilization of *Dothideomycetes* sp. NRC-SSW Chitosanase and Its Application in Chitosan Hydrolysis

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Abstract

Chitosanases are chitosan specific glycosyl hydrolases that can exploit in several biotechnological applications. In the current study, a modified bead of carrageenan and carboxymethyl cellulose was used for the covalent immobilization of *Dothideomycetes* sp. NRC-SSW chitosanase. The immobilization process was optimized and the immobilization recovery was increased up to 69%. The stepwise different bead formulations were examined under Scanning Electron Microscope, Fourier Transform Infrared Spectroscopy and Thermal Gravimetric analysis, confirming the success of the immobilization process. Additionally different properties including enzyme storage, thermal and pH stabilities were examined and an improvement in all of these properties was estimated. Although the same V_{max} value (23.8U/min/mg protein) was recorded for the free and the immobilized enzymes, the K_m was 10mg chitosan/ml and 16.67mg chitosan/ml for the immobilized and free form respectively. The activation energy for chitosan hydrolysis using the immobilized enzyme (12.497kJmol^{-1}) was less than the third of that recorded for the free one (42.196kJmol^{-1}). Furthermore, the immobilized enzyme was stable until the 5th cycle at which it retained more than 50% of its original activity. All the tested parameters confirmed that the immobilized *Dothideomycetes* sp. NRC-SSW chitosanase was more applicable for chitosan hydrolysis than the native enzyme.

Keywords: Chitosanase; *Dothideomycetes* sp. NRC-SSW; immobilization; chitosan hydrolysis

1. Introduction

Chitosan is a β-(1-4)-linked D-glucosamine linear polysaccharide with less than 20% randomly distributed N-acetyl-D-glucosamine units that commercially prepared by the deacetylation of chitin, a natural biopolymer exists in nematodes, fungi, crustaceans and other invertebrates. Chitosan possesses various biological activities as antimicrobial, anticancer and antioxidant that attributed to its different types of reactive function groups, an amino/acetamido group, primary and secondary hydroxyl groups at C-2, C-3 and C-6 positions respectively. Moreover it is a biopolymer extensively used in various food, pharmaceutical and cosmetic industries but its high molecular weight and poor water solubility are disadvantages that limited some of its applications. Its hydrolysis not only serves as a solution for these drawbacks but it delivers various biologically active compounds including glucosamine, N-acetyl-glucosamine and chitooligosaccharides [1]. Chitooligosaccharides are water soluble compounds prepared by the partial de-polymerization of chitosan or its acetylated derivative (chitin). They have been reported to possess several biological activities including prebiotic [2], antioxidant [3], anti-inflammatory and immuno-modulatory [4], anticancer [5], antibacterial [6], hypolipidemic [7] and antifungal effects [8, 9].

Chemical hydrolysis was the commercial applied method for chitosan de-polymerization but in the last few years the use of enzymes has been suggested as an alternative method from the perspective of employed mild conditions, environmental protection and high yield of the resulted product. The cost, availability and specificity of the applied enzyme are the main variables that have to be considered for the

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industrial applications of chitosan enzymatic hydrolysis [10-13]. Chitosanases are the specific chitosanolysis enzymes. Cost effective technology for the enzymatic hydrolysis of chitosan urged the efforts for the preparation of highly active and stable chitosanases, reflecting the importance of the immobilization of the produced enzymes [14-17].

Immobilization is an efficient strategy for enzymes stabilization and protection in addition to the improvement of their properties with their potential reuse. Various techniques have been reported to be used for enzyme immobilization such as entrapment, cross linking, adsorption and covalent binding [18]. Covalent binding is the most preferred method in the industrial application of enzymes as the leakage of the enzyme is reduced by strengthening of the bond between the carrier and the enzyme [19]. Covalent binding immobilization of chitosanolytic enzymes has been previously studied using silica [17, 20], agarose [14, 21], poly-acrylonitrile [22] and silica-coated magnetic nanoparticles [16].

The present study examined the immobilization of *Dothideomycetes* sp. NRC-SSW chitosanase by covalent binding to micro-carrier prepared by using a mixture of carrageenan and carboxymethyl cellulose (CMC) followed by studying the immobilization process optimization. Moreover, the enzyme thermal and operational stabilities (reusability and storage stability) as well as its kinetic constants were estimated. Finally, chitosan hydrolysis using the immobilized enzyme was examined.

