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STABILITY IMPROVEMENT OF IMMOBILIZED ALKALINE PHOSPHATASE USING CHITOSAN NANOPARTICLES

F. Jafary[1*], M. Panjehpour[2], J. Varshosaz[3] and P. Yaghmaei[1]

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
Phone: + 98 9132152195; Fax: + 98 0313 6688597
[*]E-mail: f.jafary@ymail.com; yaghmaei_ p@yahoo.com
[2]Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences,
Isfahan University of Medical Sciences
E-mail: Panjehpour@ pharm.mui.ac.ir
[3]Department of Pharmaceutics, School of Pharmacy and Novel Drug Delivery Systems
Research Centre, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: varshosaz@pharm.mui.ac.ir

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Abstract - Enzyme engineering via immobilization techniques is a suitable approach for improving enzyme function and stability and is superior to the other chemical or biological methods. In this study chitosan nanoparticles were synthesized using the Ionic Gelation method and were characterized by Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Alkaline phosphatase was successfully immobilized on the chitosan nanoparticles in optimum conditions. Chitosan nanoparticles were used because of their special properties for enzyme immobilization. This study indicated that the immobilized enzyme has improved function at high temperature and during storage. Immobilization resulted in an increased range of optimum pH and temperature, and reusability of enzyme. Furthermore, the binding efficiency calculation indicated that the immobilized alkaline phosphatase conserved 71% of its native activity. Kinetic parameter studies indicated no significant difference between the immobilized and free enzymes.
Keyword: Alkaline phosphatase; Chitosan nanoparticles; Enzyme Immobilization; Stability.

INTRODUCTION

Improvement of enzyme properties via immobilization techniques, i. e., adsorption, multipoint and multisubunit covalent binding, entrapment, etc is important in different fields. Immobilization can improve functional properties such as stability, specificity and activity of enzymes in harsh conditions. The immobilized enzyme can also remain active for long time intervals and be reused for several times in industrial reactors (Mateo *et al*., 2007; Nisha *et al*., 2012).

Alkaline phosphatase (ALP) is a hydrolase enzyme that removes phosphate groups from many kinds of molecules (EC3.1.3.1) and can be obtained from various sources [Muginova *et al*., 2007]. ALP can be used in different fields such as immunoassays and electrochemical immunosensors as a labeling enzyme in the dairy industry as an indicator of successful pasteurization, in molecular biology laboratories for preserving DNA molecules, etc. (Muginova *et al*., 2007; Kreuzer *et al*., 1999; Rankin *et al*., 2010; Rittie and Perbal, 2008). Therefore, the improvement of the enzymatic functional stability is important.

*To whom correspondence should be addressed

A variety of organic and inorganic supports are available for immobilization to ensure the highest retention of enzyme activity and stability, such as polymers, biopolymers etc. In recent years, nanoparticles have attracted more attention due to their excellent properties and interesting applications [Mateo *et al*., 2007; Liu *et al*., 2012].

Chitosan is a natural hydrophilic polysaccharide with special physical-chemical and biological properties, including non-toxicity, chelating metal ions, biodegradability, antibacterial and antifungal effects, immune system stimulation, etc. (Wang *et al*., 2011; Kong *et al*., 2010; Ravi Kumar, 2000). Therefore, it can be applied in different industries such as biomedicine, food, biotechnology, pulp and paper, agriculture, cosmetics and can be considered as an attractive candidate for a variety of technological applications, including nasal and oral delivery of polar drugs, vaccine delivery, and tissue engineering (Wang *et al*., 2011; Venkatesan and Kim, 2010, Ravi Kumar, 2000). In addition, because of chitosan's low cost and robustness, which improve enzyme resistance to chemical degradation and prevent disturbance caused by metal ions, it is considered as a suitable support for immobilizing enzymes (Krajewska, 2004).

The aim of this study was to indicate the effect of immobilization on alkaline phosphatase properties. We proposed that the immobilization could increase the stability of alkaline phosphatase (ALP) and improve its properties, which are essential for its applications in various fields mentioned earlier. Another point of this work is to demonstrate the importance of chitosan as a biological support, and its application in living organisms, unlike previous supports such as glass, silica films, polystyrene, etc. (Jafary *et al*., 2009).

In this study we synthesized chitosan nanoparticles using the Ionic Gelation method and immobilized alkaline phosphatase on them according to the method described by Bindhu and Abraham (2003). EDC (1-ethyl-3-(3-dimethylaminopropyl carbodiimide) was used for covalent immobilization of the enzyme, which is quite simple and efficient for enzyme immobilization. The properties of the immobilized enzyme were then studied.

