Saudi Journal of Medical and Pharmaceutical Sciences
Scholars Middle East Publishers
Dubai, United Arab Emirates
Website: https://saudijournals.com/

ISSN 2413-4929 (Print)
ISSN 2413-4910 (Online)

Immobilization of Pepper Chitosanase on Different Natural Carriers for Improving Enzyme Stability

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Original Research Article

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Article History
Received: 14.11.2017
Accepted: 18.11.2017
Published: 30.11.2017

DOI:
10.36348/sjmps.2017.v03i11.015

Abstract: The aimed of this work, was improved the stability of the chitosanase enzyme by immobilization with different natural carriers to enhance the economic of industrial biocatalytic process. It was used in preparation of biological active chitooligosaccharides. Pepper chitosanase extracted from (*Capsiuum annuum*) leaves was immobilized by four different immobilization methods with chitosan, DEAE-cellulose and sodium alginate. The resulted immobilized chitosanases were compared with respect to their immobilization efficiency, reusability and storage stability. Immobilization of chitosanase improved their enzymatic activity. They were 105, 83.8 and 65.2 U/g for immobilized chitosanase with entrapment, covalent and ionic binding methods, respectively. Immobilized chitosanase by covalent and ionic binding exhibited good reusability more than by entrapment method. Generally, the immobilized chitosanases showed better storage stability than that of the free one. The amount chitooligosaccharides produced by using immobilized chitosanase by ionic binding, covalent binding and entrapment methods was higher than that by adsorption method and also more than produced by free one. From a economical point of view, good reusability and storage stability are of the most important feature for the industrial application as biocatalytic.

Keywords: Chitosanase, Pepper (*Capsicum annuum*) leaves, Immobilization methods, Physical adsorption, ionic binding, covalent binding, Entrapment, Operational and storage stability

INTRODUCTION

Chitosanases (EC. 3.2.1.132) are of hydrolytic enzymes class acting on chitosan (a polymer of β-1,4-D glucose amine. Immobilized chitosanases were used for preparation of chitooligosaccharides which were used as a medical supply, as functional food and as a biological active compound (antibacterial, antitumor, immuno-stimulating ..etc.) [1-7].

Immobilization provides a physical support for enzymes and increases its stabilization. The main advantage for enzyme immobilization is its reusability for many times and the easy separation of the enzyme from the reaction mixture (substrates and products), which reduces the enzyme and enzymatic products cost tremendously. Immobilized enzymes were reusable, stable and suitable as specific industrial catalysts [8-10].

Immobilization of different enzymes such as lipase, invertase and L-asparaginase had been carried out [11-15]. Enzymes were immobilized by a variety of natural carriers used such as immobilization of inulinase on grafted alginate, immobilization of lipase into mesoporous silica and immobilization of commercial laccase on green coconut fiber [16-18]. Chitosanases were immobilized on chitin, DEAE cellulose and amylase-coated magnetic nanoparticles [19-21].

The principal methods for immobilization of enzymes were adsorption, covalent, encapsulation, entrapment and cross linking [22]. The immobilized enzyme and the substrate react in the microenvironment, while the free enzyme and the substrate react in the bulk solution environment. The stabilization of immobilized enzyme was depended on the number of bonds formed between the enzyme and the support matrix. However, when the immobilized enzyme acts on the macromolecular substrates, active site of the enzymes does not able to access with the substrates, lead to loss of enzyme activity [23-25].

In this present study, we improved the characterization of the pepper chitosanase by immobilization with different natural carriers. The immobilization efficiency, storage stability and

reusability of the immobilized chitosanase were studied and compared with that of the free one. The amount of chitooligosaccharides produced by hydrolysis of chitosan using the prepared immobilized chitosanases were determined and compared with that produced by the free one.

MATERIALS AND METHODS

Methods

Chitosan with molecular weight 300 and 600 KDa and 70-85 % deacetylated, N acetyl-glucosamine were purchased from Merck chemical Cò. Fresh pepper (*Capsicum annuum*) leaves were collected from the field. All other chemicals were of analytical grade.

