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Immobilization, Optimization and Stability of Pepper (*Capsicum annuum*) Chitosanase on Chitin.

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ABSTRACT

Free chitosanase C_N, the ammonium sulphate fraction precipitated from crude pepper chitosanase, was immobilized on chitin by using physical adsorption and covalent binding methods. We developed chitosanase immobilized on chitin by adsorption methods for 2 and 24 hat different pHs values from 5.4 to 8.0.The optimal condition for chitosanase immobilization by physical adsorption was at pH 5.4 for 24 h (IC_{N2}). While the optimal condition for immobilization by covalent method was as follows: chitin was added to free enzyme in ratio 5:2 mg/U and treated with 5ml 5% solution of glutaraldehyed (GA) as cross-linking reagent at pH 5.4 (IC_{N2}). Results showed that immobilized chitosanase prepared by covalent binding on chitin(IC_N) hadthe highest immobilization efficiency (85.2% yield and specific activity 21 U/mg) than by physical adsorption (11.3% yield and specific activity 4.72 U/mg) at optimum immobilization condition. Free chitosanases C_A and C_B(the partially purified pepper chitosanase) were immobilized on chitin by covalent binding method at the optimum immobilization condition. Immobilization efficiency of all prepared chitosanases on chitin by physical adsorption (IC_{N2}) and covalent binding (IC_A, IC_B and IC_N) were compared with respect to their immobilization activity, yield, reusability and storage stability. IC_N showed good operation stability for 10 times reuse compare to the other immobilized prepared enzymes. All immobilized chitosanases showed better storage stability (residual activity 77.2 to 94.7%) in distilled water at -4°C up to 30 days than free chitosanase (residual activity 56.2%). The amount of chitooligosaccarides produced by IC_N was higher than that produced by other prepared immobilized enzymes. IC_N is a typical example for best immobilized chitosanase and was chosen for further study.

Keywords: Chitosanase (EC 3.2.1.132), *Capsicum annuum,* Pepper leaves, Immobilization, chitin, physical adsorption, covalent binding.

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INTRODUCTION

Chitosanases (EC.3.2.1.132) represent a class of hydrolytic enzymes acting on chitosan (a polymer of β-1, 4-D glucosamine). Most of chitosanases occur in a variety of microorganisms, including bacteria and fungi [1-3]. Chitosanases are also found in vegetative parts and seeds of some higher plants [4-5].

Chitooligosaccharides (COS), the degraded products of chitosan prepared enzymatically or by chemical hydrolysis, are water soluble and possess distinctive biological activity[6]such as cytotoxicity[7], antibacterial[8], antioxidant[9], anti-inflammatory [10] and inhibition of angiogenesis [11]. Enzyme immobilization has been a popular strategy for most large scale application. Immobilization of chitosanase on solid support possess several advantages like ease of product separation and purification, repeated usage of enzyme and improvement of enzyme stability and storage. Immobilization of chitosan degrading enzyme has been reported for example on agar gel by covalent binding [12], on amylase coated nanoparticle [13], on chitin [14] and DEAE cellulose [15].

Chitin, a polysaccharide containing 2-deoxy-2-amino glucose units linked through ?-1, 4 linkages. It is hydrophilic, inert and non-biodegradable. It has been reported that every five amino groups out of six in the chitin molecule are in the acetylated form. The presence of the amino groups in the chitin molecule provides a binding site for proteins. It can be used as a solid support for the preparation of immobilized enzymes. A number of studies have shown that chitin is a good carrier for enzyme immobilization. Chitin can be used for immobilization of protease [16] α -L-arabinofuranosidase [17] and cellulase [18]. Compared with free enzyme, the immobilized enzyme has better temperature and storage stability.

In a previous study, screening experiments on different plant parts were carried out to choice the most suitable source with high chitosanase activity [5]. Pepper leaves were chosen for extraction and purification of the chitosanase enzyme [19]. In the present work, we investigated the preparation of immobilized pepper chitosanase using chitin as a support material via physical adsorption and covalent binding technique. The effect of immobilization condition on the immobilization efficiency was studied. We examined the reusability of the immobilized chitosanase and comparing the storage stability with the free enzyme.

