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Kinetic and Stability Improvement of Immobilized Pepper Chitosanase on Chitin by Covalent Bond

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Abstract: Kinetic properties and stability studies of pepper chitosanase immobilized on chitin by covalent bond for production of chitooligosaccharides was the main objective of this work. A comparison with the properties that shown by the free one was also carried out. Maximum enzyme activity was observed at 2.825 and 0.576 mg chitosan /reaction mixture for free chitosanase (FC) and immobilized chitosanase (IC), respectively. They followed Michaelis-Menten Kinetics. The values of the Michaelis-Menten constant indicated that IC exhibited higher affinity toward chitosan than that of the free one. Km value was 35.71 mg/reaction mixture for the IC and 17.86 mg/reaction mixture for the free one. The maximum velocities (V_{max}) were 12.5 and 47.62 U/mg for IC and FC, respectively. The optimum pH of IC was slightly shifted to acidic range (pH 5.6) from 5.8, which is the optimum for free one. IC exhibited high activity at wide range of temperature from 40 to 60ºC with optimum at 55ºC, while high activity of FC was found at range from 40 to 55ºC with optimum at 45ºC. The thermal and operational stabilities of both free and immobilized chitosanase were also evaluated. Results showed that the immobilization enhanced the enzyme stability. IC showed better thermal stability than the free one. It lost 5-10 % of its original activity after heating at 50°C for 1h, while FC lost 35 % after the same treatment. IC also lost 58% of its original activity after heating at 60°C for 1h, while free one lost 80 % after the same treatment. Results showed 22% rate of hydrolysis after 1 h and reached about 24 and 30 % after 2 and 24 h of hydrolysis. The end product of chitosan hydrolysis by the IC showed N-acetyl glucosamine and mixture of DP 2-6 oligomers. This improvement of the IC properties made it a potential candidate for future use in industrial application especially in chitooligosaccharides production.

Keywords: Chitosanase (EC 3.2.1.132), Pepper (*Capsicum annuum*) leaves, kinetic properties, operational and thermal stability

INTRODUCTION

Enzymes were used as catalyst to enhance the rate of the reactions at milder conditions of pH, temperature compared to chemical catalysts that require several conditions. They were co-friendly with respect to the chemical ones.

Improvement of enzyme properties via immobilization method was important in different fields. The main application of immobilized enzymes was the food and pharmaceutical industries [1-2]. Immobilization could improve functional properties such as stability of enzymes activity in harsh conditions. The immobilized enzymes could be also reused for several times. They could be easily recovered from the reaction medium [3-5].

Immobilized enzymes were defined as enzymes physically confined or localized in a certain defined region of space with increase their catalytic activities. Immobilization was mainly used when a reaction process did not require enzyme in the product. The properties of the immobilized enzyme were different from the free one. This was due to that the immobilized enzyme and the substrate react in the microenvironment, while the free enzyme and the substrate react in the bulk solution environment. This improvement of immobilized enzyme properties was also due to the change in the three dimensional conformation of the protein when linked with the support matrix. The stabilization of immobilized enzyme was depended on the number of bonds formed between the enzyme and the support matrix [6-7].

Chitosanases (EC. 3. 2. 1. 132) represented a class of hydrolytic enzymes. It catalyzed the β-1-4 glycosidic bond hydrolysis of chitosan to produce chitooligosaccharides. Most of the chitosanases from various sources reported endo-acting in nature. They were liberating predominantly mixture of dimers,

trimers and oligomers from chitosan, but exochitosanase acted on chitosan and releases D-glucosamine [8-9]. Chitosanases were used for production of bioactive chitooligosaccharides from chitosan.

As a part of our continuous search for preparation of new potent immobilized chitosanase, pepper leaves (*Capsicum* annuum) were chosen as the most suitable source for extraction chitosanase enzyme [10]**.** Pepper chitosanase was successfully immobilized on chitin by covalent binding technique [11]. The aim of this work was to provide relevant information for potential application at the industrial level of the immobilized chitosanase for chitooligosaccharides production. In the present work, we investigated the kinetic properties and the operational stability of immobilized chitosanase enzyme and comparing them with those for the free enzyme.

MATERIALS AND METHODS

MATERIALS

Chitosan with an average molecular of weight 300,000 KDa and 70-85 % deacetylated, chitin, N acetyl-glucosamine were purchased from Merck chemical Cò. Fresh pepper (*Capsicum annuum*) leaves were collected from the field. Immobilized pepper chitosanase was prepared in our laboratory. All other chemicals were of analytical grade.

METHODS

Preparation of chitosan so solution Soluble chitosan for determining chitosanase activity was prepared as follows: ten grams of chitosan powder was suspended in 400 ml distilled water and dissolved while being stirred in 5ml concentrated acetic acid. This solution was made with up to 1 L of water, and the pH was adjusted by using 1N NaOH [12].