Methods

Preparation of free pepper chitosanase

Small parts of fresh pepper leaves were homogenized thoroughly with distilled water. After 24 h stored at 5°C, they were separated by filtration through cheese cloth. Free chitosanase enzyme was precipitated by ammonium sulphate at 20-60% saturation [26]. The resulting precipitate was separated by centrifugation at 13,000 xg, 5°C, for 15 min. and dissolved in distilled water and subsequently dialyzed against distilled water for 48 h at 5°C. It was used as free chitosanase. Chitosanase activity and protein concentration were determined.

Chitosanase assay

Free and immobilized chitosanase activities were determined by quantitative estimation of the liberated reducing sugars produced from soluble chitosan [27-28]. Soluble chitosan was prepared according to the method of Choi *et al.* [29] Concentrated acetic acid was added with stirring to 10 gm chitosan suspended in 400 ml distilled water. The solution was completed to 1 L with water. The concentration of the soluble chitosan was adjusted to 1%, using 0.05 M sodium acetate buffer, pH 5.8. Reaction mixture contained 0.9 ml of 1% soluble chitosan, adequate amount of free and immobilized chitosanase and 1 ml of 0.05 M sodium acetate buffer, pH 5.8 was incubated at 40°C for 1.0 h. Terminated the reaction by heating the reaction mixture in boiling water bath for 10 min. The concentration of reducing sugar (chitooligosacharides and glucosamine) produced in the supernatant was determined by dinitrosalicylic acid method using glucosamine as standard [30].

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. The Specific activity of chitosanase was 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*., [31] using a bovine serum albumin as a standard.

Immobilization methods of pepper chitosanase enzyme on different natural carriers by different methods

Physical adsorption method

Immobilization of chitosanase on 300 and 600 KDa chitosan was carried out according to method of Woodward [32]. Free chitosanase solution (400 U) in 2.0 ml of o.2 M acetate buffer, pH 5.8 was mixed separately with1.0 gm of chitosan 300 and 600 KDa and incubated at 4°C. After 2 and 24 h, the immobilized chitosanase was washed three times with acetate buffer, pH 5.8 to remove the unbound free chitosanase.

Ionic binding

DEAE-cellulose (1.0 g) was equilibrated with 0.2 Macetate buffer pH 5.8 and incubated with 2 ml of free chitosanase solution (400 U/g carriers) for 2 h [32]. The unbound enzyme was removed by centrifugation and washing with 0.2 M acetate buffer, pH 5.8.

Covalent binding

One g chitosan was shaken in 5ml 0.1M HCl containing different concentration of glutraldehyde ranged from 2 to 5% for 2 h at 30°C. Chitosan was precipitated by addition of one ml of 0.1M NaOH and were collected by filtration and washed with distilled water. The wet chitosan was mixed with 2.0 ml of free chitosanase solution with stirring for 1.0 h at 30°C. The unbound free chitosanase was removed by washing with distilled water.

Entrapment

1. Entrapment in calcium-alginate beads.

Entrapment of chitosanase in calcium-alginate was carried out according to Fraser and Bickerstaff [33]. Free chitosanase solution (400 U) were mixed with 1.0 gram of sodium alginate at different concentration ranged from 0.5 to 3 %, separately. The alginate-enzyme mixture was made into beads by dropping the alginate solution in 0.15 M calcium chloride as crosslinking agent. After 20-30 min , alginate beads were collected and washed with 0.2 M acetate buffer, pH 5.2 to remove the unbound free chitosanase.

2. Entrapment in carrageenan.

Entrapment of the chitosanase in carrageenan was carried out using the method of Iborra *et al*. [34]. Free chitosanase solution (200 units in acetate buffer, 0.2M, pH 5.8) were mixed with 20 ml of carrageenan solution with different concentration from 1 to 3 % at 4 °C. Then, this solution was then dropped into a stirred cold solution of 0.3 M potassium chloride. After 10

mins. beads were filtered off and washed with distilled water.

Activities of all prepared immobilized chitosanase were determined. Free chitosanase activity and protein concentration were determined in each wash solution which contained unbound free chitosanase.