MATERIALS AND METHODS

Materials

Chitosan with an average molecular of weight 300,000, chitin, glucosamine, chitobiose and tetramers were purchased from Merch chemical Cò. Chitosanase was prepared in our laboratory. Other chemicals were of analytical grade.

Methods

Preparation of chitosanase enzymes:

Partially purified chitosanases C_N, C_A and C_B were prepared from fresh pepper leaves as described previously [5&19].

Preparation of the crude chitosanase from pepper leaves:

Healthy fresh pepper leaves were collected and cleaned thoroughly with distilled water. They were sliced into small parts and homogenized in Braun multimix Mx 32 with distilled water at 5°C. The resulting homogenate was filtrated through cheese cloth and dialyzed against distilled water for 48h at 5°C.The resulting dialysates were centrifuged (13,000 xg, 5°C) for 15 min and the supernatant was used as the crude enzymes.

Preparation of chitosanase C_N:

Adequate volume of the prepared crude enzyme was treated with different concentrations of ammonium sulphate (0-20 and 20-60% saturation); each fraction of 20-60% was obtained by centrifugation (13,000 xg, 5°C) for 15 min. The resulting precipitate at 20-60% was dissolved in appropriate amount of

distilled water and dialyzed exhaustively against distilled water for 2 days at 4°C to get rid of the excess of ammonium sulfate. Undissolved protein was removed by centrifugation before enzyme assay. Enzyme activity and protein concentration were determined. The ammonium sulphate fraction at saturation 20 to 60% was used as chitosanase C_N.

Preparation of chitosanase C_A and C_B:

Sephadex G-100 powder was previously soaked in distilled water for two days. Fine particles must be removed by decantation. Sephadex G-100 column (1.2 x 50 cm) was equilibrated with 0.01M acetate buffer, pH 5.8 before used. The concentrated enzyme chitosanase C_N was applied to the top of the column and the protein was eluted with the same buffer at a flow rate of 15ml/h. The most active fractions were collected. Two peaks with chitosanase activity (C_A and C_B) were obtained.

Preparation of soluble chitosan:

Soluble chitosan for determination chitosanase activity and production of chitosan oligosaccharides (COS) and low molecular weight chitosan was prepared as follow: 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 [20].

Chitosanase assay:

Chitosanase activity was determined by quantitative estimation of the reducing sugars (chitooligosacharides and glucosamine) produced from chitosan.

Free chitosanase assay:

The activity of free chitosanase$C_N$at 40°C was determined by a 3,5- dinitrosalicylic acid method DNS using glucosamine as standard [21].

Immobilized chitosanase assay:

Chitosanase activity was measured by using reaction mixture contained 0.9 ml of 1% soluble chitosan (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. The reaction mixture was incubated at 40°C for 1.0 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 sugar in the supernatant was determined by DNS method using glucosamine as standard [21].

Enzyme unit:

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

Protein Determination:

Protein concentration was determined by the method of Lowry *et al*., [22] using a bovine serum albumin as a standard.

Immobilization methods of chitosanases enzymes (C_N, C_A and C_B) by:

Physical adsorption method:

It was carried out according to Woodward [23].Chitin were incubated each with the enzyme in ratio 5:2 mg/U dissolved in 2.0 ml of o.2 M acetate buffer (pH 5.8) at 4°C for 2 h and 24 h. The unbound enzyme was removed from the carriers by washing three times with acetate buffer (pH 5.8). Chitosanase activity and

protein concentration were determined in wash solution (unbound enzyme). The immobilized chitosanase activity was determined in the precipitated enzyme.

Covalent binding:

One g chitin was shaken in 5ml 0.1M HCl containing 2, 3, 4 or 5% (v/v) glutraldehyde (GA) for 2 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 enzyme solution (400 U chitosanase C_N, C_A and C_B) in ratio 5:2mg/U. After being shaken for 1.0 h at 30°C, the unbound enzyme was removed by washing with distilled water. Chitosanase activity and protein concentration were determined in wash solution (unbound enzyme). The immobilized chitosanase activity was determined in the precipitated enzyme (U/mg).