Preparation of free chitosanase Chitosanase enzyme was extracted from fresh pepper leaves and FC was prepared using the procedure reported in previous paper 10. Briefly, healthy fresh pepper leaves were collected and homogenized in distilled water at 5°C. The resulting homogenate was dialyzed against distilled water for 48h at 5°C. The resulting dialysates were centrifuged and the supernatant was treated with ammonium sulphate. Ammonium sulphate fraction with 20-60% saturation was used as FC. Enzyme activity and protein concentration were determined.

Preparation of immobilized chitosanase Chitosanase was immobilized on chitin by covalent bond using the procedure reported in previous paper 11. Briefly, One gm chitin was shaken in 5ml 0.1M HCl containing 5% (v/v) glutraldehyde (GA) for 24 h at 30°C. The solubilized chitosan was precipitated by addition of one ml of 0.1M NaOH. The precipitates were collected by filtration and washed with distilled water to remove the excess GA. The wet chitin was mixed with 2.0 ml of free chitosanases FC (400 U). After being shaken for 1.0 h at 30°C, the unbound enzyme was removed by washing with distilled water.

Measurement of free and immobilized chitosanase activity The reaction mixture of 0.9 ml of 1% soluble chitosan dissolved in 0.05 M sodium acetate buffer, pH 5.8, adequate amount of immobilized chitosanase and 1 ml of 0.05 M sodium acetate buffer, pH 5.8 was incubated at 40°C for 1.5 h. The reaction was stopped by boiling in water bath for 10 min. The mixture was centrifuged at 3000 rpm for 15 min. The concentration of reducing sugars produced from chitosan was measured by dinitrosalicyclic acid method (DNS) using glucosamine as standard [13]

One unit of chitosanase was defined as the amount of enzyme that could liberate one µmole of reducing sugar per h under the standard assay conditions using glucosamine as standard. The specific activity of chitosanase is expressed as units per milligram protein**.** The activity of chitosanase value was the average values of three repeated measurements.

Protein determination The protein concentration was determined according to the method using bovine serum albumin as a standard [14].

Effect of different pH's on the enzymes activities Small aliquots of the prepared enzymes FC and IC were assayed with two buffering solutions, namely 0.1 M acetate (pH 4.5-5.8) and 0.1 M phosphate (pH 6.0-8.0), for recording pH profile under the standard assay conditions.

Effect of different temperatures on the enzyme activities The activities of the prepared enzymes FC and IC were determined at different incubation temperatures ranged from 30-70°C.

Effect of the reaction time on enzyme activities The prepared enzymes FC and IC were incubated with the substrate for different time intervals up to 21 h, for recording time profile under the standard assay conditions.

The mode of action of the prepared chitosanase TLC techniques were used to analyze the end product and identify the mode of action of the prepared chitosanases [15]**.** Chitosan hydrolysate and D-glucosamine, as standard, were dissolved in deionized water and were subjected to one dimensional

thin layer chromatography chromatogram on aluminium sheet of silica layer. One micro-litter of each sample was applied to the chromatoplate with the micropipette in the usual manner. The eluted solvent was n-propanol: water: concentrated ammonia (7:2:1 v/v/v). Sugar spots were visualized by charring with 10% sulphuric acid in ethanol.

Effect of different enzyme concentrations The enzyme activity of the prepared chitosanase enzymes FC and IC was estimated at different concentrations ranging from 0.353 to 3.53 mg enzyme / reaction mixture and 0.096 to 0.96 mg enzyme /reaction mixture, respectively. The products were estimated and their amount calculated as µg glucosamine/ reaction mixture. Relation between enzyme concentrations and reaction products was plotted.

Effect of different substrate concentrations on enzyme activities Different substrate concentrations were incubated at different concentrations ranging from 1.8 to 18 mg/ reaction mixture for the FC $_{and}$ from 2 to 20 mg/ reaction mixture for the IC. Then the enzymatic activities were plotted against substrate concentrations.

Determination of Michaelis' constant (K_m) and maximum velocity (V_{max}) The K_m and V_{max} values of the FC and IC toward chitosan were determined [16].

Enzymes stabilities

Thermal stability of the chitosanase enzymes Small aliquots of the prepared enzymes FC and IC were preheated at different temperatures ranging from 30 to 70°C for varying time intervals; 30, 60 min. The remaining enzyme activities were then assayed using the standard assay conditions. Residual activity was expressed as a percentage of the initial activity under the standard assay conditions.

Operation stability of the immobilized chitosanase Immobilized chitosanase 0.5 g carrier was added to 10 ml 1% chitosan solution in water bath 40°C for 1.0 h. At the end of the reaction time, the immobilized chitosanase was collected and washed with distilled water and resuspened in 10 ml of freshly prepared substrate to start a new run. The supernatants were assayed for immobilized chitosanase activity. This process was repeated for 10 times. Residual enzyme activity was calculated a percentage of its initial one. Degree of hydrolysis was calculated a percentage of hydrolysate concentration produced to the chitosan concentration.