2. Experimental

2.1. Materials

Chitosan of different molecular weights (medium and low), glucosamine, carrageenan and carboxymethyl cellulose were obtained from Sigma-Aldrich, Saint Louis, USA. Silica gel plates were purchased from Merck, Darmstadt, Germany. All other chemicals were of analytical or HPLC grade.

2.2. Chitosanase production

Crude chitosanase used in this study was prepared as previously described by Hashem *et al*., [5] under submerged fermentation using *Dothideomycetes* sp. NRC-SSW (accession no. **LC467498**). Briefly, the fermentation was performed using the optimized medium composed of (g/L) chitosan (medium molecular weight), 30; K_2HPO_4, 1.5; $MgSO_4$, 0.4; KCl, 4.0; yeast extract, 18.5 and $FeSO_4$, 0.01 (pH 5.5) then incubated at 30 ºC for 4 days at 180 rpm. At the end of the fermentation period, the culture was centrifuged at 5000 rpm for 10 min.

2.3. Partial purification of chitosanase

Ethanol fractional precipitation was applied and the fractions resulted from 30-90 % ethanol concentration were obtained. Each fraction was assayed for chitosanase activity and protein content [5].

2.4. Chitosanase activity and protein content determination

The activity of the enzyme was determined in a reaction mixture of 500µL of the crude enzyme with 500 µL of 1 % soluble chitosan (pH 5), incubated for 30 min at 50 °C [23]. The total amount of reducing sugars was determined using DNS method [24]. One unit of the enzyme was defined as the amount of enzyme that released 1 µmol of D-glucosamine per minute under the conditions described above.

The protein content was determined using Lowry-Folin method against a bovine serum albumin standard [25].

2.5. Preparation of Carrageenan and carboxymethyl cellulose beads

Carrageenan and CMC at different ratios (2:1, 1:1, and 1:2) were used to form beads through dropping the solution of the mixture in 0.05 M ferric chloride ($FeCl_3$) using 100 µm nozzle of Innotech Encapsulator. The formed beads were left for 3h in $FeCl_3$ for hardening then treated with 4 % polyethylene imine (PEI) at pH 9.5 for 3 h. After washing the beads with distilled water, they were treated for 3 h with 2.5 % glutaraldehyde (GA). The beads in the final form washed thoroughly with distilled water then used in the next experiments.

2.6. Preparation of immobilized chitosanase

The enzyme (50 U) was added to 1 g of the above prepared beads and left overnight at room temperature. The unbound enzyme was removed by washing and the activity of the immobilized enzyme was determined by incubating the beads with chitosan (1 %) at 50 ºC for 30 min. After that, DNS method [24] was used to determine the amount of the released reducing sugars in the supernatant. The immobilization recovery was calculated as follow:

Immobilization recovery (%) = [I/A] x 100 Eq. (1)

where I is the immobilized enzyme (U/g beads) and A is the added enzyme (U/g beads).

2.7. Central composite design for optimization of the immobilization process

Statistical optimization using central composite design (CCD) with two variables (loading time X_1 and enzyme concentration X_2) was performed [26]. The second order polynomial function to correlate relationship between independent variables and response of immobilized units per gram beads was as follow:

$$Y_{Activity} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_1 \beta_2 X_1 X_2 \quad \text{Eq. (2)}$$

where $Y_{Activity}$ is the predicted amounts of chitosanase per gram beads (i.e., U/g beads). β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are quadratic coefficients, β_{12} is cross product coefficients.

2.8. Qualification of the immobilization process

The beads after each formulation step were subjected to Fourier transform infrared analysis (FT-IR) using FTIR-8300, Shimadzu, Japan and thermal gravimetric analysis (TGA) using PERKIN ELMER TGA7 in addition to scan under scanning electron microscope using SEM, FEG250, Quanta.

2.9. Effect of pH and temperature

The effect of pH on the activity and stability of the immobilized and the free (partial pure) enzyme was investigated in pH range from 7 to 8 using 0.2 M Tris HCl buffer.

The activity of the immobilized and the free enzyme were investigated at the optimum pH at temperature range from 45 to 70 ºC. The activation energy (E_a) for the immobilized and the free enzyme was calculated from the slope of Arrhenius plot, as given in the following equation:

$$\text{Slope} = -E_a/R \qquad \text{Eq. (3)}$$

in which R is the gas constant.

Thermal stability of the free and the immobilized enzyme was investigated in absence of the substrate at different temperatures (35 to 50 ºC) for different intervals of time (15 to 120 min) then the residual activity of the free and immobilized enzyme was estimated at the optimum conditions previously specified for each enzyme.