Efficiency measurement:

Efficiency of different carriers used for immobilization was determined by calculations:

1. Free enzyme bound (U) = free enzyme added (U) - free enzyme unbound (U)
2. Immobilization yield (%) $= \frac{\text{Immobilized chitosanase activity (U)}}{\text{Free chitosanase bound (U)}} \text{ x } 100$
3. Degree of hydrolysis (D.H.)% = $\frac{\text{Chitooligosaccharides concentration (mg)}}{\text{Chitosan concentration (mg)}} \text{ x } 100$
4. Specific activity (S.A.) $= \frac{\text{Chitosanase activity (U)}}{\text{Protein concentration (mg)}}$
5. Purification fold $= \frac{\text{Specific activity of immobilized chitosanase}}{\text{Specific activity of free chitosanase}}$

Operation stability of the immobilized chitosanases:

Immobilized chitosanase 0.5 g carrier was added to 10 ml 1% chitosan solution in a water bath 50ºC 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 chitosanase activity. It was repeated for 10 times.

Storage stability of free and immobilized chitosanase:

The free immobilized enzymes were stored in distilled water at -4ºC for 30 days. The residual activity were measured by the standard assay procedure described earlier. The activity of the free and immobilized chitosanase were expressed as a percentage of its residual activities compared to the initial activity.

Production of chitooligosaccharides from chitosan:

ChitooligosaccaridesCOS_N, COS_A and COS_Bwere prepared by using 0.4 g carrier of immobilized chitosanase(IC_N , IC_A and IC_B) respectively**,**8 ml acetate buffer pH 5.4 and 8 ml soluble chitosan 1 %**.** The reaction mixture was incubated at 55^{o}C for 2 h. After that this mixture was centrifuged and the supernatant were taken to determine the amount of Chitooligosaccarides by DNS method using glucosamine stander [21].

Statistical analysis:

Data are expressed as the mean ± standard error (SE) from at least three experiments.

RESULTS AND DISCUSSION

Pepper leaves was a good source of chitosanase enzyme as described previously [5].It is a good economic source as it is an agriculture waste. Chitosanase from pepper leaves was partial purified by using ammonium sulphate precipitation (20-60%) saturation followed by gel filtration on Sephadex G-10[19]. The partial purification of chitosanase gave three chitosanases, ammonium sulphate chitosanase (C_N) and two Sephadex G-100 chitosanases (C_A and C_B) with specific activity 4.2, 34.6 and 24.05 U/mg, respectively.

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In the present study, three chitosanases (C_A, C_B and C_N) were immobilized on chitin by physical adsorption and covalent binding methods. The general output of the efficiency of immobilization process represented in immobilization yield (%), chitoansanase activity (U/g carrier), specific activity (U/mg) and degree of hydrolysis (%) of the immobilized enzyme were determined and compare with the free one.

Immobilization by physical adsorption:

Effect of different incubation times on immobilization efficiency.

Figure (1) recorded the effect of incubation time of chitosanase C_N with chitin carrier for 2 h and 24 h. Immobilization yield of chitosanase IC_{N2} (10.8±0.025 %) and purification fold (2.4 times) at 24h incubation time of immobilization were higher than that at 2h incubation time IC_{N1} (6.62±0.288 % and 1.047 times, respectively).Thus immobilization efficiency increase with increase time of incubation.