Effect of some metal ions on the enzyme activities The effect of metal ions on the chitosanase activity were studied. The salts which were used namely, $HgCl_2$, AgCl, $CoCl_2$, NaCl, $ZnSO_4$, $MnSO_4$, $CuSO_4$, $AgNO_3$, $FeSO_4$ and Hg $(NO_3)_2$ at concentration 0.1 mM per reaction mixture. Residual activities in the presence of the salts were compared with that of the controls (without salts).

RESULTS AND DISCUSSION

Immobilized chitosanase had many biological activities useful for the industrial application. The most important one was its potential used for enzymatic production of chitooligosaccharides from chitosan. Chitooligosaccharides had different biological activities such as antimicrobial, antidiabetic, anti-inflammatory and antioxidant [17-20].

Optimum pH was determined by individual changing the pH values from 4.5 to 8 in the reaction mixture (Figure 1). The optimum pH of the IC was slightly shifted to acidic range (pH 5.6) from 5.8, which was the optimum for free one. While**,** Zheng and Xiao[21] reported that the optimal pH of immobilized chitosanase with DEAE cellulose by crosslinking was shifted from 5.0 to 4.0.

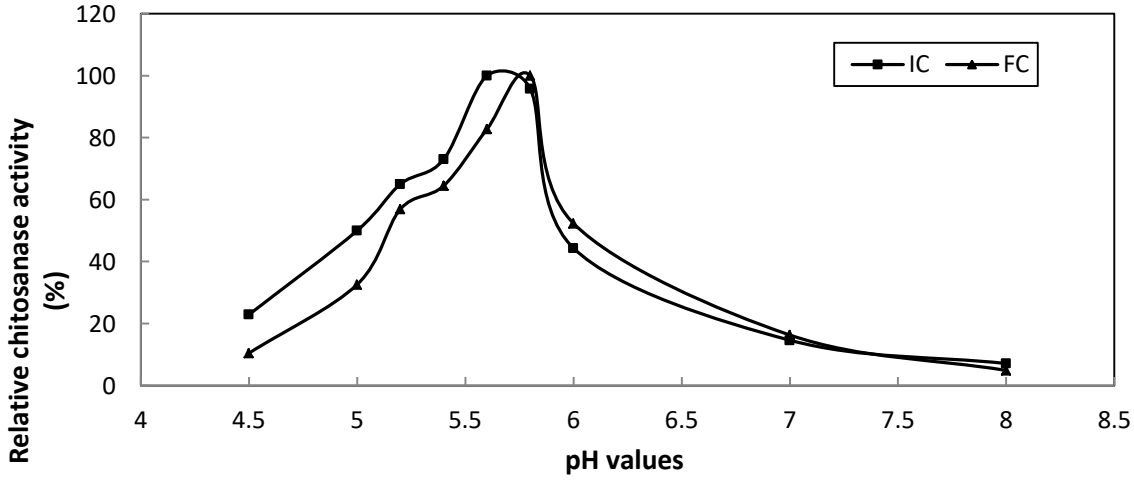

Fig-1: Effect of different pH values on the activity of free (FC) and immobilized (IC) chitosanases.

The effect of different temperatures from 40 to 70°C on FC and IC activities was shown in figure (2). IC showed maximum activity over the temperature range from 40 to 60°C with optimum at 55°C, while FC showed maximum activity over the temperature range from 40 to 50°C with optimum at 45°C. The increase in optimum temperature could be explained by alteration of the physical and chemical properties of the IC. The results were lower than those reported for free and immobilized chitosanase isolated from *Penicillium* sp. ZD-Z1 (50 and 60°C, respectively) [21]. The variations in the optimum temperature value may depend on the type of support materials and immobilization methods.