2.10. Effect of chitosan concentration

The enzyme activity was determined using different chitosan concentrations ranged from 5-15 mg/ml at the optimal conditions previously specified for each enzyme. The maximal activities (V_{max}) and Michaelis-Menten constants (K_m) for the free and the immobilized chitosanases were calculated from Lineweaver-Burk plot.

2.11. Effect of different additives

The enzyme activity in the presence of different metal ions (Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Li^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, Zn^{2+}) and EDTA at a final concentration of 0.5 mM was examined. Different concentrations of the most effective metal ion were also investigated in concentration range from 0.1 to 10 mM.

2.12. Enzyme reusability

The reusability of the immobilized enzyme in chitosan hydrolysis was estimated by evaluating the amount of the released reducing sugars after each cycle. Additionally, the storage stability of the immobilized enzyme was evaluated by determining the activity of the enzyme every week for 1month after its immersion in distilled water and storage at 4 ºC.

2.13. Catalytic efficiency of the immobilized enzyme

2.13.1. Chitosan hydrolysis

The efficiency of the immobilized chitosanase in the hydrolysis of chitosan in compare to the partial pure enzyme was estimated in a reaction mixture with enzyme/substrate ratio of 0.05 U/mg incubated at 50 ºC for 5 h [5].

2.13.2. Evaluation of the hydrolysis products

Chitosan hydrolysis products were analyzed using thin layer chromatography (TLC) in which propanol: water: ammonia (7: 2: 1 v/v) was used as the mobile phase [27] then visualization was performed using diphenyl amine-aniline spraying reagent [28]. Additionally, high performed liquid chromatography (HPLC) was carried out using glucosamine as a standard.

3. Results and discussion

3.1. Enzyme activity

Chitosanases are specific glycosyl hydrolases for chitosan hydrolysis [29]. The genotype NRC-SSW of *Dothideomycetes* sp. was reported in our previous study to be a good producer of chitosanase [5]. It produces an extracellular chitosanase activity of 21.5 U/ml in the presence of chitosan as inducer with a specific activity of 3.4 U/mg protein as the total protein content was 6.3 mg/ml.

3.2. Partial purification

Ethanol precipitation process of the produced enzyme at 60 % ethanol concentration resulted in 4.91 fold increase in the specific activity with 47.16 % of the recovered activity. However, this fraction lost most of its activity by either air drying or lyophilization and even by refrigeration for more than 24 h. Reuse, easy and storage stability are the real domains for the biotechnological industrial application of enzymes. Immobilization is one of the biotechnological processes that used to satisfy one or all of these urged needs [15, 17, 30]. Therefore in the current study, immobilization was examined to overcome the un-stability storage problem of *Dothideomycetes* sp. NRC-SSW chitosanase. The fraction precipitated at ethanol concentration of 30-70 % was used in the immobilization process. The specific activity of this fraction was 8.3 U/mg protein.

3.3. Enzyme immobilization on carrageenan-carboxymethyl cellulose beads

Covalent immobilization of the produced chitosanase was carried out using Carrageenan: CMC beads at different ratios. Low immobilization recovery (almost 4 %) was observed for all the formulations but it was noticed that the carrier in the ratio 2:1 (Carrageenan: CMC) was harder than the others and the unbound enzyme activity disappeared. The low immobilization recovery may be resulted from the loss of the enzyme during the immobilization process and/or the binding of the enzyme may take place by the way that inhibited its active sites. Santos-Moriano *et al*., [14] explained this effect by the hiding of the enzyme active sites during the immobilization process. In our attempt to overcome the low immobilization recovery, the addition of chitosan (enzyme substrate) during the enzyme loading was studied and the result indicated that the immobilization recovery was increased by about 4 times. A gradual increase in the immobilization recovery was achieved by the addition of different amounts of chitosan up to 0.03 g chitosan / gram of beads as shown in Fig. 1.

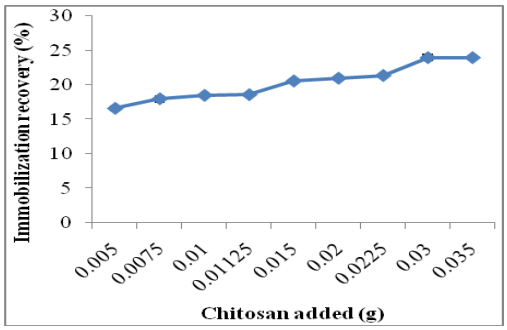

Fig. 1. Effect of addition of different concentration of chitosan.