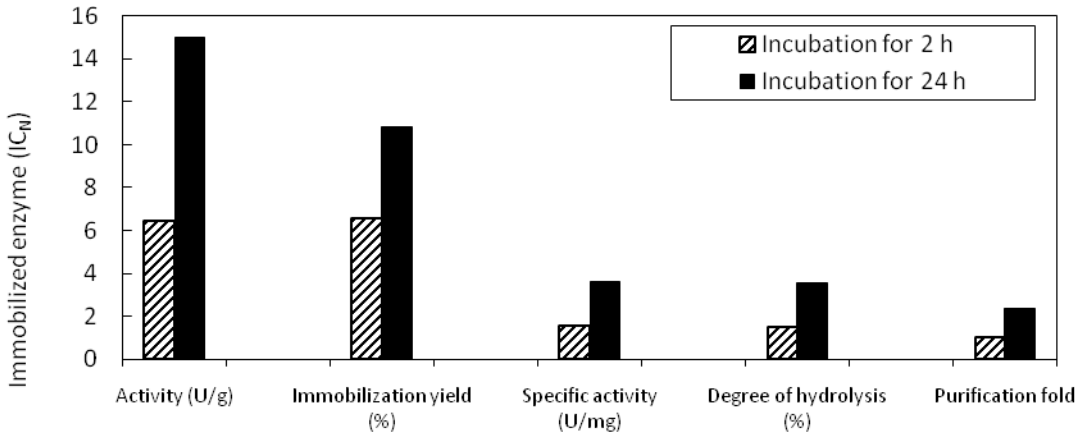

Figure (1): Immobilization efficiency of the immobilized chitosanse (IC_N) by physical adsorption method on chitin for 2 h and 24 h.

Effect of different pHs on immobilization efficiency:

Figure (2) reported immobilization of chitosanase IC_N on chitin at different pHs values. Results indicate that the best pH value for immobilization was (pH 5.4) With immobilization yield 11.3±0.025% and purification fold 4.72 times.

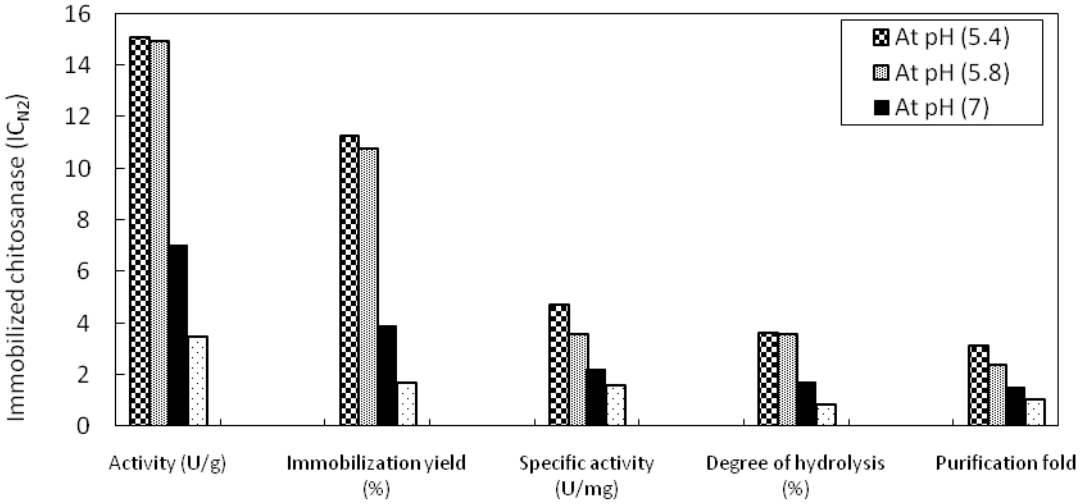

Figure (2): Immobilization efficiency of the immobilized chitosanse by physical adsorption method on chitin at different pH's for 24 h.

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Effect of free chitosanase bound concentration on immobilization efficiency:

The immobilization activity of IC_{N2} decreased with increasing the concentration of free chitosanase bound to the carrier (Table 1). This may be due to decrease the porosity of the surface on carrier which lead to decrease of immobilized enzyme activity.

Table (1): Immobilization of chitosanase on chitin by physical adsorption at different times and at different pH's

Immobilization pH	Immobilization time (h)	Free chitosanase bound (U/g carrier)	Immobilized chitosanase activity (U/g carrier)
5.8	2	97.6	6.46±0.281
5.4	24	133.5	15.1±0.033
5.8	24	138.25	14.96±0.035
7.0	24	180.5	7.02±0.035
8.0	24	208.5	3.51±0.035

In general, immobilization of chitosanase on chitin by physical adsorption showed low immobilization efficiency. This could be due to weak binding between the carriers and the chitosanase by the physical adsorption method. This is similar to that reported by Esawy et al., [24] and contrary to that reported by Mostafa [25]. However, lipase enzyme was immobilized successfully by physical adsorption on tricalcium phosphate gel and active carbon with high specific activity than the free one [26].

