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Immobilization of thermoalkalophilic recombinant esterase enzyme by entrapment in silicate coated Ca-alginate beads and its hydrolytic properties

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ABSTRACT

Thermoalkalophilic esterase enzyme from Balçova (Agamemnon) geothermal site were aimed to be immobilized effectively via a simple and cost-effective protocol in silicate coated Calcium alginate (Ca-alginate) beads by entrapment. The optimal immobilization conditions of enzyme in Ca-alginate beads were investigated and obtained with 2% alginate using 0.5 mg/ml enzyme and 0.7 M $CaCl_2$ solution. In order to prevent enzyme from leaking out of the gel beads, Ca-alginate beads were then coated with silicate. Enzyme loading efficiency and immobilization yield for silicate coated beads was determined as 98.1% and 71.27%, respectively and compared with non-coated ones which were 68.5% and 45.80%, respectively. Surface morphologies, structure and elemental analysis of both silicate coated and non-coated alginate beads were also compared using Fourier Transform Infrared Spectroscopy (FT-IR) and Scanning Electron Microscope (SEM) equipped with Energy-dispersive X-ray spectroscopy (EDX). Moreover, silicate coated alginate beads enhanced reusability of esterase in continuous processes compared to non-coated beads. The hydrolytic properties of free and immobilized enzyme in terms of storage and thermal stability as well as the effects of the temperature and pH were determined. It was observed that operational, thermal and storage stabilities of the esterase were increased with immobilization.

1. Introduction

Esterases (EC 3.1.1.1) belong to the enzyme class of hydrolases and catalyze the hydrolysis of ester bonds, generating alcohol and carboxylic acids. They play an important role in a variety of biotechnological, industrial, environmental and pharmaceutical applications because of their many useful properties [1–3]. Especially, esterases from extremophiles having excellent features are great importance for the utilization of enzyme in a variety of reactions [4–6]. Thermoalkalophilic esterases can perform better in a number of commercial applications compared to other enzymes because of their surprising high resistance against heat and pH.

Although enzymes are excellent tool in a wide range of biotechnological, biomedical, pharmaceutical and industrial applications due to their important catalytic properties, the utilization of them as a free form is not always suitable and optimal for these processes because of their low stability, inhibition by high concentrations of substrates and products, low activity and selectivity toward non-natural substrates under nonconventional conditions, and so on. Enzyme immobilization technology may be an effective way not only to overcome these unsuitable characteristics but also to allow reuse of enzymes for many reaction cycles of industrial processes. Immobilization of enzymes may offer additional benefits over free enzymes such as possibility of continuous process, controlled product formation, rapid termination of reactions and ease of enzyme removal from the reaction mixture [7].

There are a large number of support materials and methods for the immobilization of enzyme. Entrapment is one of the simplest methods available for enzyme immobilization under mild conditions compared with other immobilization methods [8]. Ca-alginate beads are one of the most commonly used carriers in the entrapment immobilization of enzymes because of their significant advantages such as good biocompatibility, low cost, availability and resistance to microbial attack [9]. However, there are some disadvantages using this method and support material as well including low mechanical strength, large pore size and leakage of enzyme from beads [10]. Until now, various methods have been proposed to optimize the entrapment efficiency such as coating the surface of alginate beads with cross-linked biopolymers [11–14].

Since esterase enzymes are special interest in a variety of biotechnological applications and utilization of them as a free form is not suitable, in this study, for the first time we have used thermoalkalophilic recombinant esterase enzyme from Balçova Geothermal region [15] for immobilization studies. Enzyme was immobilized effectively via a simple and cost-effective protocol using Ca-alginate by entrapment. The effects of immobilization conditions on enzyme loading efficiency and immobilization yield have been examined and various parameters like concentration

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of alginate, $CaCl_2$ and enzyme were determined in details. In order to overcome leakage of entrapped enzyme, Ca-alginate beads were coated with silicate. As far as we aware, the immobilization of a thermoalkalophilic enzyme by these matrixes has not been reported in the literature. For this purpose, using the optimum immobilization conditions of esterase enzyme in silicate coated Ca-alginate beads, we have determined the hydrolytic properties of immobilized enzyme in terms of storage, operational and thermal stability as well as the effects of the temperature, pH and bead size on the activity of the immobilized esterase and compared with the free enzyme.