The immobilization process was optimized using CCD for two variables, loading time (X_1) and enzyme concentration (X_2). The results (Table 1) clarify that the conducted optimum condition was the loading of 10 U/g beads for one hour. These conditions increased the immobilization recovery to 69 % with 12.6 U/mg protein specific activity. The increase in the loading enzyme concentration did not improve the immobilization recovery that may be attributed to the increase in the enzyme on the beads surface that hamper the diffusion of the substrate to the active sites of the enzyme [17, 31]. The immobilization recovery in this study was higher than 25 % reported by Santos-Moriano *et al*., [14] but similar to that reported by Sinha *et al*., [22]. The R^2 value (0.926) estimated by the multiple regression analysis of the results (Table 2) indicated that the statistical model can explain 92.6 % of the variability in the response. Edwards *et al*., [32] reported that the accuracy of the applied model can be confirmed from its R^2 value (R^2 value > 0.9 indicated the accuracy of the model). Moreover, the accuracy of the applied model was confirmed from the low value (7.27 %) of the absolute average deviation (AAD) that calculated as follow:

$$AAD = \{[\sum_{i=1}^{p}(|Yexp - Yprd|/Yexp)]/P\} \times 100 \quad \text{Eq. (4)}$$

where P, Yexp and Yprd are the number of the experiment, observed and predicted immobilized chitosanase activity respectively. The suitable values of R^2 and AAD indicated that the applied model depicts the correct behavior and it applied successfully in the optimization process [33].

The results of the analysis of variance (ANOVA) of the regression model demonstrated the significant of the model, as an evident from the high F value (17.518) and the low P value (0.0008). The second-order regression equation provided the levels of the enzyme activity as a function of loading time (X_1) and amount of loading units (X_2) was:

$$Y_{Activity} = -0.0573 - 0.0283X_1 + 0.7755X_2 - 0.0005X_1^2 - 0.0122X_2^2 - 0.0016X_1X_2 \quad \text{Eq. (5)}$$

where $Y_{Activity}$ is the predicted amount of chitosanase per gram beads (U/g beads).

The three-dimensional response surface has been plotted on the base of the conducted model equation to investigate the interaction among the variables as shown in Fig. 2 and the residual analysis (Fig. 3) indicated that the residuals were symmetrically and constantly spread throughout the response range, ensuring that the model is correct on average for all the observed results. All the above results reflected the accuracy and applicability of the central composite design for the optimization of chitosanase immobilization process. Central composite design was used previously for optimization of enzyme loading [34-36].

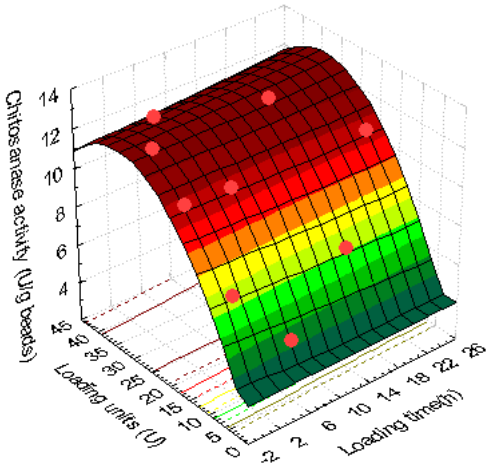

Fig. 2. The effect of the enzyme concentration and the immobilization time on the immobilized chitosanase activity.

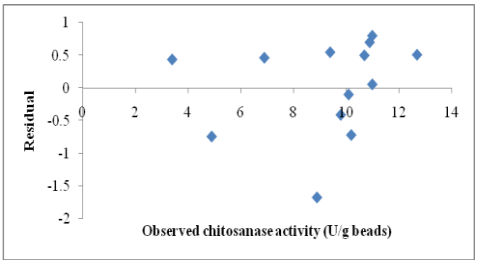

Fig. 3. Residual plot.

3.4. Fourier Transform Infrared (FTIR)

The beads at the steps of preparation, activation and immobilization were subjected to FT-IR analysis in the wavelength region of 400-4000 cm^{-1} (Fig. 4). The spectra of the aminated beads showed a broad peak that corresponding to amine group at 3427 cm^{-1}, indicating that the beads become aminated and ready for activation as shown in curve (B). While these beads after activated with glutaraldehyde showed two new peaks. The first characteristic one was at 1640 cm^{-1} that referred to ketone group (C=O) that found naturally in glutaraldehyde and the another peak was at 1455 cm^{-1} that is related to (C=N-) group that resulted from the interaction that occurred between amine group (NH_2) of the aminated beads and (C=O) group of glutaraldehyde as shown in curve (C). On the other hand, the curve (D) showed increase in amine peak that indicated the increment in the amine concentration which found naturally in the enzyme. All these results ensured that the enzyme was covalently immobilized to the beads. These results were in agreement with the other published results [37].