MATERIALS AND METHODS

Reagents

p-Nitrophenyl phosphate, Alkaline phosphatase from bovine intestinal mucosa with nominal activity of 1000 U/mg (one unit of ALP is defined as the amount of enzyme which hydrolysis 1 μM of *p*-nitrophenyl phosphate to *p*-nitrophenol per min at 25 °C and pH 9.5) and low molecular weight chitosan (MW ≤ 6000 D) having > 85% degree of deacetylation were obtained from Sigma Aldrich. Sodium tripolyphosphate, acetic acid and all other reagents were purchased from Merck. All reagents used in this work were of analytical grade and were used without further purification.

Preparation of Chitosan Nanoparticles

Preparation of chitosan nanoparticles was performed according to the procedure of Vaezifar *et al.* (2013). Chitosan powder (5 mg) was dissolved in 1.0% (w/v) acetic acid. Sodium tripolyphosphate solution with a concentration of 1 mg/mL was prepared by dissolving in distilled water. Then the tripolyphosphate (TPP) solution was dropped continuously into the chitosan solution stirred with a magnet stirrer. The TPP addition was continued at room temperature until the spontaneous formation of chitosan nanoparticles. The turbidity point was detected using a laser pointer and, then, the suspension obtained was centrifuged at 20.000 rpm for 10 min. Finally the chitosan nanoparticles were washed extensively with distilled water to extract any impurity and were dried at 70 °C for 24 h. The morphological characterization and particle size distribution were determined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively. In addition, FT- IR analysis was applied.

Fourier Transform Infrared Spectroscopy

The FT-IR spectrum was obtained using potassium bromide pellets on a spectrometer (JASCO FT/IR-6300, Japan) in the range of 500-5000 cm^{-1} with resolution of 4.0 cm^{-1}.

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) (Seron Technology AIS 2500, India) was used for morphological characterization of the original chitosan powder and chitosan nanoparticles. The gold coating was performed by spraying gold powder. Samples were suspended in water via sonication for 3 min to make a dilute suspension. A drop of original chitosan dilute suspension was placed on a glass slide and, after drying, was investigated.

Transmission Electron Microscopy

In order to verify the particle size distribution of the chitosan nanoparticles Transmission Electron

Microscopy (Philips CM12 TEM, operated at 120 KV) was used. The nanoparticles were suspended in deionized distilled water and ultrasonically dispersed. The nanoparticle dispersions were applied on a copper grid-supported carbon film.

Enzyme Immobilization and Assay

The immobilization performed in this work was not optimized for ALP (Bindhu and Abraham, 2003). Some modifications were performed in steps to optimize the work. The following steps were applied for ALP immobilization. 1 mg of ALP was added to 1 mL of 0.1 M Tris-HCl buffer pH 9.5, and, then, the enzyme solution was added to 5.0 mg of chitosan nanoparticles. Finally, 25 μL of EDC (concentration of 100 mg/mL) was added to the solution. The mixture was slowly stirred for 48 h at 4 °C; the solids were separated and washed with the same buffer to remove the untreated enzyme. This step continued until no enzyme activity was detected in the washes. The obtained solids were tested for enzymatic activity.

Enzyme Activity Assay

The activity assay of alkaline phosphatase was carried out according to the method which was used by Zubriene *et al.* (2002). The assay was performed at room temperature using *p*-nitrophenyl phosphate as substrate and measuring the absorbance of the liberated *p*-nitrophenoxide ion at 405 nm by quantitative spectrophotometric method. 100 μL of enzyme solution was added to 900 μl of *p*-nitrophenyl phosphate solution (1 mM) in 0.5 M Tris-HCl buffer (pH 9.5) containing 1 mM of $MgCl_2$. The mixture was incubated at 37 °C for 10 min and the absorbance was measured. The activity assay for immobilized enzyme was carried out in the same condition. The reaction was interrupted before spectrophotometric measurements to separate immobilized enzyme from the reaction mixture. Each test was performed three times for statistical validity.

Enzyme Binding Efficiency

After enzyme immobilization under the optimum condition, the solid particles were separated from the solution. Then the supernatant was collected, dialyzed against water and lyophilized. The protein content was detected using the Lowry method by dissolving the lyophilized sample in sodium phosphate buffer. The binding efficiency (amount of retained protein that remains active) was considered as the ratio of the percentage of immobilized enzyme and the percentage of retained protein. This experiment was carried out three times for statistical validity.