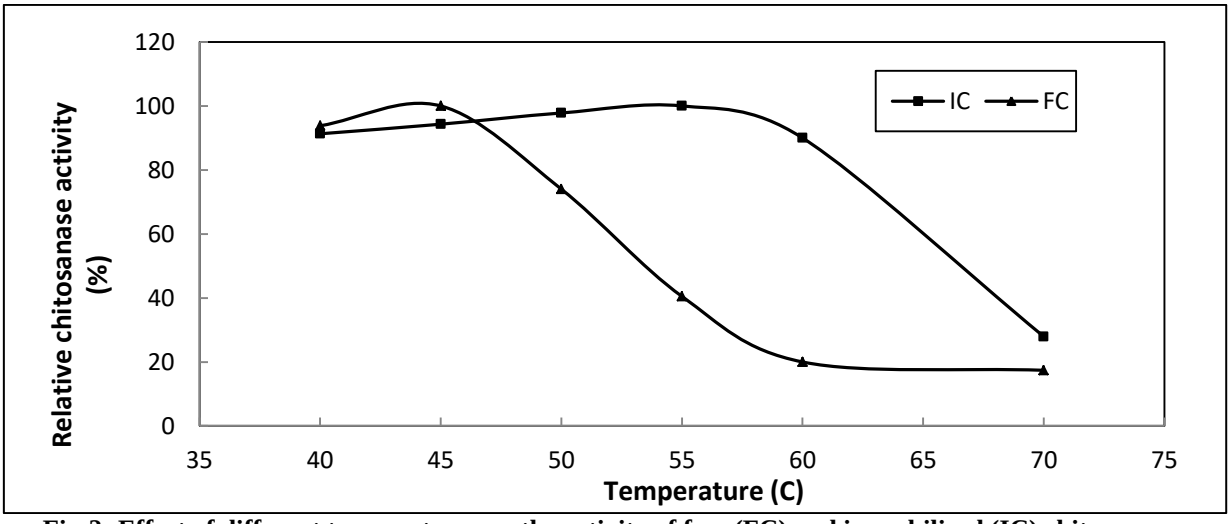

Fig-2: Effect of different temperatures on the activity of free (FC) and immobilized (IC) chitosanases

The activity of the IC was a linear function of incubation time up to 1.5 h beyond this time, there was no increase in activity (Figure 3). Figure (3) showed a typical hydrolysis curve obtained under experimental conditions, resulting in an increase in enzyme activity as a function of reaction time. This result was similar with those of **Gao [22]**. This could be explained by that the degradation of the oligosaccharides and production of more smaller oligosaccharides may be equal, so there was no observed difference in activity. This explanation was confirmed by the results of our study on the effect of time on the degree of hydrolysis of the IC.

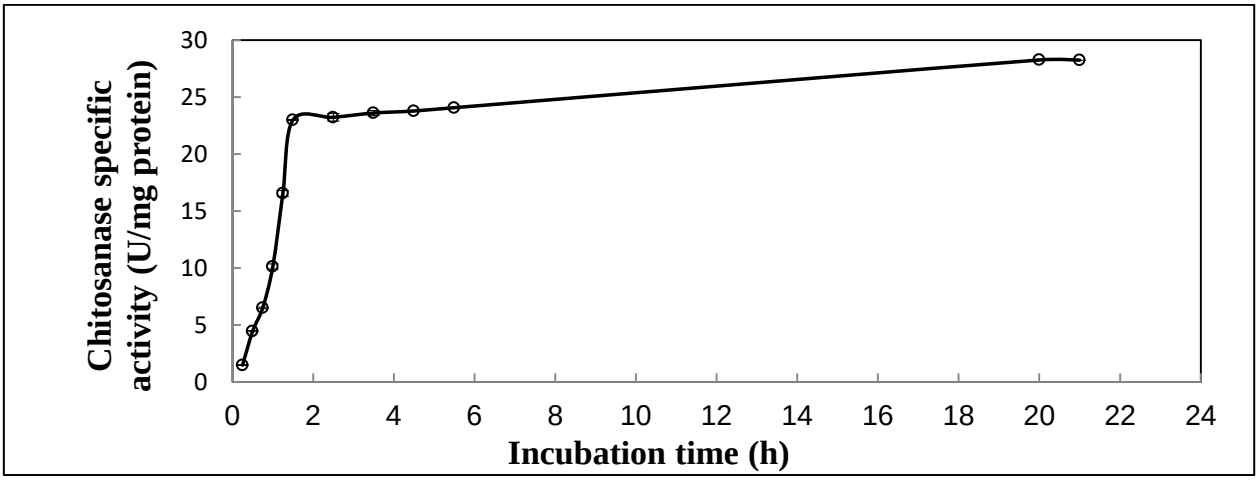

Fig-3: Effect of different incubation times on the activity of immobilized chitosanase (IC).

TLC was carried out to investigate the mode of action of the prepared IC. Figure (4) illustrating the TLC picture of standard D-glucosamine and chitosan hydrolysate (chitooligosaccharides). Chitooligosaccharides and D-glucosamine and oligomers (DP 2 to 6) were the end products of hydrolysis of chitosan by the prepared IC. Thus, the prepared immobilized enzyme had mixture of endo and exo chitosanase type. Little spots were observed between bands. We speculated that these tiny spots

were corresponding to partially acetylated chitooligosaccharides. Cabrera and Cutsem, [15] recorded the same result.