Efficiency of immobilization was determined:

1- Immobilization yield (%) $= \frac{\text{Immobilized chitosanase activity (U)}}{\text{Free chitosanase bound (U)}} \text{ x } 100$

Where, free chitosanase bound = [Free chitosanase added (U) – Unbound free chitosanase (U)]

2- Degree of hydrolysis (D.H.) % $= \frac{\text{Chitooligosaccharides concentration (mg)}}{\text{Chitosan concentration (mg)}} \text{ x } 100$

Production of chitooligosaccharides using the prepared immobilized chitosanase

Reaction mixture contain 0.1g immobilized enzyme, 0.9 ml 1% soluble chitosan and 1.0 ml acetate buffer, pH 5.8 was incubated for 1.0 h at 40 °C. The amount of chitooligosaccharides supernatant was determined.

Operation stability of the immobilized chitosanases

Reaction mixture, contained 0.5 gm immobilized chitosanase and 10 ml 1% soluble chitosan was incubated in a water bath at 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 reaction was repeated for 10 times. Chitosanase activity was determined.

Storage stability of immobilized chitosanase

The free and immobilized enzymes were stored in distilled water and 0.5 M acetate buffer, respectively, at -4ºC for 30 days. The residual activities of the free and immobilized chitosanases were measured by the standard assay procedure as described before. They were expressed as a percentage of the residual activity compared to the initial activity.

Statistical analysis

Data are expressed as the mean ± standard error (SE) from at least three experiments. Standard error of the mean was represented by errors bars.

RESULTS AND DISCUSSION

The use of free enzymes in industrial applications has been limited mainly due to high enzyme cost, their instability and irrecoverability. Looking for carriers have low economic cost and lead to greater product purity was always the aim for many researchers. Industrial enzymes are widely immobilized in natural and synthetic polymer.

In the present work, *Capsicum annuum* (pepper) chitosanase was immobilized on different natural carriers using different methods of immobilization such as physical adsorption, ionic binding, covalent binding and entrapment. The immobilization efficiency was calculated to choice the best carries and method. It based on determination immobilization yield, immobilization activity, specific activity and degree of hydrolysis represent.

Physical adsorption on chitosan

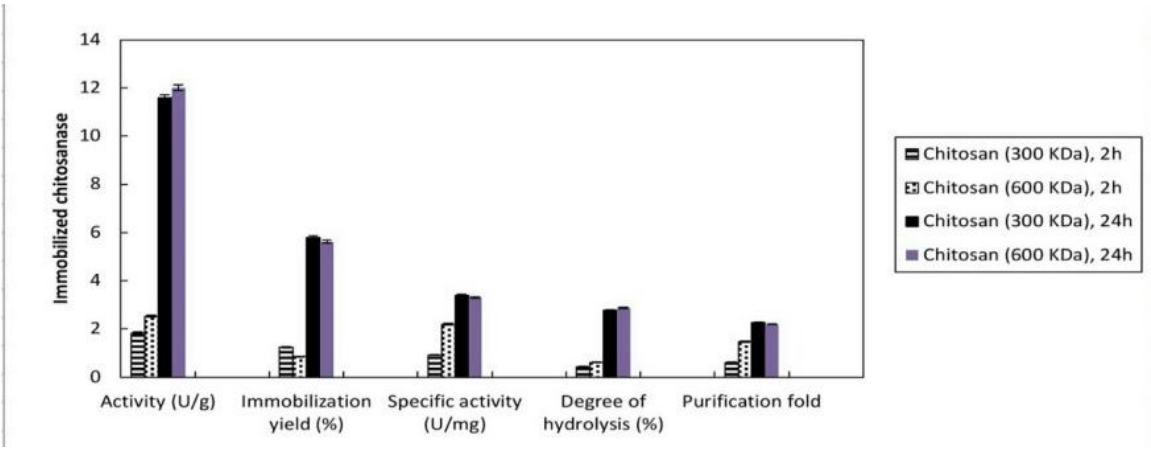

Fig-1: Immobilization efficiency of immobilized chitosanase by physical adsorption method on chitosan.

Ionic bonding with DEAE-cellulose

Immobilization of free chitosanase by physical adsorption on chitosan 300.0 and 600.0 KDa) was carried out. Efficiency of chitosanase immobilization on chitosan for 24 h immobilized time was higher than that with 2 h (Figure 1). The resultant of adsorption also showed that increase of free enzyme bound by 1.46 times lead to increase of the immobilization by 4.15 times. The preparation of immobilized chitosanase by the adsorption method had been reported [35]. Immobilization of chitosanase by physical adsorption showed lowest immobilization efficiency than that by other immobilization methods.