3.5. Thermal Gravimetric analysis (TGA)

The results of the thermal gravimetric analysis and the temperature at which 50 % of the beads have been degraded (T_{50} %) during the different immobilization steps were illustrated in table (3). The gradual increase in the temperature resistance of the formed beads clarified the success of the immobilization process. The TGA of Carrageenan-CMC beads was 119 °C compared to aminated one that was 209 °C and activated one that was 246 °C. However it became 288 and 477 °C after immobilization. This obvious improvement could be explained by the interaction between poly-anions (-COO-) of the carrier and poly-cations ($-NH^{3+}$) of PEI. Activation using GA showed further increase in TGA that could be attributed to the formation of strong covalent bonds due to Schiffs base formation between the free amine group of PEI and GA. These results were in agreement with other published results [35].

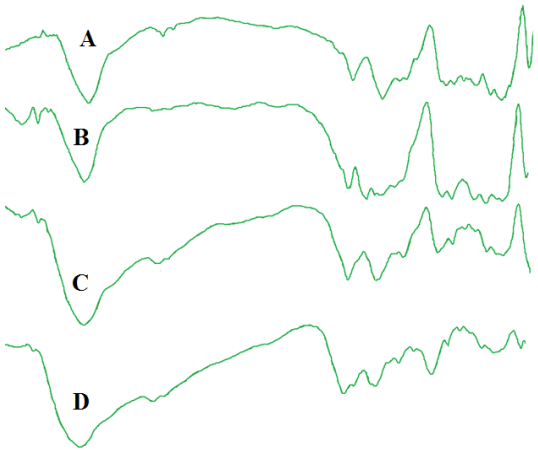

Figure (4): FT-IR spectra of Carrageenan-CMC beads (A), aminated beads (B), activated beads (C) and immobilized one (D).

3.6. Scanning Electron Microscope

The results shown in Fig. (5) clarified the changes in the beads surface after each step of the immobilization process, for example in (A) the surface was not smooth and had some aggregates and these aggregates increased the surface area for more reaction with PEI, in (B) the accumulation of amine particle on the surface of the beads covered all of these aggregates. Also as shown in (C) the aggregated particles increased indicating the accumulation of GA and the beads become ready to react with amine groups found in the enzyme as shown in (D). This result agrees with other published result [38].

3.7. Effect of pH and temperature

It is well known that the pH and the temperature are important variables that influence the enzyme activity. The pH can either change the ionization of the enzyme-substrate complex or it can alter the protein structure of the enzyme [39]. The results shown in Fig. 6 indicated that the immobilization slightly shifted the optimum activity of the enzyme from pH 7.2 to 7.5. Additionally, the immobilized enzyme retained 100 % of its activity at pH 7.5 and retained more than 80 % of its activity at pH 8 for 1 h. Similar results have been conducted by Wang *et al*., [40] using a combination of sodium alginate and cellulose as a composite carrier for the immobilization of *Pseudomonas* sp. CUY8 chitosanase.

Table 1. CCD for the immobilization of chitosanase

Trial	X_1 (h)	X_2 (U/g beads)	Experimental chitosanase activity (U/g beads)	Immobilization recovery (%)	Predicted chitosanase activity (U/g beads)
1	-1(1h)	-1(10)	6.9	69.0	6.4
2	-1(1h)	+1(30)	12.7	42.3	12.2
3	+1(16h)	-1(10)	4.9	49.0	5.6
4	+1(16h)	+1(30)	10.2	34.0	10.9
5	-∞(1/4h))	0(20)	8.9	44.5	10.6
6	+∞(24h)	0(20)	9.4	47.0	8.9
7	0(6h)	-∞(5)	3.4	68.0	3.0
8	0(6h)	+∞(40)	11.0	27.5	10.9
9	0(6h)	0(20)	9.8	49	10.2
10	0(6h)	0(20)	10.7	53.5	10.2
11	0(6h)	0(20)	10.1	50.5	10.2
12	0(6h)	0(20)	11.0	55	10.2
13	0(6h)	0(20)	10.9	54.5	10.2