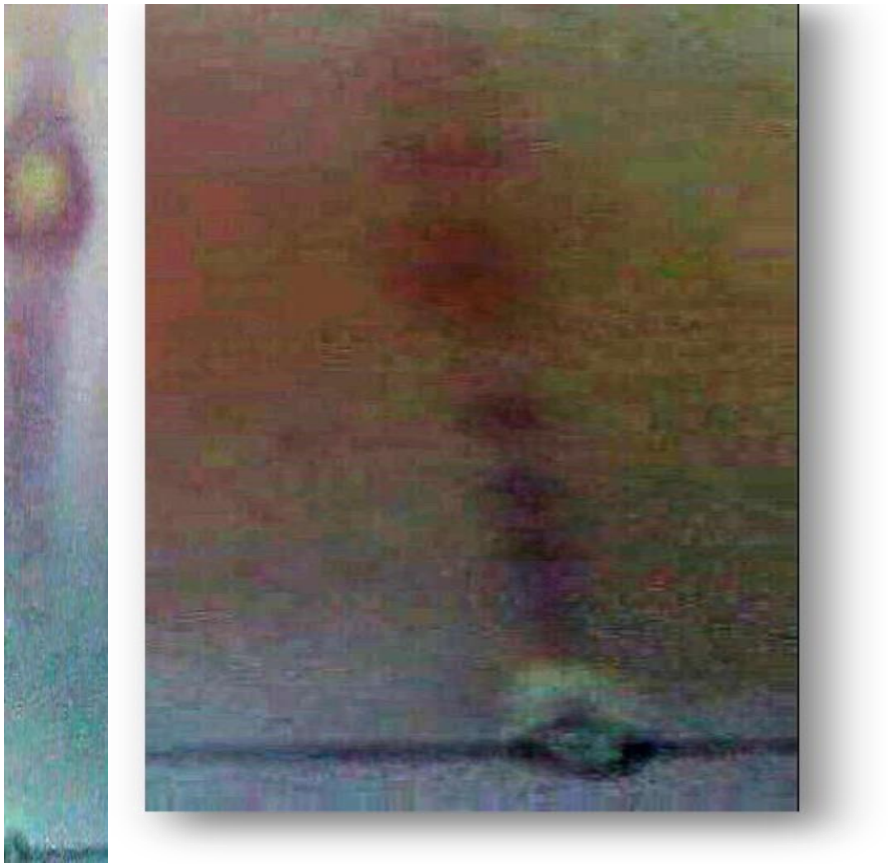

Fig-4: TLC analysis of chitosan hydrolysate and standard D-glucosamine.

The chitosanases activities were estimated at different concentrations for both free and immobilized one. Their activities were increased proportionally in a linear relationship with the increasing of enzyme concentration up to 0.576 mg protein/reaction mixture for IC and 2.825 mg protein/reaction mixture for FC (Figure 5). Chitosanase activities were calculated as mg glucosamine per reaction mixture.

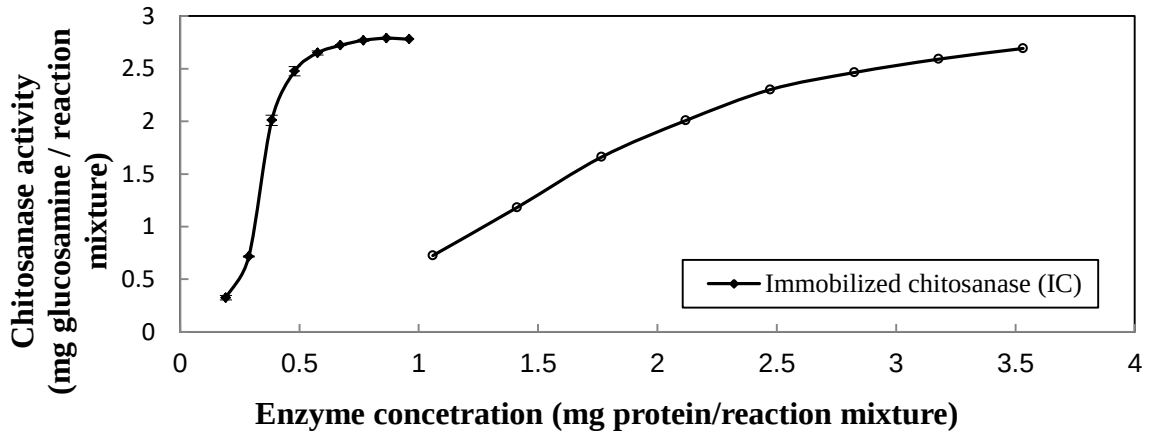

Fig-5: Effect of different concentrations of the free (FC) and immobilized (IC) chitosanases on their activities.

Linear relationships were observed between chitosan and the specific activities of the FC and IC up to 18 mg/reaction mixture for both free and immobilized chitosanase as shown in figures (6 and 7). They followed Michaelis-Menten Kinetics. The K_m values of the FC and IC were 35.71 and 17.86 mg/reaction mixture, respectively with the using of soluble chitosan (Figures 6 and 7). The V_{max} for the free and immobilized chitosanases were 12.5 and 47.62 U/mg, respectively. The K_m value of the immobilized enzyme was lower than that of the free enzyme, which means that the immobilized enzyme had more affinity towards chitosan than that of the free one. However**,** Zeng and Zheng [23] reported that the K_m value of the *penicillium* sp.ZDZ1 chitosanase immobilized on chitin by covalent binding (17.66 g/l) was higher than that of the free one (2.611 g/l). Kusano *et al.* [24] explained that the increase of immobilized enzyme K_m than the free one led to a decrease in the flexibility of the enzyme molecule.