Immobilization by covalent binding:

Table (2) and figure (3) showed immobilization of three chitosanases (IC_A, IC_B and IC_N) with chitin by covalent bindingusing 5% GAas crosslinking reagent. The concentration of GA used for three chitosanases was similar to that of *Penicillium* Sp. ZDZ1 immobilized chitosanase [14]and *Bacillus* immobilized alkaline protease [16] obtained similar results. L-asparaginase was efficiently immobilized by covalent binding with activated carbon with immobilization of 73.6% at 5% GA [27].Moreover, Gauthier *et al*., [28] reported that increasing the length of the spaces group contributed to increase in the stability of immobilized protease on agarose. However, Zhou *et al*., [29] reported that 2% GA was sufficient for enzyme immobilization. Immobilized chitosanase (IC_N) gave the highest activity 201±0.983 U/g carriers and immobilization yield 85.2 %, with degree of hydrolysis 48.14% and represented a typical example for pepper leaves chitosanase immobilization.

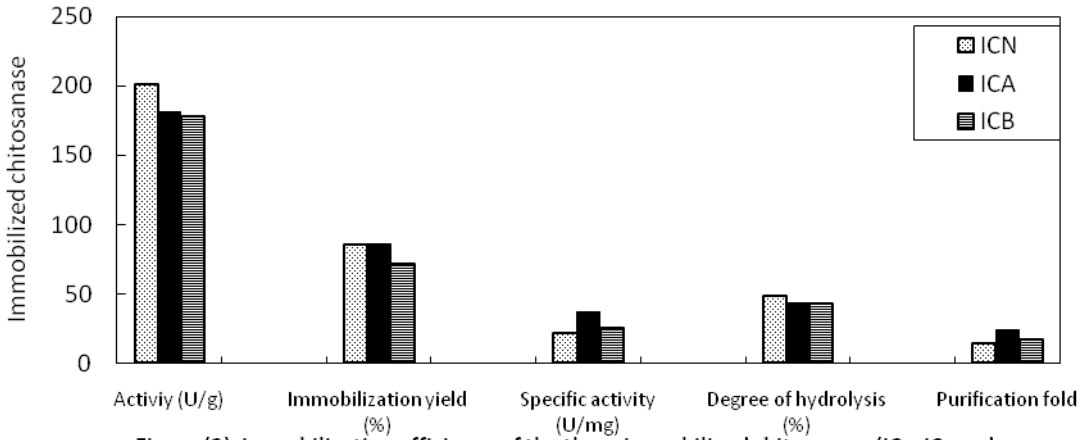

Figure (3): Immobilization efficiency of the three immobilized chitosanses (IC_N, IC_A and IC_B) by covalent binding with chitin.

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Table (2): Immobilization of chitosanases (C_N,C_A and C_B) with chitin by covalent binding.

Chitosanase enzyme type	Free chitosanase bound activity (U/g carrier)	Immobilized chitosanase activity (U/g carrier)
IC_N	236	201±0.983
IC_A	210	181.4±0.703
IC_B	248	177.4±0.4215

Immobilization yield of chitosanase by covalent binding was higher than of physical adsorption by 7.54 times. In case of covalent binding method almost 59.9% of used chitosanase was immobilized on chitin, whereas in case of the physical adsorption method with chitin (at the same enzyme concentration added 400U) was about 33% of enzyme was immobilized. Good immobilization efficiency by covalent binding might have been due to the formation of stable crosslinking between the carrier and the enzyme through a spacer group (GA).