Table 2. Analysis of CCD

Term	Regression coefficient	Standard error	t- test	P-value
Intercept	-0.05734	1.636929	-0.03503	0.973035
X_1	-0.02833	0.184866	-0.15326	0.882517
X_2	0.775478	0.123393	6.284623	0.00041
X_1^2	-0.00051	0.005748	-0.08905	0.931538
X_2^2	-0.01215	0.002478	-4.9041	0.001745
X_1X_2	-0.00159	0.006079	-0.26157	0.801181

Summary of the model	
Multiple R	0.962288
R^2	0.925998
Adjusted R^2	0.873139
Standard Error	0.94001

Table 3. TGA and $T_{50\%}$ data

Type	TGA (ºC)	$T_{50\%}$ (ºC)
Carrageenan-CMC	119	347
Carrageenan-CMC + PEI	209	361
Carrageenan-CMC + PEI +GA	246	371
Carrageenan-CMC + PEI +GA+Enzyme	288 & 477	451

It was quite clear from Fig. 7 that the immobilization had no effect on the optimal activity of the enzyme; 60 ºC was indicated for both forms. No variation in the optimum temperature between the immobilized and the free chitosanase reported by Song *et al*., [20], suggesting that the temperature did not affect the activity of the immobilized enzyme since it did not depend on the changes in the folding structure of the enzyme that affected its potent solubility. While Wang *et al*., [40] reported that the immobilized enzyme had optimum temperature lower than that of the free enzyme.

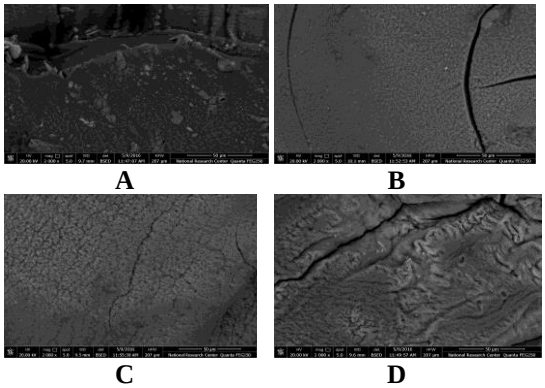

Fig. 5. SEM of Carrageenan-CMC beads (A), aminated beads (B), activated beads (C) and immobilized one (D).

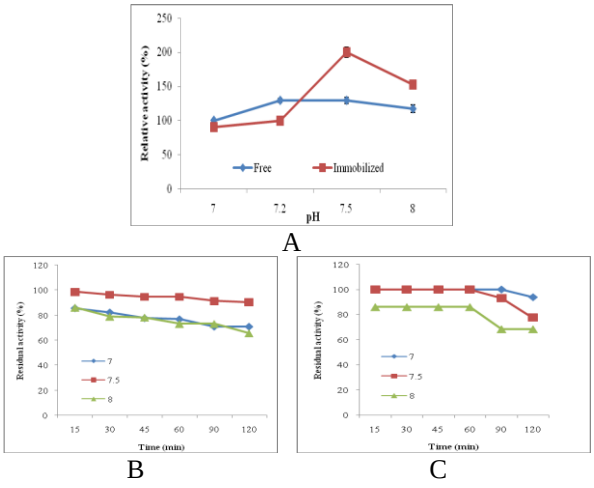

Fig. 6. The optimum pH profile for the free and the immobilized enzyme (A) at which the control is pH 7. The pH effect on the free (B) and immobilized (C) enzyme for different incubation periods.

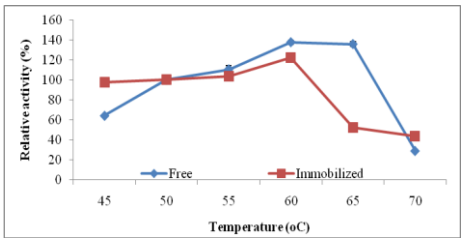

Fig. 7. The temperature profile of the free and the immobilized enzyme (50°C is the control).

Thermal stability profile (Fig. 8) indicated that the immobilization process improved the enzyme stability at temperature ranged from 40 °C to 45 °C with a little effect at 50 °C. The results indicated that the immobilized enzyme retained 74 % of its activity after 2 h at 40 °C in compare to 34 % for the free enzyme and at 45°C the immobilized enzyme retained 52% of its activity after 1 h in compare to 34 % for the free enzyme. Qin *et al.*, [41] reported that chitosanases are temperature sensible, its denaturation starts at 30 °C. de Medeiros Dantas *et al.*, [17], Song *et al.*, [20]), Wang *et al.*, [40] and Wang *et al.*, [16] reported that covalent immobilization of chitosanases improved the stability of the enzymes in compare to the free forms, being in agreement with the results of the current study.