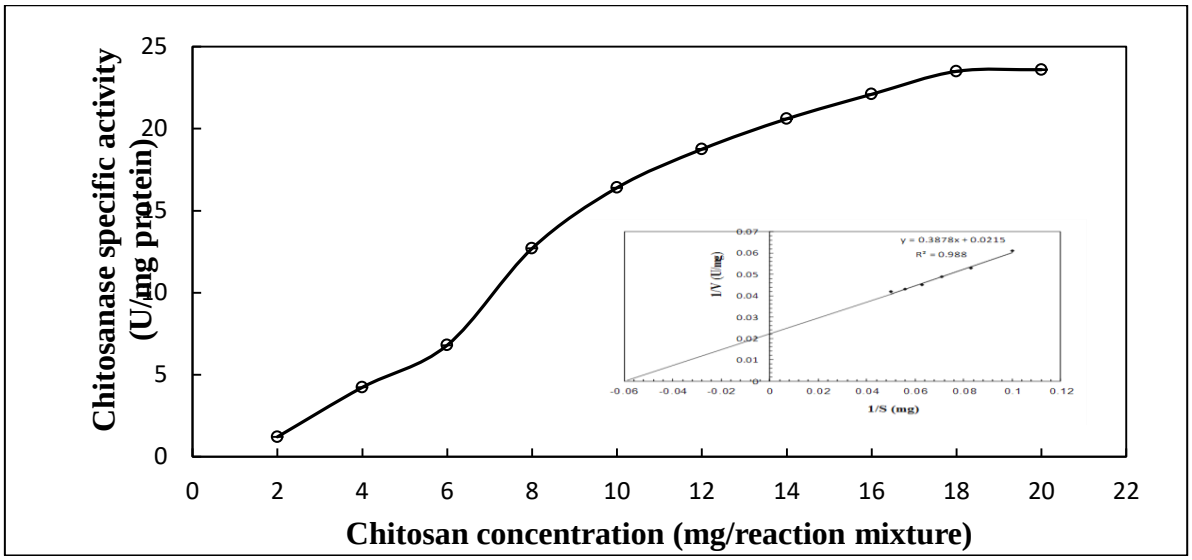

Fig-6: Effect of different substrate concentrations on the activity of immobilized chitosanase (IC)

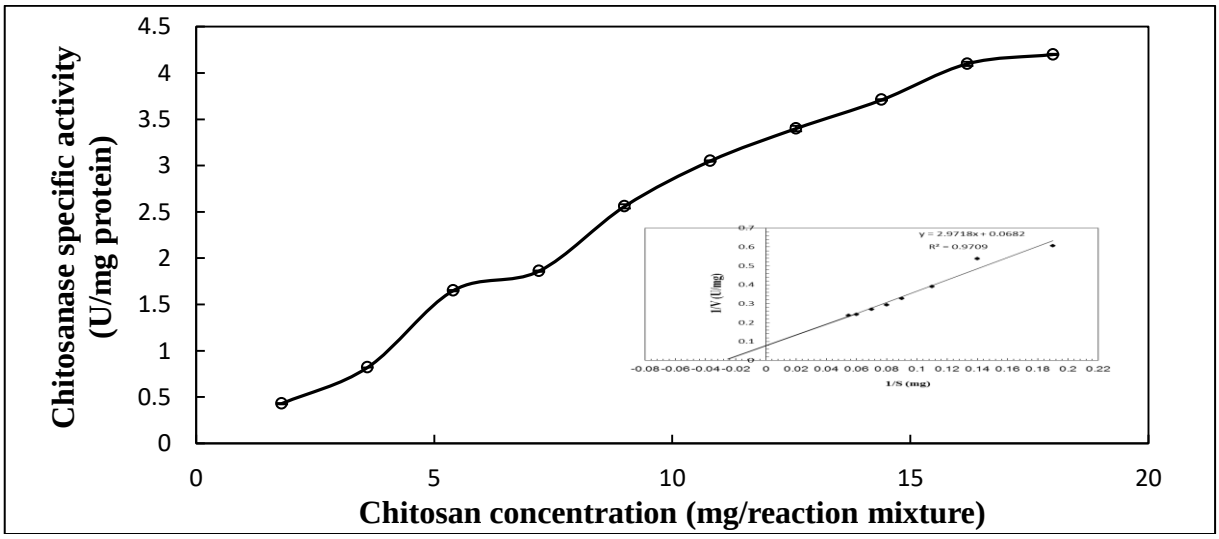

Fig-7: Effect of different substrate concentrations on the activity of free chitosanase (FC).