2. Materials and methods

2.1. Enzyme preparation

Thermoalkalophilic esterase from *Geobacillus* sp. isolated previously from *Balçova* (*Agamemnon*) Geothermal Site in *İzmir* of *Turkey* was used in this study [15]. Expression of the recombinant enzyme in *Escherichia coli* and purification using one step affinity chromatography were carried out according to Tekedar and Şanlı-Mohamed [15] procedures.

2.2. Immobilization of thermoalkalophilic esterase enzyme in Ca-alginate beads

The most efficient condition of thermoalkalophilic esterase enzyme in Ca-alginate beads were investigated under various % sodium alginate, $CaCl_2$ and enzyme concentration. Alginate (1–4%, w/v) was mixed with Tris–HCl buffer (0.1 M, at pH 8.0) for 30 min at 50 °C. The solution was waited in 4 °C for 10 min to eliminate bubbles. Esterase enzyme (0.01, 0.1, 0.5 and 1 mg/ml) was mixed with alginate solution (total 10 ml) then the mixture was stirred thoroughly to ensure complete mixing for 10 min. As soon as the mixed solution was dripped into 75 ml of $CaCl_2$ solution (0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 M) with a syringe, Ca-alginate beads were formed. After 30 min of hardening, the beads (more or less 500 beads) were separated from the calcium chloride solution by vacuum filtration and washed on a filter thrice with distilled water. Enzyme loading efficiency (LE) and immobilization yield (IY) was determined according to the equations indicated below:

$$\mathrm{LE} = \frac{\text{total esterase} - \text{free esterase}}{\text{total esterase}} \times 100 \quad (1)$$

$$\mathrm{IY} = \frac{\text{specific activity of immobilized Est.}}{\text{specific activity of free Est.}} \times 100 \quad (2)$$

2.3. Coating Ca-alginate beads with silicate

After immobilization of esterase enzyme in Ca-alginate beads, beads were waited in 4 ml n-hexane and 6 ml TEOS (tetraethyl orthosilicate) for 24 h at room temperature and washed thrice with distilled water.

Silicate coated alginate beads of three different sizes were generated by changing the size of a needle. The diameter of beads was determined with the formula as shown below:

$$\text{increment in volume} = \frac{4}{3} \times \pi \times (\text{bead diameter})^3 \times \text{number of beads} \quad (3)$$

Surface structure analysis of esterase immobilized non-coated and silicate coated alginate beads was performed using scanning electron microscope (SEM). The elemental analysis of the non-coated and silicate coated alginate beads were investigated by energy-dispersive X-ray sepectroscopy (EDX). Structural changes in functional groups of alginate beads after coating with silicate were characterized using Fourier Transform Infrared Spectroscopy (FT-IR). Silicate coated and non-coated alginate beads were dried for 1 h at 50 °C prior to operation.

2.4. Hydrolytic activity determination of thermoalkalophilic esterase

The esterase activity of free and immobilized enzyme was assayed spectrophotometrically using *p*-nitrophenyl acetate (pNPC$_2$) as a substrate having one of the best hydrolytic activity among the variety of *p*-nitrophenyl (*p*-NP) esters [15]. In case of free enzyme, the assay mixture (1 ml) contained 5 μl (50 mM) pNPC$_2$ substrate and 0.1 mg/ml purified enzyme and initial rates were estimated by measuring the increase in absorbance at 400 nm as a function of time at 55 °C. For the hydrolytic activity of immobilized enzyme, ten coated beads were placed in a solution of 2985 μl Tris–HCl buffer and 15 μl substrate (50 mM pNPA) and incubated for 5 min at 55 °C and the increase in absorbance at 400 nm was recorded. Specific activity of immobilized esterase was expressed as initial rate per mg protein entrapped in the beads.