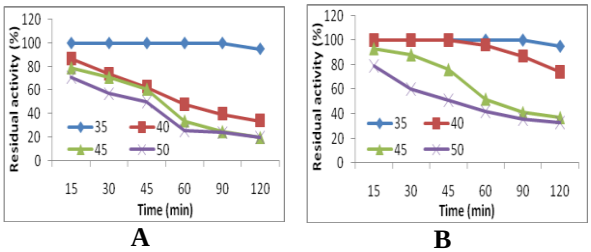

Fig. 8. Thermal stability of the free (A) and immobilized (B) enzyme.

The effect of the immobilization on the enzyme activation energy (E_a) was studied by Arrhenius plot (Fig. 9). The calculated E_a indicated that the energy required to form the enzyme-substrate complex for the free enzyme (42.196 kJmol^{-1}) was 3.3 times higher than that required for the immobilized one (12.497 kJmol^{-1}). This criterion makes the immobilized enzyme more suitable for its industrial applications hence it needs lower activation energy which will be reflected on the total cost of the industrial processes.

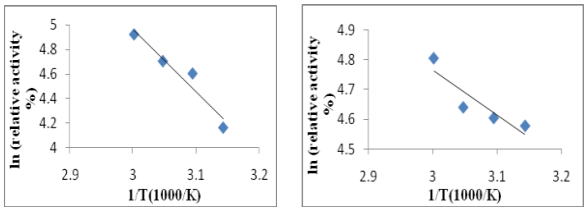

Fig. 9. Arrhenius plot for the free (A) and the immobilized (B) enzyme.

3.8. Kinetic parameters

According to Lineweaver-Burk plot (Fig. 10), the maximal activity (V_{max}) and Michaelis-Menten constant (K_m) for both the free and immobilized enzymes were calculated. The V_{max} (23.8U/min/mg protein) was found to be the same for both forms (free and immobilized) while the K_m was 16.67 and 10mg chitosan/ml for the free and the immobilized enzyme respectively. The lower the K_m value indicated the higher affinity of the enzyme towards the used substrate that may be attributed to the electrostatic interaction between the carrier and the substrate [20, 40].

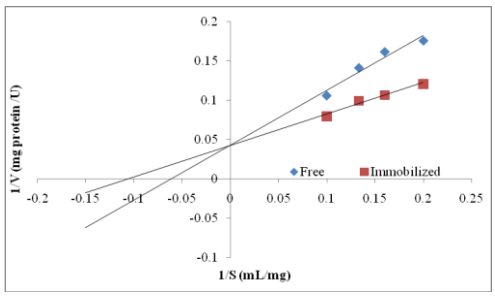

Fig. 10. Lineweaver-Burk plot.

3.9. Effect of metal ions on free and immobilized enzyme

The effect of the addition of various metal ions in the reaction mixture of the free as well as the immobilized enzyme was studied. It was indicated that Mn^{2+} was a strong activator as it increased the chitosanase activity of the free and the immobilized enzyme by 142.6 and 29.7% respectively while EDTA and Cu^{2+} decrease the activity of the free enzyme by 76.4 and 77% respectively without affecting the immobilized enzyme (Fig. 11A). By

examining the effect of different concentrations of Mn^{2+} the results demonstrated that the activity reached its maximum at 5mM concentration for both of the free (214.2%) and the immobilized (150.7%) enzyme (Fig. 11B). It has been reported that metal ions had an important role in the enzyme catalytic mechanism and/or act as a stabilizer to the active site of the enzyme. When participating in the catalytic mechanism, metal cations can bring specific functional group together in the appropriate orientation for the reaction. The different effect of cation upon catalytic action of the enzyme may be due to their ability to adopt different geometrics in the same site in the absence of substrate [42]. The stimulatory effect of Mn^{2+} on chitosanases was also reported by other authors [43-45].