Properties of the Immobilized ALP

The Optimum Temperature

The optimum temperature of native and immobilized enzyme was detected by heating them in a water bath for 1 min at different temperatures ranging from 20 to 50 °C in 0.1 M Tris buffer, pH 9.5. Then reaction was started by adding the substrate solution and stopped after 1 min by separation of immobilized enzyme from the reaction mixture via filtration. The reaction containing free enzyme was stopped by adding the stop solution (90 μl of 0.1 M NaOH and 0.1 M EDTA).

The Optimum pH

To determine the optimum pH for free and immobilized ALP, the activity was measured in different pH buffer solutions, ranging from 7 to 11, changing the buffer solution as follows: 0.1 M sodium phosphate buffer (pH 7.0 to 8.0) and 0.1 M Tris buffer (pH 9.0 to 11.0).

Thermal Stability

Thermal stability studies were performed via suspending both the native and immobilized enzymes in 0.1 M Tris buffer, pH 9.5, and incubating the mixture in a water bath at 50 °C and 60 °C for different time periods. After the appropriate time, the mixture was immediately transitioned to an ice bath and then assessed for the remaining activity.

Stability During Storage

To evaluate the storage stability of immobilized enzyme, the native and immobilized enzymes were stored at room temperature (approximately 30 °C) and at 4 °C in 0.1 M Tris buffer, pH 9.5. Then, at regular periods of time, aliquots were tested to measure the enzyme activity according to the method described previously.

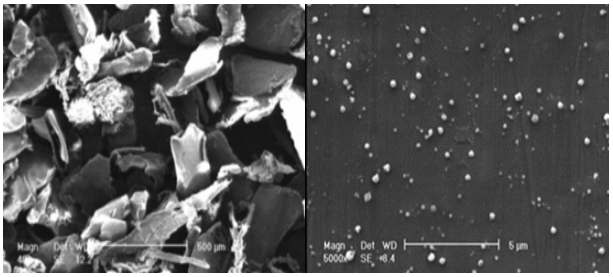
Determination of the Michaellis-Menten Constants

Determination of the Michaellis-Menten constants (Km and V_{max}) were carried out by the Lineweaver-Burk method at different concentrations of *p*-nitrophenyl phosphate ranging from 5×10^{-4} to 1×10^{-2} mol L^{-1} in 0.1 M Tris buffer, pH 9.5, at room temperature.

RESULTS AND DISCUSSION

Characterization of Chitosan Nanoparticle

The SEM and TEM images of chitosan and chitosan nanoparticles are shown in Fig. 1(a), Fig. 1(b) and Fig. 2, respectively. The morphology of chitosan powder is flakes but the chitosan nanoparticles are round. The TEM images of chitosan nanoparticles show that the size of the particles is under 100 nm.



(a) (b)

Figure 1(a), 1(b): SEM images of chitosan and chitosan nanoparticles.

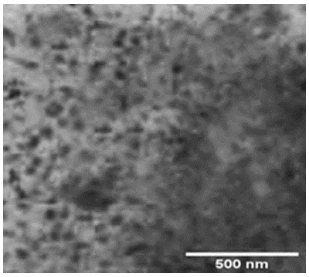


Figure 2: TEM images of chitosan nanoparticle.

Analysis of FT-IR Spectra

The FT-IR spectra of chitosan and chitosan nanoparticles are illustrated in Fig. 3. There were no major differences in the FT-IR spectra of chitosan powder and chitosan nanoparticles. In both of them the band at 3436 cm^{-1} was assigned to NH_2 and OH stretching vibrations. The bands at 1657 cm^{-1}, 1598 cm^{-1} and 1320 cm^{-1} were attributed to Amide I, - NH_2 bending and Amide III, respectively (Vaezifar *et al*., 2013; Zhang *et al*., 2012). The only difference was observed at 1155 cm^{-1} in chitosan nanoparticles. The peak of P=O appeared at 1155 cm^{-1} and could be assigned to the linkage between ammonium ions and phosphate groups (Zhang *et al*., 2012).