Thermal stability curves of FC and IC at different temperatures (40, 50, 60 and 70°C) for 30 and 60 min were shown in figure (8). The FC and IC maintained all initial activity after preincubated at 40ºC for 1 h and lost only about 5-10% of its activity at 50ºC. The IC enzyme was retained 43% of its activity at 60ºC for 1 h, while the free one retained only 20% of its activity at the same temperature. The FC had a lower resistance to high temperature compared to the IC. The higher optimal reaction temperature and improved thermal stability up to 60ºC for the IC than the free one confirmed the results that covalent binding enhanced thermal stability. Improved thermal stability may be due to the restricted conformational change of the chitosanase upon immobilization, as suggested by Martinek *et al.*, [25]. Thermally stable enzymes

enhanced by immobilization, represented valuable tools for reasonable of biotechnological processes.

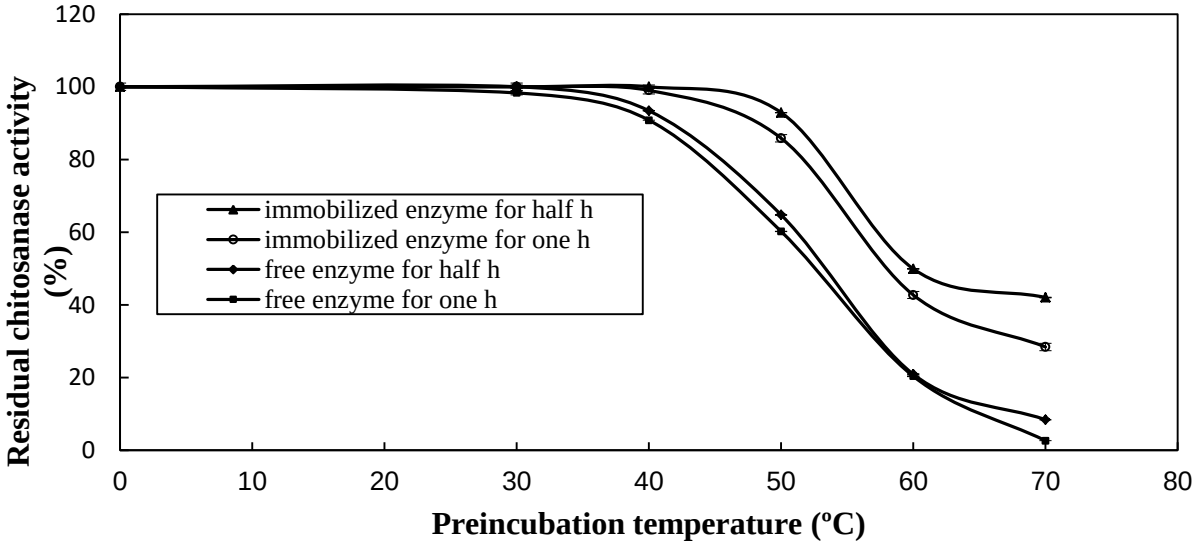

Fig-8: Thermal stability of the free (FC) and immobilized (IC) chitosanases after pre-incubation with different temperatures for half and one hour. Residual activity was expressed as a percentage of the initial activity before pre-incubation.

Due to the high cost of enzymes, they should be reused for several times to be competitive with chemical catalysts. Ideally, the immobilization of the enzymes should provide, beside thermal and storage stabilities, a high operational stability. Reusing of IC for several times with different incubation times (1, 2 and 24 h) was shown in figures (9&10). Results indicated that long incubation time of the enzyme resulted in inhibition of enzyme activity. The residual activity of immobilized chitosanase after the first run for 1 h, 2 h and 24 h were 97.3, 90.99 and 16% of the initial activities, respectively. At repeating the batches, degree of hydrolysis and enzyme activity after 24 h were very low, but in case of 1 and 2 h incubation times they were decreased gradually till 10 and 4 times, respectively. Degree of hydrolysis of the immobilized chitosanase at incubation time for 24 h (30%) was higher than that at incubation time for 1 h (22%) and 2h (24%) at first run by 8 and 6 %, respectively. This means that the stability of the IC was highly affected by increasing the time of incubation. This decrease in the activity is a consequence to different factors as the progressive decay in mechanical resistance of the immobilized enzyme prepares (support-enzyme).