2.5. Characterization of hydrolytic properties of free and immobilized thermoalkalophilic esterase

The effect of temperature was tested on hydrolytic properties of free and immobilized thermoalkalophilic esterase by measuring esterase activity at different temperatures (30, 40, 50, 55, 60, 65, 70, 75, 80 and 90 °C), after 5 min of reaction in Tris–HCl buffer (0.1 M, pH 8.0), and using pNPC$_2$ as substrate. Thermal stability of free and immobilized thermoalkalophilic esterase was evaluated as residual activity under standard assay conditions, after incubation in absence of substrate for 60 min at 40, 50, 60, 70, 80 and 90 °C in Tris–HCl buffer (0.1 M, pH 8.0). Enzyme activity prior to incubation was defined as 100% at each assayed temperature.

The effect of pH on hydrolytic activity of free and immobilized esterase was determined using pNPC$_2$ as substrate at different pH values (4, 5, 6, 6.5, 7, 8, 9, 10, 11 and 12), after 5 min of reaction at 55 °C. The pH stability was determined by measuring the residual activity under standard assay conditions, after pre-incubation of immobilized esterase for 60 min at pH 6.0, 7.0, 8.0, 9.0, 10 and 11.0. Enzyme activity prior to incubation was defined as 100% at each assayed pH.

The reusability of immobilized esterase enzyme with silicate coated versus non-coated alginate beads was performed by conducting the activity measurement at time intervals of 15 min. After each activity measurement, beads were separated by filtration and washed thrice with 5 ml distilled water. Then fresh Tris–HCl buffer (0.1 M, pH 8.0) and pNPC$_2$ substrate were added to the beads and the next activity measurement was carried out and compared with the first run. Procedure was repeated for 10 times for silicate-coated and non-coated alginate beads separately. The first run activity was defined as 100%.

Storage stability of immobilized esterase was investigated by incubation of alginate beads at 4 and 25 °C for 3-month period and the activity measurement under standard assay condition was performed at time intervals of ten days. Enzyme activity prior to incubation was defined as 100%.

Kinetic parameters of immobilized esterase were determined using Lineweaver–Burk plots and assuming that the reactions followed a simple Michaelis–Menten kinetics. Lineweaver–Burk curves were obtained for *p*NPC2 at six different substrate concentration (0.05, 0.1, 0.3, 0.5, 0.7 and 1 mM) using standard enzyme assay.

Table 1
The effect of alginate, $CaCl_2$ and esterase concentration on immobilization of thermophilic esterase.

	Enzyme loading efficiency (%)	Immobilization yield (%)
Alginate concentration (%) (0.5 M $CaCl_2$, 0.1 mg/mlesterase)		
1	54.3	22.9
2	68.1	44.1
3	72.8	39.9
4	79.0	37.2
$CaCl_2$ concentration (M) (2% alginate, 0.1 mg/mlesterase)		
0.1	65.0	27.9
0.3	66.8	38.8
0.5	68.1	43.2
0.7	68.5	45.8
0.9	68.0	45.1
1.1	67.9	44.3
Esterase concentration (mg/ml) (2% alginate, 0.7 M $CaCl_2$)		
0.01	45.0	32.5
0.1	68.5	45.8
0.5	69.1	45.5
1.0	68.9	45.0

3. Results and discussion

In this study, thermoalkalophilic recombinant esterase enzyme was used for immobilization by entrapment in coated Ca-alginate beads. Heterologous expression of esterase enzyme was achieved in *E. coli* and the enzyme was efficiently purified to homogeneity before immobilization procedure. As a result of heterologous expression and purification of esterase, it has been indicated that approximately 30.0–50.0 mg of purified active protein can be isolated with high specific activity [15].