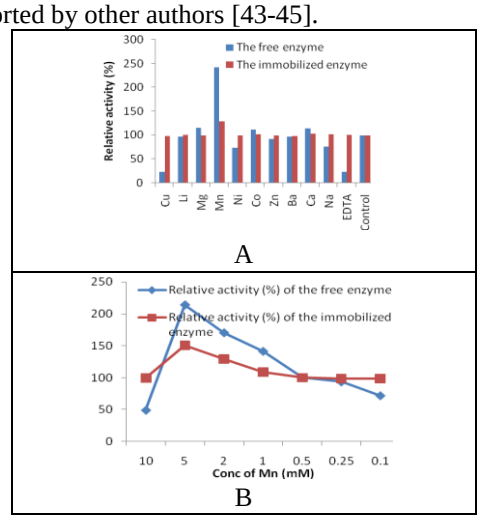

Fig. 11. The effects of the addition (A) of various metal ions and EDTA (B) different concentrations of Mn (concentration of 0.5mM is the control) on the activity of the free and the immobilized enzymes.

3.10. Enzyme reusability

The prepared immobilized enzyme after the 2nd cycle preserved 78% of its initial activity and retained more than 50% of its activity after the 5th cycle. The dropping in the activity may be attributed to the blocking of the binding active sites of some of the enzyme by the released sugars during the hydrolysis process [20] or the leakage of the enzyme from the carrier [46]. It is worthy to mention that the immobilized enzyme showed improved storage stability as it retained 100% of its activity for more than one month at 4°C otherwise it dried.

3.11. Chitosan hydrolysis

The efficiency of the immobilized *Dothideomycetes* sp. NRC-SSW chitosanase in chitosan hydrolysis was examined and the hydrolysis reaction mixture was analyzed by TLC (Fig. 12) and HPLC (Fig. 13). The results of TLC analysis indicated that the immobilized enzyme revealed the same activity to that reported for the free enzyme [5]. Additionally, HPLC analysis confirmed the hydrolysis of chitosan without the detection of glucosamine, suggesting the endo-acting activity of the enzyme. The retention time for glucosamine was 3.061min.

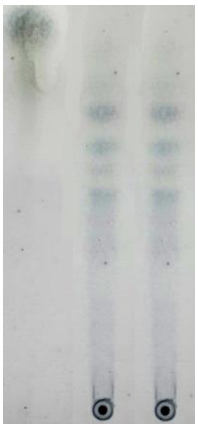

Fig. 12. TLC analysis (Lan 1: standard glucosamine. Lan 2: hydrolysis product using free enzyme. Lan 3: hydrolysis product using immobilized enzyme).

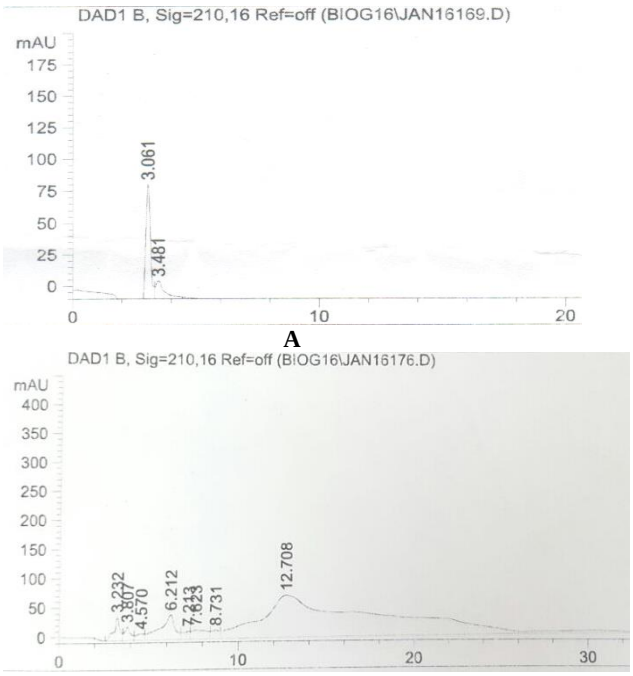

Fig. 13. HPLC analysis of (A) standard glucosamine and (B) COS mixture.

4. Conclusion

In the current study, covalent binding immobilization of *Dothideomycetes* sp. NRC-SSW chitosanase using carrageenan-CMC beads in the ratio 2:1 has successfully achieved. The immobilized enzyme possessed higher storage and thermal stability than the free form. Additionally, it possessed lower activation energy and higher affinity for chitosan hydrolysis. Furthermore, it was successively reused for more than 5 cycles with high retaining activity. Moreover, it revealed the same pattern for chitosan hydrolysis similar to the free enzyme. These criteria confirmed the success of the immobilization process producing immobilized chitosanase

applicable in chitooligosaccharide biotechnological industry.

5. Conflict of interest

There are no conflicts to declare.

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