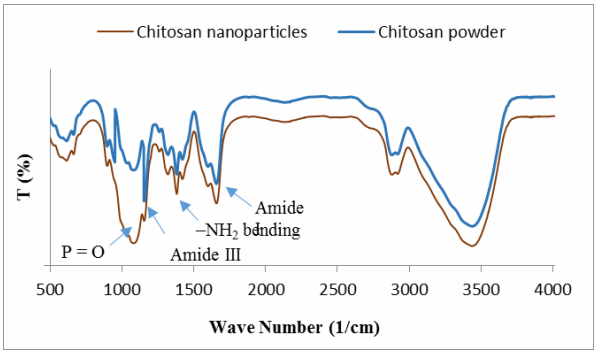


Figure 3: FT-IR spectra of chitosan and chitosan nanoparticles.

Efficiency of Enzyme Immobilization

The binding efficiency of the immobilized enzyme was determined as the ratio between the percentage of immobilized enzyme and the percentage of retained protein. The results are summarized in Table 1. As is obvious 54.6% of the available enzyme was immobilized and 76.23% of the protein was retained. Determination of the binding efficiency indicated that 71% of the immobilized protein maintained its native activity. Similar investigations were performed by Hirano and Miur (1979) and Zubriene *et al.* (2003). They used chitosan polymer and chitosan microparticles to immobilize alkaline phosphatase, respectively. The binding efficiency for chitosan polymer was 61% and for chitosan microparticles was 49.5%. The results indicate that the chitosan nanoparticles, because of their high surface in comparison to chitosan polymer and chitosan microparticles, are better for immobilization.

Table 1: Efficiency of immobilizing alkaline phosphatase onto chitosan nanoparticles.

Total enzyme		Immobilized enzyme		Total protein		Retained protein		Binding efficiency[b]
U[a] (1000 U/mg)	%	U(546 U/mg)	%	mg	%	mg	%	
1000±11.8	100	546±9.3	54.6%	1.0±0.4	100	0.762±0.06	76.23%	0.71

Average ± standard deviation (n = 5)
[a]U means the enzyme unit
[b]Binding efficiency calculated as the percentage of U(54.6%) / percentage of retained protein (76.23%).

The Optimum Temperature

The optimum temperature curve is depicted in Fig. 4. In contrast to the free enzyme, the immobilized enzyme does not show an optimum temperature, but a maximum activity over the temperature range 30-50 °C. This observed change in optimum temperature was probably because of the restricted mobility of the immobilized enzyme, which is caused by the immobilization procedure. Similar results have been reported in other publications for the immobilization of enzymes onto chitosan polymer and chitosan nanoparticles (Hirano and Miur, 1979; Tang *et al*., 2006).

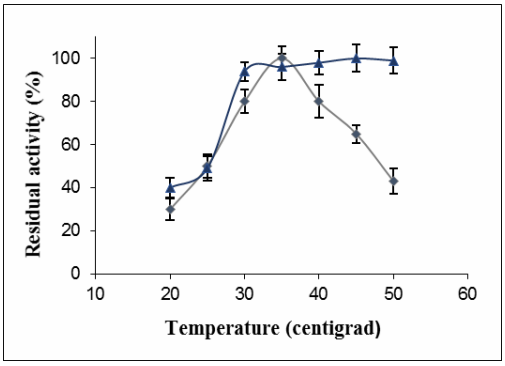


Figure 4: Optimum temperature of the free (◆) and immobilized (▲) enzymes.

The Optimum pH

The effect of pH on the native and immobilized enzyme activities is shown in Fig. 5. In contrast to the free enzyme, the immobilized enzyme does not show an optimum pH, but a maximum activity over the pH range 7.75-9.5.

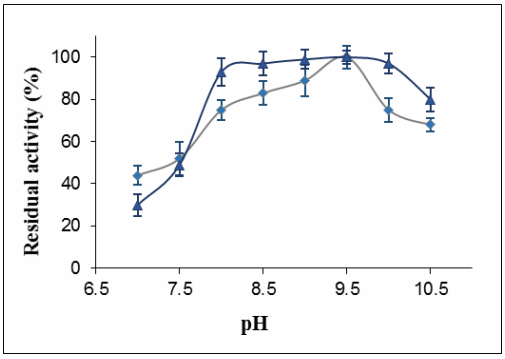


Figure 5: Optimum pH of the free (◆) and immobilized (▲) enzymes.

Similar results have been reported in the literature (Hirano and Miur, 1979; Tang *et al*., 2006). In addition, beta-glucosidase exhibited broader pH and temperature ranges when immobilized on chitosan beads using the crosslinking-adsorption-crosslinking method (Zhou *et al*., 2013).