3.1. Optimum conditions for esterase immobilization

In the case of entrapment of enzymes, optimization of the immobilization conditions improves their utilization [16], therefore different experimental conditions were studied for determining the optimum parameters for esterase immobilization. Since sodium alginate and $CaCl_2$ concentration are major parameters for enzyme entrapment in gel beads due to gelation formation by cross-linking between alginate and Ca^{2+} ions, the immobilization process of purified thermoalkalophilic esterase enzyme was carried out varying concentration of alginate solution 1–4% (w/v) using 0.01–1 mg/ml esterase and 0.1–1.1 M $CaCl_2$ solution. The results of enzyme loading efficiency and immobilization yield for each parameter are shown in Table 1.

As is seen from the table, elevated alginate concentration has improved enzyme loading efficiency progressively whereas the best yield of immobilization was obtained at 2% (w/v) sodium alginate. This increase in loading efficiency of esterase with high alginate concentration may be the results of strong tightly cross-linked gel formation and entrapment of the enzyme [17]. Low percent immobilization yield was obtained with l% and more than 3% alginate concentrations, 22.9% and 39.9%, respectively. At 1% alginate concentration, esterase may leak out of beads due to soft, unstable and flaccid beads formation during immobilization [18]. The decrease in activity of entrapped esterase using more than 3% alginate concentration during bead formation could be explained as diffusion limitation of substrate in to entrapped enzyme [19]. Therefore, 2% alginate concentration was selected in our studies.

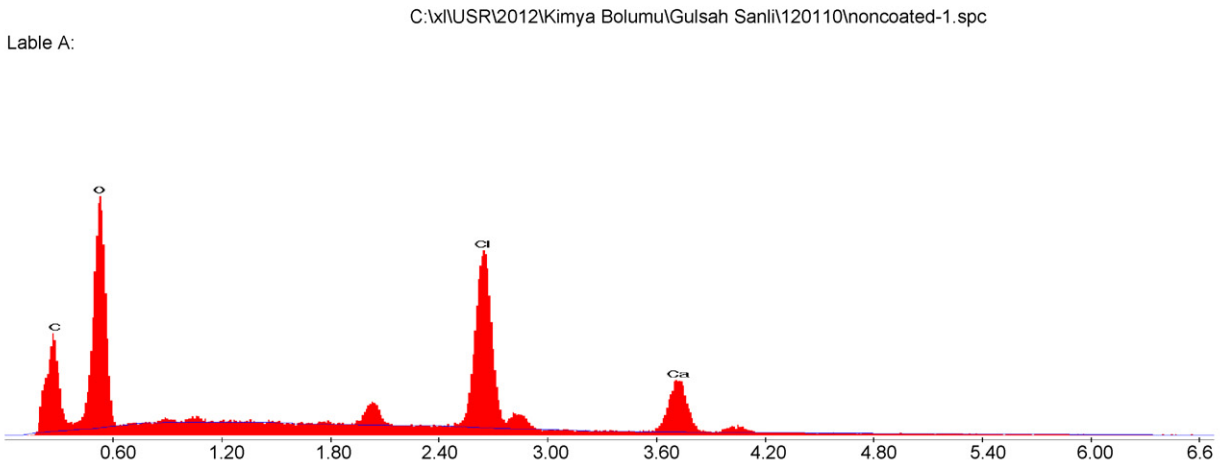
The effect of $CaCl_2$ solution on enzyme loading efficiency and immobilization yield was studied and the maximum yield was observed at 0.7 M $CaCl_2$ solution (Table 1). There was almost no effect of $CaCl_2$ concentration on enzyme loading efficiency whereas the immobilization yield was small at below 0.5 M $CaCl_2$ concentrations due to unstable cross-linked gel formation and dispersion of beads in a short time.