DEAE-cellulose was used for the immobilization of free pepper chitosanase by ionic biding. Immobilization of chitosanase by ionic binding gave immobilization yield 27.4±0.183 % with immobilization activity 83.77±0.562 U/g carrier, which was higher than by physical adsorption on chitosan. Figure (2) represented the immobilization efficiency of the immobilized chitosanase with DEAE cellulose.

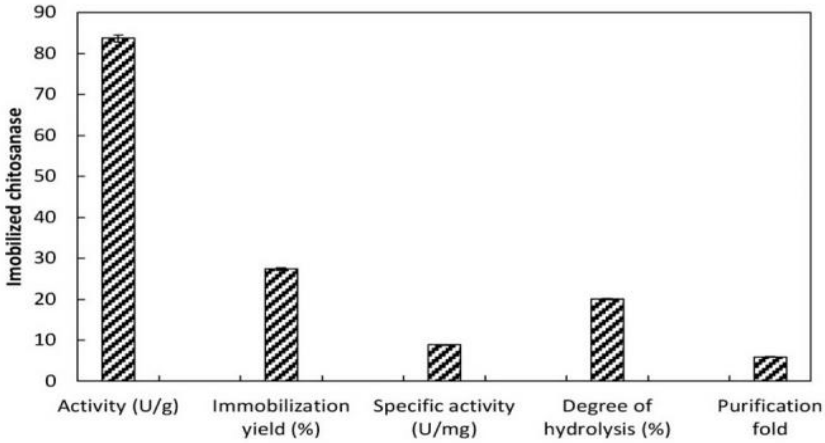

Fig-2: Immobilization efficiency of immobilized chitosanase by ionic bonding method on DEAE: cellulose

Covalent binding on chitosan

Immobilization of free chitosanase with chitosan by covalent binding at different concentrations of glutaraldehyde (GA) ranging from 2 to 5 % was investigated (Figure 3). The immobilization efficiency of the enzyme which was immobilized with chitosan was increased with increasing concentration of glutaraldehyde and was accomplished with slight decrease in free chitosanase bound percent. The optimal concentration of glutaraldehyde for immobilization free chitosanase with chitosan was 5%. The immobilization efficiency of chitosanase with chitosan at best glutaraldehyde concentration (5 %) was as follow: immobilization yield 23.5 ± 0.10 %, immobilization activity 65.2 ± 0.208 U/g carrier, purification fold 4.53 times and degree of hydrolysis 15.61 %.

This may be explained by the more free amino groups on the chitosan, the more amino group crosslinked by glutaraldehyde and consequently the more glutaraldehyde will be needed for immobilization. This lead to increase immobilization efficiency. The concentration of glutaraldehyde used for chitosanase immobilization was similar to that of immobilized glucoamylase [36]. However, Zhou *et al*. [37] and Spagna *et al*. [38] reported that 2 and 0.5%, respectively glutaraldehyde was sufficient for enzyme immobilization.

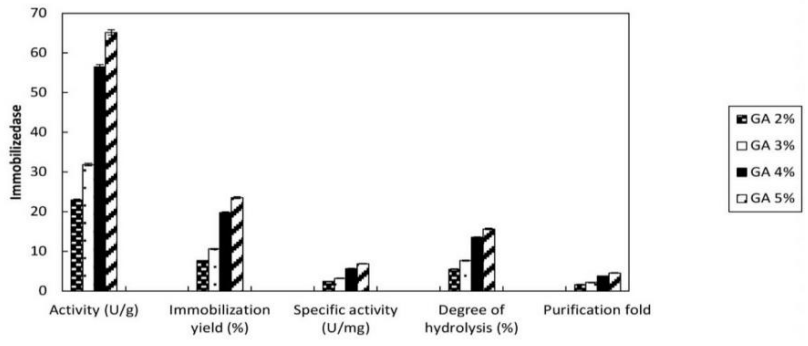

Fig-3: Immobilization efficiency of immobilized chitosanase by covalent binding method on chitosan at different glutraldehyde concentrations