Effect of Temperature on Enzyme Stability

The thermal stability curves of free and immobilized ALP at 50 °C and 60 °C are shown in Fig. 6. The free enzyme has a lower resistance at high temperature compared to the immobilized enzyme. The immobilized enzyme kept less than 80% of its initial activity after 120 min at 60 °C and approximately 90% of its activity at 50 °C after the same time period while the free enzyme kept only 30% of its activity after 120 min at 60 °C and maintained 60% of its initial activity at 50 °C during the same period of time. This enhancement in denaturation resistance of the immobilized enzyme was probably due to the multipoint attachment acquired in the immobilization procedure, which increases the enzyme rigidity and protects it from unfolding.

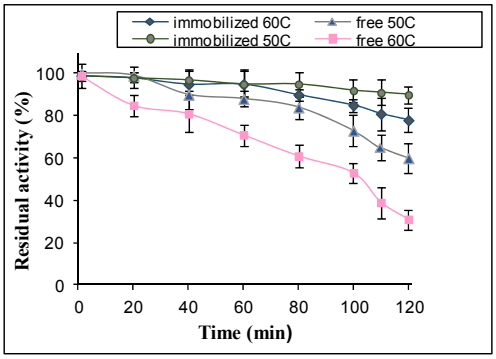


Figure 6: Thermal stability of the free and immobilized enzymes at 50 °C and 60 °C.

Similar result was obtained by Tang *et al.* (2006) using chitosan nanoparticles for immobilization of neutral proteinase. The loss of activity of immobilized enzyme was less than 5% and 10% after 120 min at 40 °C and 50 °C, respectively, but the free enzyme activity decreased significantly during this time. Egwim *et al.* (2012) reported that lipase immobilized onto chitosan beads had improved thermal, operational and storage stabilities. In a study performed by Kamburov *et al.* (2011) on covalent immobilization of trypsin on chitosan macrobeads, immobilization led to the increase of the enzyme heat resistance. Adriano *et al.* (2005) indicated that the immobilization of penicillin G on chitosan activated by glutaraldehyde can increase the stability of the enzyme 4.9-

fold at 50 °C and 4.5-fold at pH 10.0. Kuo *et al.* (2012) immobilized lipase on chitosan-coated Fe_3O_4 nanoparticles activated with EDC as coupling agent. The reusability and operational stability of lipase were increased by immobilization. The immobilized enzyme had wider thermal and pH ranges.

Stability of Immobilized Enzyme During Storage

The measurement of the immobilized and free enzyme activities during storage at 4 °C and 30 °C indicated that the stability of the enzyme was increased by the immobilization process. It should be added that the immobilized enzyme kept its full activity for 30 days at 4 °C and was active after 10 days of storage at 30 °C and then began to lose its activity gradually. The native enzyme retained more than 85% of its initial activity at 4 °C and lost 100% of its activity at 30 °C by 30 days. In addition, reusability evaluation gave remarkable results; the activity of immobilized enzyme was retained 100% after 6 reuses. The results are shown in Fig. 7 and Fig. 8, respectively. Natural proteinase immobilized onto chitosan nanoparticles also showed improved storage stability.

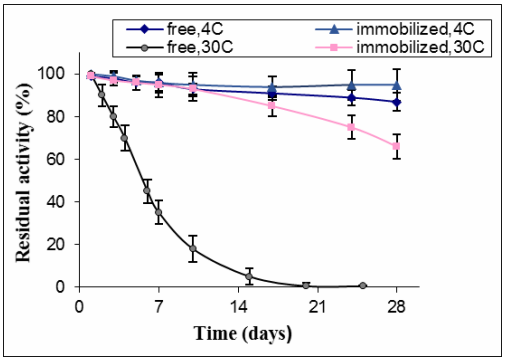


Figure 7: Storage stability of the free and immobilized enzymes.

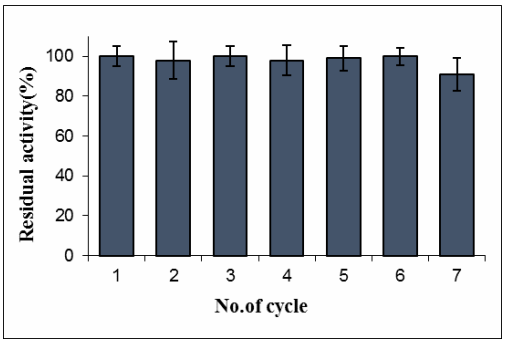


Figure 8: Reusability of immobilized ALP on chitosan nanoparticle.