Different concentrations of esterase were used and the best enzyme loading efficiency and immobilization yield was observed as 68.5% and 45.8%, respectively with 0.1 mg/ml esterase. At higher protein concentration, a saturation of immobilization was observed so that esterase concentration was fixed as 0.1 mg/ml for subsequent experiments. Consequently, the optimal conditions of esterase immobilization for the best activity were observed with 2% alginate using 0.5 mg/ml enzyme and 0.7 M $CaCl_2$ solution. Although alginate as a biopolymer has been used to prepare microspheres as enzyme stabilization carriers which are utilized in industrial applications, it has some disadvantages such as mechanical instability which results in enzyme leakage out of polymeric membrane due to porous structure of alginate [13]. In fact, it was reported that if the entrapped enzyme leakage out of beads during the course of time as a result of the large pores of the alginate matrix is eliminated, enzyme activity could be improved [17,19]. In order to investigate leakage problem of entrapped esterase in alginate beads, alginate beads were coated with hexane and TEOS. The results have demonstrated that coating the surface of alginate beads with hexane and TEOS has enhanced immobilization efficiency and immobilization yield by 45% and 21%, respectively due to enzyme stabilization and prevention of enzyme leakage from the alginate beads. Similar to our results here, esterase from *Rhyzopus oryzae* has enhanced enzyme immobilization parameters after silica coating that was attributed to the enzyme leakage prevention with better stability during enzymatic reaction.

The silica coating of alginate beads was characterized by several methods including FT-IR, SEM and EDX. The elemental analysis of the non-coated and silicate coated alginate beads were investigated by EDX analysis showing the presence of elemental Si in silicate coated alginate beads (Fig. 1). Changes in functional groups of alginate beads after silicate coating were characterized using FT-IR spectroscopy (Fig. 2). In the case of silicate coated alginate beads, new and sharper bands appeared at 1083 cm^{-1}, 965 cm^{-1} and 796 cm^{-1} that can be correlated with Si—O—Si stretching, which is also consistent with the studies of Hwang et al. [13]. The differences in surface morphologies of both silicate coated and non-coated alginate beads were also investigated using scanning electron microscope (SEM). SEM micrographs were shown for silicate coated and non-coated beads in Fig. 3. It was found out that the surface of silicate-coated alginate beads was different than non-coated alginate beads having a very compact, smooth and tight structure. In contrast, non-coated alginate beads surface have had some pores which may be the reason of enzyme leakage displaying lower enzymatic activity. Other researchers have also reported that surface modification of immobilized enzyme may prevent the leakage and keep the activity of enzyme by forming a physical barrier [16,17,20].

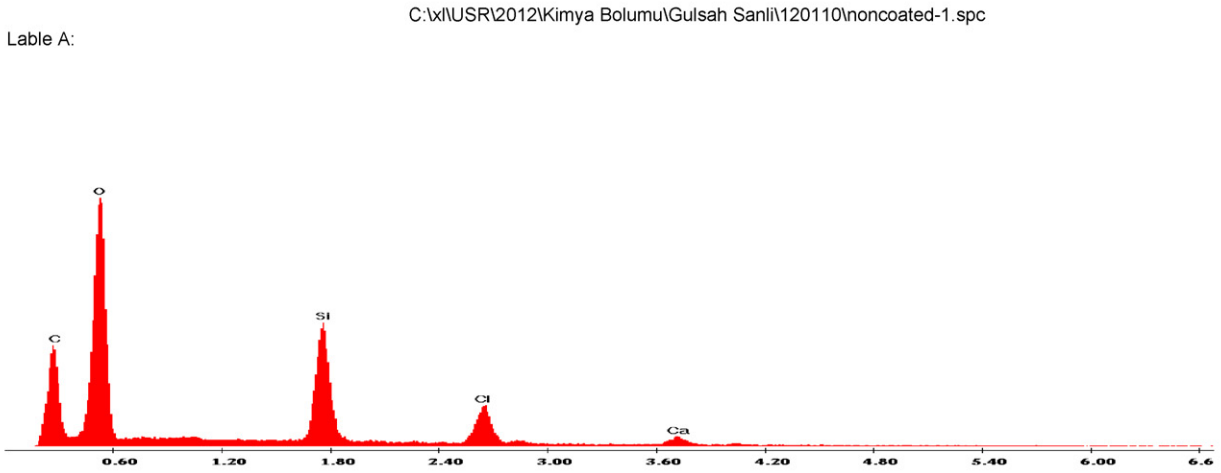
Operational stability of an immobilized enzyme is very important parameter that determines the possible application of enzyme in large scale processes to reduce the operation cost in practical applications. Therefore, operational stability of entrapped esterase in both silicate coated and non-coated alginate beads was determined as cycles number of enzymatic reaction carried out in Tris–HCl buffer (0.1 M, pH 8) at 55 °C for 5 min, using *p*NPC2 as substrate. Specific activity of the immobilized enzyme in both cases was determined after each cycle of enzymatic reaction (Fig. 4). After three subsequent cycles, more than 80% of the enzyme activity in silicate coated alginate beads was maintained indicating a good operational stability of the immobilized enzyme. Meanwhile, immobilized esterase in non-coated alginate beads displayed a minor possibility of reuse with significant loss of original enzymatic activity after three subsequent cycles. Similar results was observed with other immobilized enzymes and reported that soft