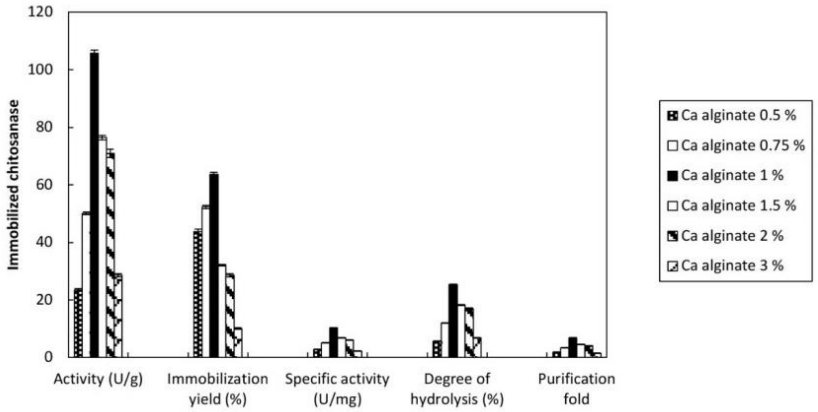

Fig-4: Immobilization efficiency of immobilized chitosanase by entrapment method at different sodium alginate concentrations

Entrapment of chitosanase in calcium alginate beads

Entrapment of free pepper chitosanase in calcium alginate beads with different concentrations (0.5, 0.75, 1, 1.5, 2 and 3%) was carried out (Figure 4). Immobilization efficiency of immobilized chitosanase was increased with increasing concentration of sodium alginate up to 1%. When the concentration of sodium alginate was higher than 1%, immobilization efficiency was decreased. Immobilization efficiency values at 1% sodium alginate were found to be immobilization activity 65.2 ± 0.28 U/mg, immobilization yield 63.7±0.277 %, purification fold 6.87 times and degree of hydrolysis 25.32 %. While results of Immobilization of chitosanase by entrapment in carrageenan indicated the unsuitability of this carrier.

Figure (5) summarized the immobilization efficiency of the chitosanase by different methods. Immobilization of chitosanase by physical adsorption showed lowest immobilization efficiency than that by other immobilization methods. 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*. [39] and contrary to that reported by Mostafa [40]. However, lipase enzyme was immobilized successfully with high specific activity than by physical adsorption on tricalcium phosphate gel and active carbon [12]. Immobilization of chitosanase by ionic binding was higher than by physical adsorption on chitosan. Among those four immobilization methods, covalent binding method was the optimal method for chitosanase immobilization on chitosan at 5% glutaraldehyde with high immobilization efficiency. Immobilization with chitosan and in alginate beads were the best carriers. Similarly, immobilization of chitosanase purified from *Bacillus* sp. R2 with covalent and ionic binding was the best techniques [41].

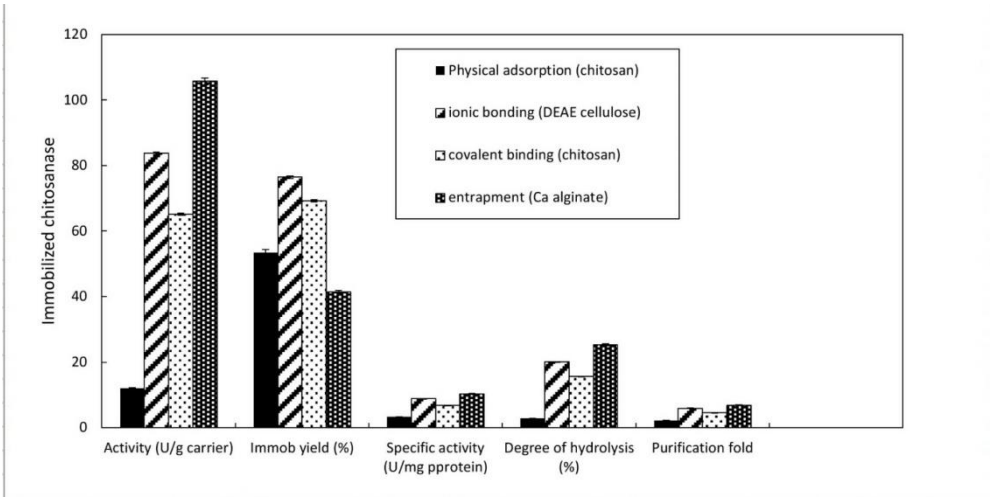

Fig-5: Summarize the immobilization efficiency of immobilized chitosanases by different methods and carriers

Production of chitooligosaccharides using the prepared immobilized chitosanase

The amount chitooligosaccharides produced by using the prepared immobilized chitosanases by ionic binding, covalent binding and entrapment methods was higher than by adsorption method and also more than produced by free one (Table 1).