The immobilized enzyme had higher activity than the native enzyme during storage at 4 °C and 30 °C (Tang *et al.*, 2006). In another study, Zebrine *et al.* (2003) indicated that the storage stability of β-galactosidase immobilized on microchitosan was very high. The immobilized enzyme kept 90% of its initial activity after 240 days at 4 °C. Pullulanase and maltogenase immobilized on chitosan microparticles had lower stability. Alkaline phosphatase immobilized onto magnetic nanoparticles showed similar result. Immobilized alkaline phosphatase was found to be more active (at least after a 16-week storage period at 4 °C) in comparison to the free enzyme (Saiyed *et al.*, 2007). Park *et al.* (2013) indicated that the reusability and storage stability of lysozyme-CLEA were increased via immobilization onto electrospun chitosan nanofibers.

Michaellis-Menten Parameters

The kinetic parameters and Lineweaver – Burk plots of free and immobilized enzymes are shown in Table 2 and Fig. 9. As shown, there is no statistical difference between the kinetic parameters of the free and immobilized enzymes. The slight increase in K_m after immobilization means that the immobilized enzyme had less affinity for the substrate. This is because of the restricted access to the active site of the immobilized enzyme which occurs during the immobilization process.

Table 2: Kinetic parameters of free and immobilized enzyme.

Parameters	Free ALP	Immobilized ALP
K_m and $K_{m.app}$ (mol L^{-1})	0.21±0.05	0.38±0.06
V_{max} and $V_{max.app}$ (mol L^{-1} min^{-1})	0.60 ± 0.04	0.87±0.08

Values are the mean ± standard deviation (n=3).

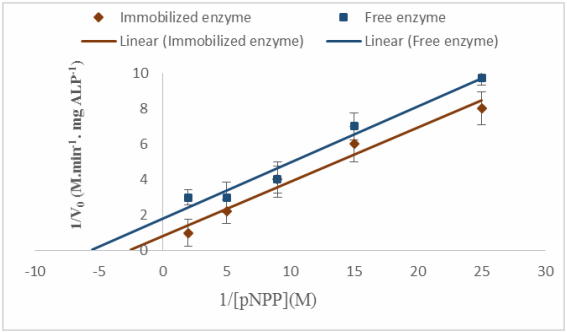


Figure 9: Lineweaver–Burk plots for the free and immobilized enzymes. Substrate concentration varied from 5×0^{-4} to 1×10^{-2} mol L^{-1}.

The K_m value of neutral proteinase immobilized onto chitosan nanoparticles was higher than its free form which is similar to our result (Tang *et al*., 2006).

Dong *et al.* (2011) in their research also indicated that immobilization of glucose oxidase on a modified chitosan led to an increase in K_m and decrease in V_{max}, so the affinity of immobilized enzyme to substrate was decreased. According to Krajewska *et al.* (1989) urease immobilized on chitosan membrane had improved storage stability and reusability; the Michaelis constant of immobilized urease was about five times higher than that of the free enzyme.

CONCLUSIONS

Alkaline phosphatase (ALP) is a metaloenzyme and has a variety of industrial applications. Therefore, improvement of its functional stability via immobilization techniques is important, especially from the economic point of view.

In the present study ALP was efficiently immobilized onto chitosan nanoparticles using covalent attachments and EDC as crosslinker. A higher immobilization yield (71%) was observed in comparison to other kinds of chitosan used for immobilization of ALP (chitosan microparticles (49.5%) and chitosan polymer (61%)). Chitosan nanoparticles showed a satisfactory capability to preserve the enzyme (76.23%) and to maintain its activity (54.6%). The Immobilized enzyme had higher stability in comparison to the free enzyme at high temperature. In contrast to the free enzyme, the immobilized enzyme remained active over a broader pH (7.75-9.5) and temperature (30-50 °C) range. Immobilization also resulted in an increase in the storage stability of the enzyme, but no significant difference in kinetic parameters between the free and immobilized enzymes was observed. In addition, the reusability of immobilized enzyme was increased by immobilization, so that after 6 operational cycles it kept its full initial activity.

The efficiency of immobilization depends on the properties of the enzymes and supports. However, this study and previous studies on immobilization of various enzymes onto chitosan and its derivatives indicated their suitability as supports for immobilization.

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