Operation stability of immobilized chitosanases:

Operation stability of theimmobilized chitosanase (IC_{N2}) by physical adsorption at pH 5.4 and 5.8 was showed in figure (4). The initial activity of immobilized chitosanase decreased by 62% for pH 5.4 and 52% for pH5.8 at first run while the relative activities in the third reactions were decrease from the second one by 8 and 11 %for pH 5.4 and 5.8, respectively. Thus, the decrease in activities from the second to the rest reaction were relatively small (decrease gradually) compared with that from the first to second reaction. The deactivation kinetics cannot be decreased by a simple first – order model. We attribute to weak bound between enzyme and carrier by physical adsorption which lead to leak out of enzyme and consequently poor reusability.

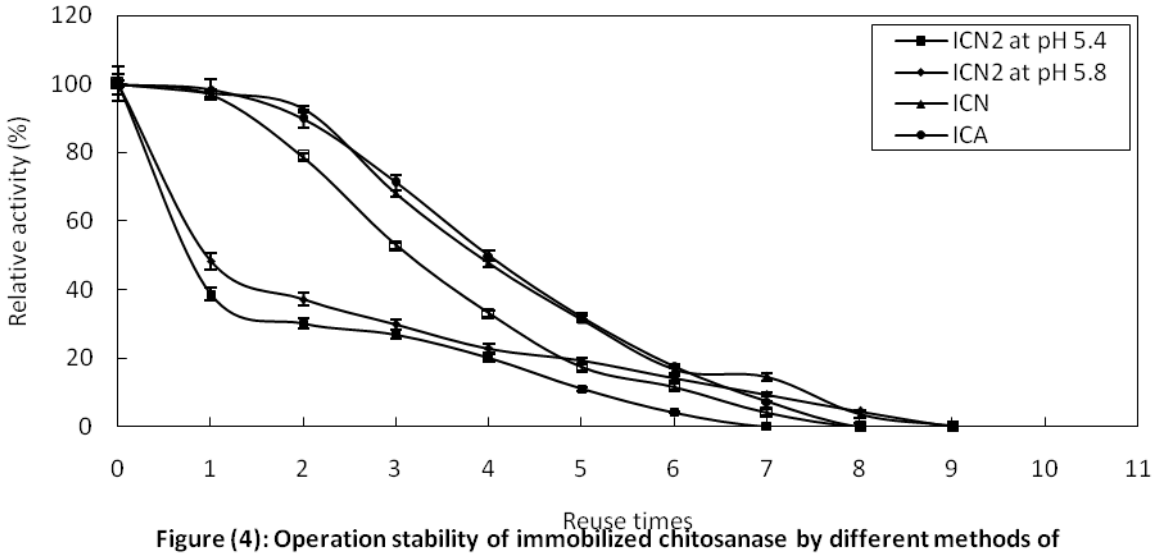

Figure (4): Operation stability of immobilized chitosanase by different methods of immobilization.

Glutraldehyde as cross linking agent for attaching enzyme (IC_A, IC_B and IC_N) with chitin by covalent binding showed good reusability with gradual loss in activity (Figure 4). The results show that the chitosanase immobilized on chitin by covalent binding can be easily recovered and used repeatedly under the conditions of our experiments. The loss of activity may be caused by the deactivation of the enzymes during the hydrolysis reaction. They loss less than 50 % of its initial activity after five times batch reaction.Sinha *et al*., [30] recorded the same result using immobilized chitosanase on PAN nanofibers.

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Storage stability of free and immobilized chitosanases:

The free and immobilized chitosanase were stored in distilled water at -4°C and the residual enzyme activity was measured after 30 days. Figure (5) indicated that the activity recovery of the immobilized chitosanase (IC_A, IC_B and IC_N) was higher (77.2 to 94.7%) than that of the free one (56.2%). Zeng and Zheng (2002) reported that the activity recovery of immobilized chitosanase on chitin by cross linking reaction was more than 80% after 20 days. These results confirm that chitosanase is significantly stabilized by immobilization.