A.Non-coated Ca-alginate beads



Element	Weights %	Atomic %
C	17.05	32.49
O	20.38	29.16
Cl	35.08	22.65
Ca	27.49	15.70
Total	100.00	100.00

B. Silicate-coated Ca-alginate beads



Element	Weights %	Atomic %
C	26.79	40.37
O	32.37	36.62
Si	19.41	12.51
Cl	13.89	7.09
Ca	7.54	3.40
Total	100.00	100.00

Fig. 1. EDX spectrum of silicate-coated and non-coated Ca-alginate beads.

surface of non-coated beads compare to silicate coated alginate beads may be the reason for leakage of enzyme from the beads that results in loss of enzymatic activity after subsequent cycle in non-coated Ca-alginate beads [16,17]. The results prove that coating alginate beads with silicate is important for utilization of esterase in continuous processes. Compared to our results here, enhanced operational stability results by silicate coating were obtained by others in which entrapped enzyme was reused more than 10 times

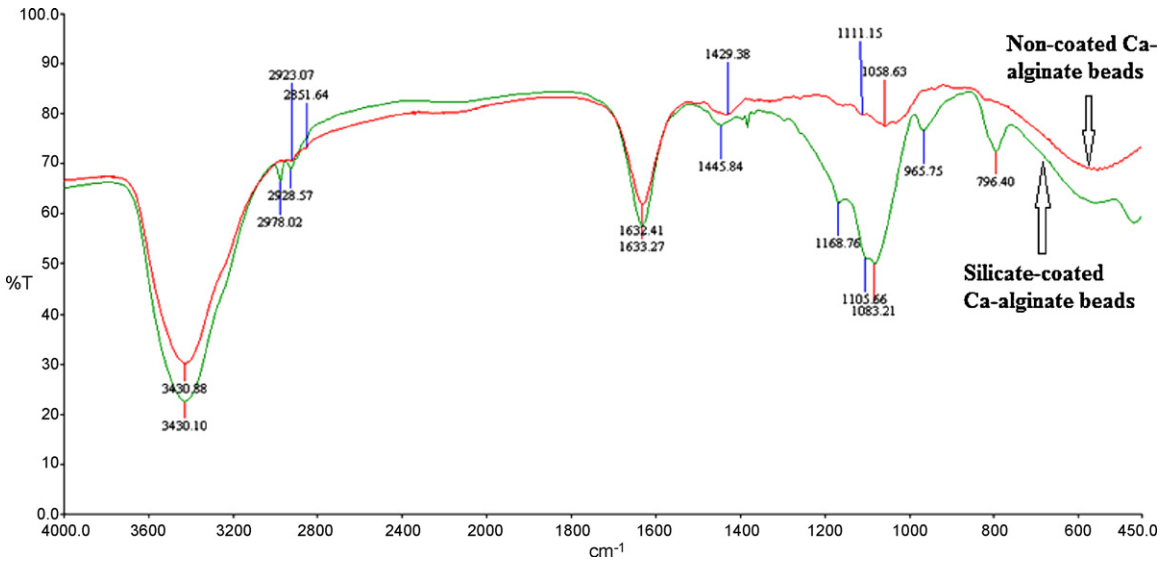


Fig. 2. FT-IR spectra of silicate-coated and non-coated Ca-alginate beads.