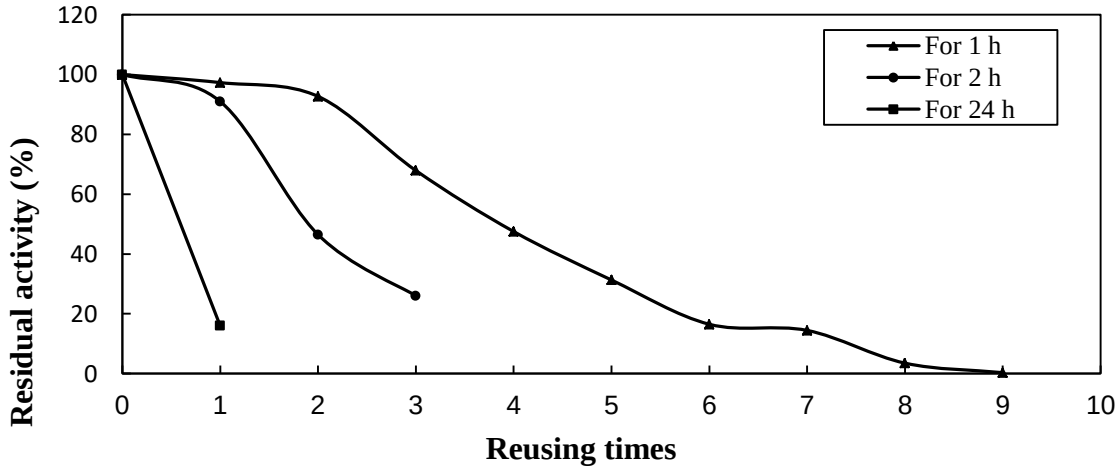

Fig-9: Reusing of the immobilized chitosanase (IC). At different incubation times. Residual activity was expressed as a percentage of the initial activity.

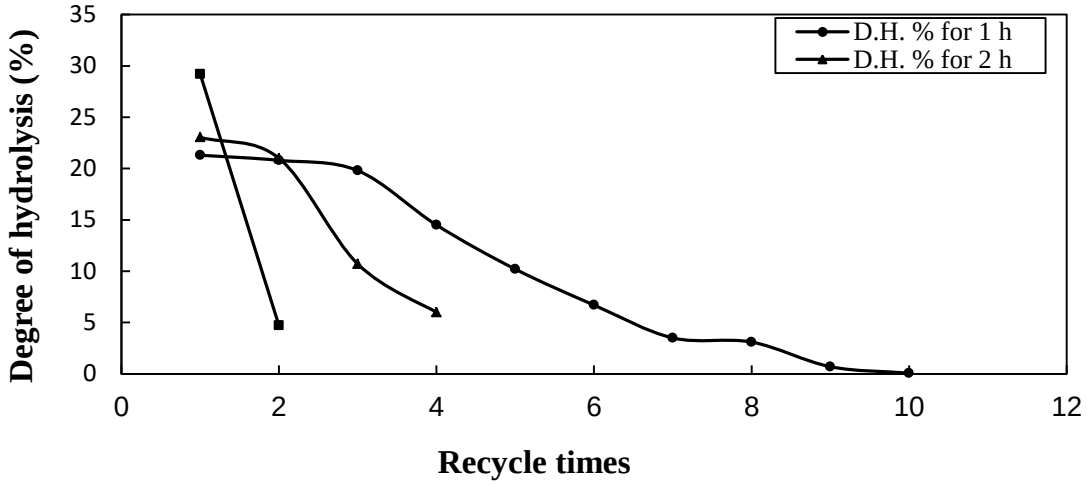

Fig-10: Degree of hydrolysis percent of the immobilized chitosanase (IC) at different incubation times.

Different inhibition values of the FC and IC activities were observed with addition of 0.1mM $CoCl_2$, $NiCl_2$, $HgSO_4$, $AgNO_3$, $FeCl_2$, $CuSO_4$ and $ZnSO_4$ to the reaction mixture (Table 1). While addition of 0.1 mM of $MnCl_2$ and NaCl to the reaction mixture, increase the activity of IC by 2.6 and 10 %, respectively, and increase the activity of FC by 1.2 and 13.3, respectively. The activation effect of Na^{+1} on the free enzyme was less than on the immobilized enzyme. This may be due to the protection of the immobilized enzyme by the immobilization matrix and hence the inhibiting reagents become less accessible to the enzyme active site as mentioned by Akosy *et al.* [26].

Table-1: Effect of some metals on the activity of the free (FC) and immobilized (IC) chitosanases

Metals	Residual activity (%)	
	IC	FC
Control	100	100
Mn^{+2}	102.6	101.2
Co^{+2}	28.6	31.9
Ni^{+2}	30	23.8
Hg^{+2}	80.5	80.1
Ag^{+1}	42.7	49.3
Na^{+1}	110	113.3
Fe^{+2}	42.1	51.2
Cu^{+2}	73.6	78.1
Zn^{+2}	29.5	25.3

* Residual activity was expressed as a percentage of the initial chitosanase activity without adding metals.

CONCLUSION

In general, the immobilized chitosanase on chitin by covalent bond showed significant advantages over free one. The temperature and operation stability of the immobilized enzyme were better than that of free one. The operation process was very simple and it is very good for large-scale industrial applications.

Conflict of interest: There is no conflict of interest among the authors in carrying out the work.

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