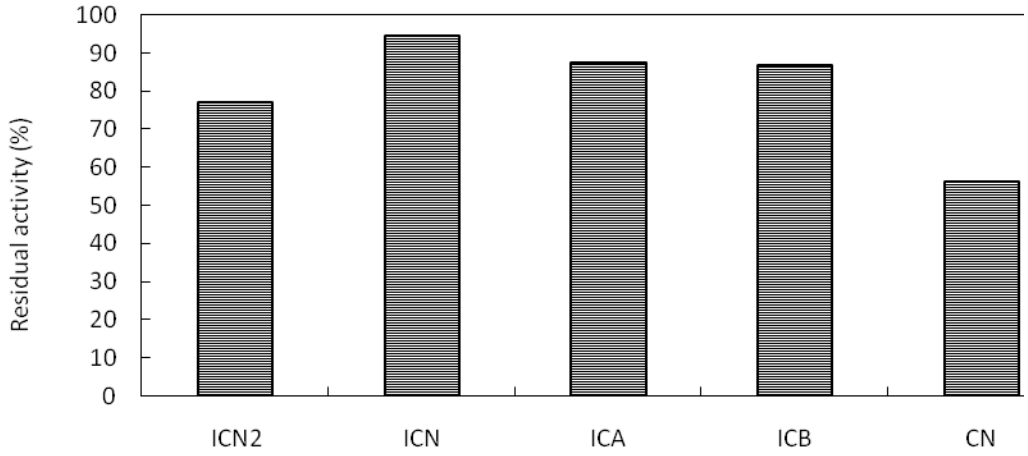

Figure (5): Storage stability of immobilized chitosanase stored at -4°C for 30 days.

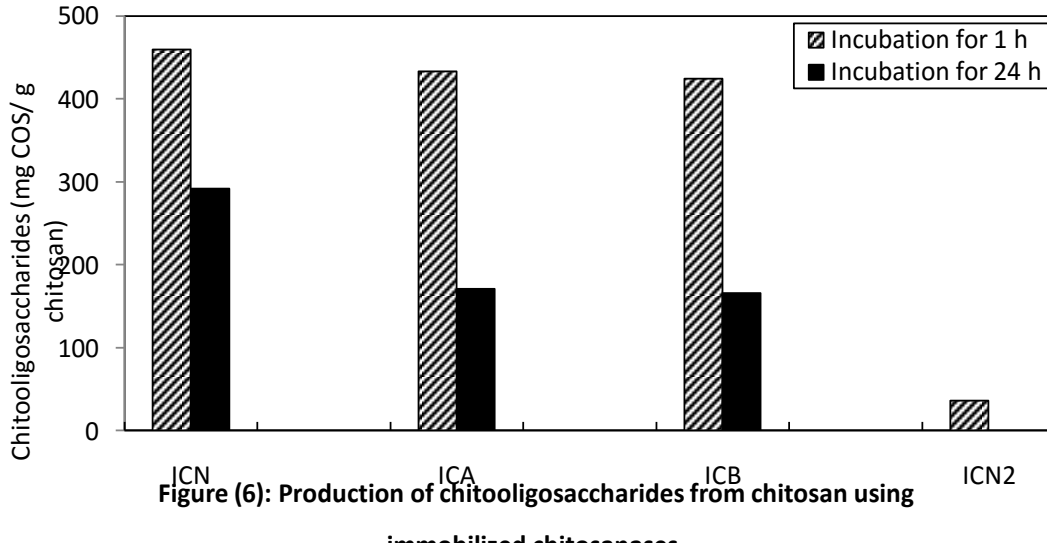

Figure (6): Production of chitooligosaccharides from chitosan using immobilized chitosanases

Production of chitooligoosaccharieds:

Generally, the immobilized chitosanase(IC_A, IC_B and IC_N)showed significant advantages over immobilized chitosanase IC_{N2}In the hydrolysis reaction of chitosan using the immobilized chitosanase (IC_A, IC_B and IC_N), physiologic active chitooligoosaccharaides were produced which is higher than that produced by using C_{N2} by 12.7 times (Figure 6).

CONCLUSIONS

Highly stable chitosanases (IC_A, IC_B and IC_N) immobilized on chitin with high activity and efficiency was prepared by covalent binding. The operation and storage stability of the prepared immobilized enzymes were much better than of free enzyme. Chitin is very cheap and immobilization process is very simple. It is very good

for large-scale industrial application. Thus, the prepared immobilized chitosanase present here has potential for being utilized in various industrial processes.