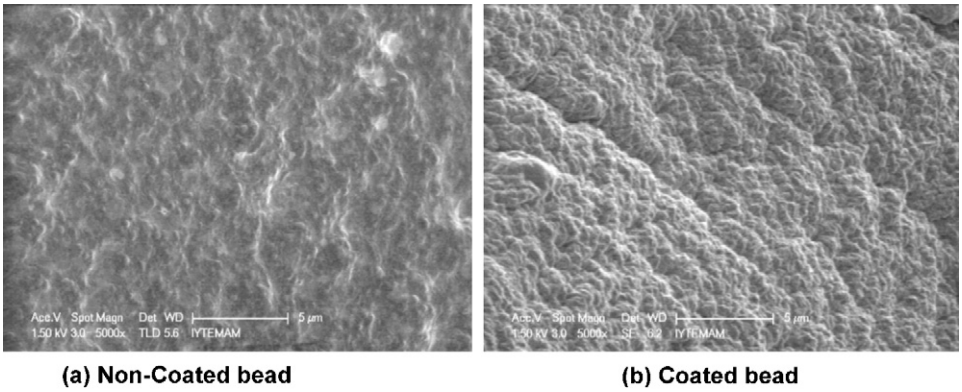


Fig. 3. Comparison of silicate-coated and non-coated Ca-alginate beads surface by SEM.

after silicate coating with no significant loss of enzyme activity [13,14].

The size of silicate coated alginate beads were investigated in terms of esterase activity as an important immobilization parameter in the case of entrapment of enzymes. The best esterase activity was observed with 0.5 mm diameter beads, the smallest size obtained in our studies (Fig. 5). As other studies have reported that enzymes in smaller beads show higher catalytic activity due to reduced substrate transfer resistance and/or increase in surface area [17,21].

3.2. Characterization of free and immobilized thermoalkalophilic esterase

The hydrolytic potential of free and immobilized thermoalkalophilic esterase were investigated by several parameters such as pH, temperature, thermal stability and storage stability.

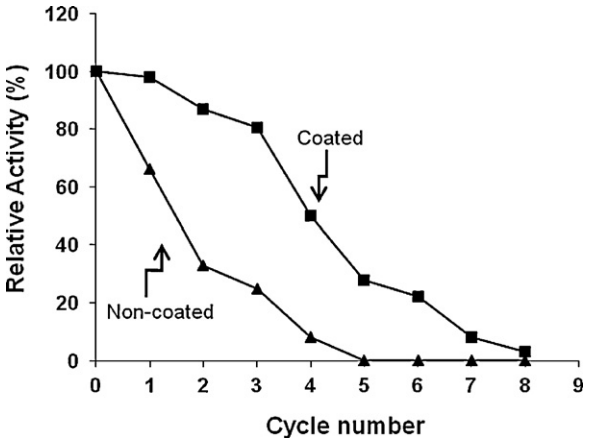


Fig. 4. Reusability of entrapped thermophilic esterase in both silicate coated and non-coated alginate beads.

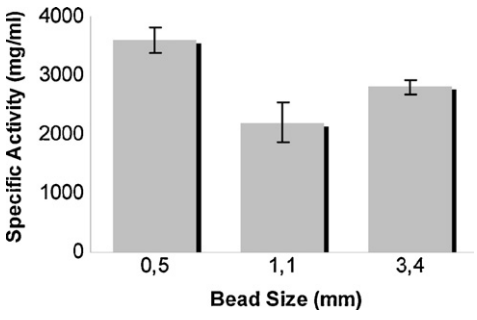


Fig. 5. Effect of different bead sizes on specific activity of thermophilic esterase.