Table-1: Production of chitooligosaccharides from chitosan by the prepared immobilized chitosanases

Immobilized chitosanase by different method	Chitooligosaccharides production	
	(mg/100ml)	Times
Physical adsorption	162.8	0.53
Ionic binding	903.2	2.9
Covalent binding	703.0	2.38
Entrapment	1044.7	3.43
Free chitosanase	304.6	1.0

Operation stability of the prepared immobilized chitosanase by different immobilization methods

Hydrolysis of chitosan by the prepared immobilized chitosanases were repeated for ten times. After each reaction, the residual enzyme activity was determined. Immobilized chitosanase by covalent and ionic binding showed good operation stability (Figure 6). The weak bound between enzyme and carrier by physical adsorption may be lead to leak out of enzyme and consequently poor reusability. Glutaraldehyde as cross linking agent for attaching enzyme with chitosan by covalent binding showed good reusability. This may be due to the stable nature of the bonds formed between chitosanase and chitosan. Similarly, Zeng and Zheng [20] reported that covalent binding chitosanase with chitin gave a good operation stability. The enzyme activity loss was less than 20 % after 10 times batch reaction.

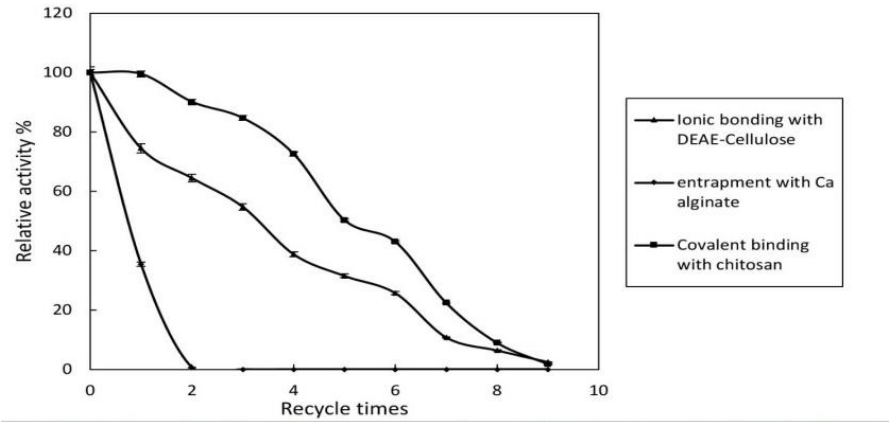

Fig-6: Operation stability of immobilized chitosanases by different methods and carriers. Initial activity was taken as 100%

Storage stability of the immobilized chitosanase by different methods

The immobilized and free chitosanases were stored in distilled water at -4ºC for 30 days. The residual enzyme activities were measured after 30 days and were compared with the initial one (Table 2). The residual activities of all immobilized chitosanase were higher than that of the free chitosanase. Free chitosanase lost about 44 % of its initial activity, while immobilized one lost about 15-25 %.

Table-2: Storage stability of free and immobilized chitosanases stored in distilled water for 30 days at -4°C

Method of immobilization	Initial activity (U)	Residual activity	
		(U)	(%)
Physical adsorption	15.1 U/carrier	11.66 U/carrier	77.2
Ionic binding	83.77 U/carrier	69.14 U/carrier	82.5
Covalent binding	65.2 U/carrier	57.05U/mg	87.5
Entrapment	96.9 U/gel	71.7 U/gel	74.03
Free chitosanase	28.25 (U/ml)	15.87 (U/ml)	56.2

CONCLUSION

Generally, immobilized chitosanases especially by covalent binding on chitosan with 5 % glutraldehyde as cross linkage, ionic binding with DEAE-Cellulose and alginate beads had higher activity, operation and storage stability. The immobilization of the enzyme causes an increase in enzyme rigidity which lead to increase its stability. The operation stability and storage stability of the immobilized chitosanase were much better than free one. The attractive results of this study made immobilized chitosanase contributed for possible application in industrial processes.

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