REFERENCES

[1] Chen X, Xia W, Yu X. Food Res Intern 2005; 38: 315-322.
[2] Jung WJ, Kuk JH, Kim KY, Jung KC, Park RD. Protein Expr purify 2006; 45: 125- 131.
[3] Wang J, Zhou W, Yuan H, Wang Y. Carbohydr Res 2008; 343: 2583-2588.
[4] Ouakfaoui SE, Asselin A. Phytochem J 1992; 31: 1513-1518.
[5] El-Sayed EM, El-Sayed ST, Shousha WG, Shehata AN, Omar NI. J American Sci 2011; 7: 713-721.
[6] Xia W, Liu P, Zhang J, Chen J 2011; 25:170–179.
[7] de Assis CF, Silveira RFM, de Oliveira RM, Costa LS, Rocha HAO, de Macedo GR, Santos ES. Bioprocess Biotech 2012; 2: 2.
[8] Park JK J. Natur Appl Sci 2013; 1: 47-58.
[9] Li K, Xing R, Liu S, Li R, Qin Y, Meng X, Li P. Carbhydr. Polym 2012; 88: 896-903.
[10] Olivier B, Jean-Yves R. Drugs Aging 2007; 24(7): 573-580.
[11] Wu H, Aam BB, Wang W, Norberg AL, Sorlie M, Eijsink VGH, Du Y. Carbohydr. Polym 2012; 89: 511-518.
[12] Ichikawa S, Tanako K, Kuroiwa T, Hiruta O, Sato S, Mukataka S *J. Biosci. Bioengin* 2002; 93(2):20l-206.
[13] Kuroiwa T, Noguchi Y, Mitsutoshi YN, Seigo S, Mukataka S, Ichikawa S. Process Biochem 2008; 43: 62-69.
[14] Zeng J, Zheng LY. *Process Biochemistry* 2002; 38:531-535.
[15] Zheng LY, Xiao YL. J Chem Eng 2004; 21: 201-205.
[16] Abedel-Nby A, Ismail AMS, Ahmed SA, Abdel-Fattah AF. Bioresource Technol 1998; 64: 205-210.
[17] Spagna G, Andreani F, Salatelli S. Process Biochem 1998; 33(1):57-62.
[18] Zheng LY, Zeng J, Zhu JF. Chem React Eng Technol 2001; 17(4):344 -348.
[19] El-Sayed EM, El-Sayed ST, Shousha WG, Shehata AN, Omar NI. Australian J Basic Appl Sci 2012; 6(4): 1-15.
[20] Choi YJ, Kim EJ, Piao Z, Yun YC, Shin YC. (2004). Appl Environ Microbiol 2004; 70: 4522-4531.
[21] Miller GL. Anal Chem 1959, 31: 426–428.
[22] Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. J Biol Chem 1951; 193: 265-275.
[23] Woodward J. Immobilized cells and enzymes: a practical Approach, 1985, p. 44, Oxford, IRL, Press Limited, England.
[24] Esawy MA, Mahmoud AR, Fattah FA. Brazil J Chemical Engineer 2008; 25: 237-246.
[25] Mostafa FAA. (2006). Microbiological and biochemical studies on bacterial fructosyltransferase. Ph.D Thesis, Cairo, Egypt, Faculty of science, Ain-Shams University.
[26] Fyiad AA, El-Sayed ST. Advances of food sciences 2007; 29, 2: 94-99.
[27] Moharam ME, Gamal AM, El-Sayed ST. J American Sci 2010; 6, 8: 131-140.
[28] Gauthier SF, Vuillemard JC, Lizotte N. J Food Biotechnol 1991; 5: 105-117.
[29] Zhou H, Yang Z, Ouyang P. J Nanjing Univ Chem Technol 1999; 21(2):68 - 71.
[30] Sinha. S, Kumar R, Dhakate SR, Chand S. Food. Engineer Biotechnol 2011; 9:119-124.