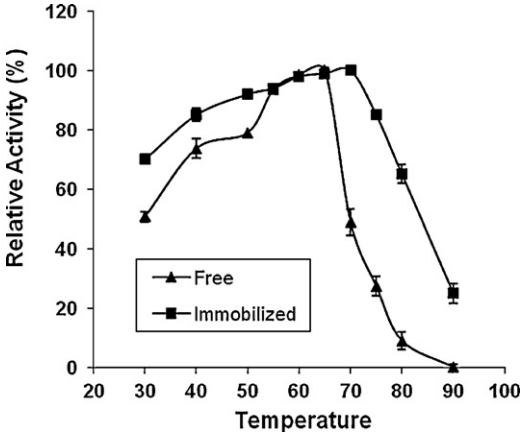


Fig. 6. Effect of temperature on the activity of free and immobilized thermophilic esterase.

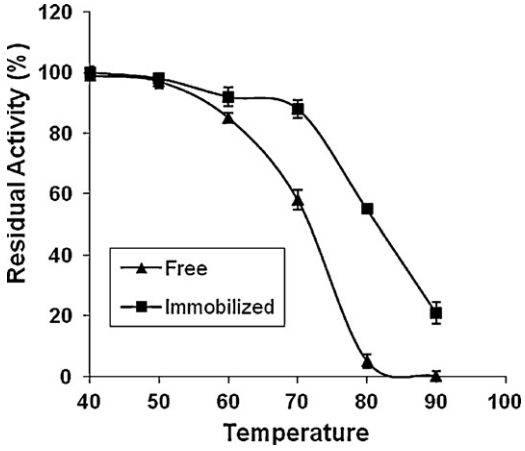


Fig. 8. Thermal stability of free and immobilized thermophilic esterase.

The temperature dependence of the activities of both free and immobilized esterase were studied in the temperature range of 30–90 °C and temperature profiles of free and immobilized esterase are presented in Fig. 6. The optimum temperature for free esterase was 65 °C. After immobilization of esterase in silicate coated alginate beads, it was found that the optimum temperature for immobilized esterase was shifted to 70 °C. The reason of this might be explained as physical limitation of esterase within micro spaces formed by entrapment inside of the beads and easy diffusion of the substrate to the active site at high temperature [22]. Therefore, the immobilized esterase showed its catalytic activity at a higher reaction temperature compared to that of the free enzyme. Results also suggest that the immobilization matrix might be able to protect the enzyme against denaturation at higher temperatures as was observed by other studies [19,22].

The effect of pH on the activity of the free and immobilized thermoalkalophilic esterase was assayed in the pH range of 4.0–11.0. Fig. 7 shows the response of free and immobilized esterase as a function of pH. As can be seen from figure that free esterase exhibited maximum activity at pH 9 whereas the maximum activity of immobilized esterase was observed at pH 8. In the case of silicate coated alginate beads, the optimum pH shifted by 1.0 unit toward the lower pH region (Fig. 7). Similar results were also observed with other studies and this behavior was attributed to the polymeric structure of Ca-alginate gel beads [19,23].

Thermal stability of enzyme is one of the important properties for its industrial applications that could be improved via immobilization. The effect of temperature on the stability of free and immobilized thermoalkalophilic esterase in coated Ca-alginate beads was determined by incubating free and immobilized esterase in the absence of substrate at 40, 50, 60, 70, 80, 90 °C for 1 h. Residual activity as a function of temperature is illustrated in Fig. 8. As can be seen from the figure, with increasing temperature immobilized esterase were inactivated at a much slower rate than those of the free esterase. The free and immobilized esterase remained completely stable for 1 h at 40–50 °C and 40–55 °C, respectively. The stability of free and immobilized enzyme after 1 h at 60 °C has begun to deviate and at 70 °C, the free and immobilized esterase retained their activity about to a level of 58, 88% during 1 h incubation period, respectively. After 1 h at 80 °C and 90 °C, the immobilized esterase retained 55% and 21% of its original activity, respectively, whereas the free one retained only 5% and 0%, respectively (Fig. 8). It is often found out that immobilized enzyme has a higher thermal stability than that of free enzyme because of restriction of conformational movement in immobilized enzyme [24]. The better stability of an immobilized enzyme compared with the free form could be explained by the esterase location inside the micro space of the support, where the enzyme is protected against alterations of the microenvironment.

Storage stability is a very important parameter for the application of an enzyme on the commercial scale and it is well known that enzyme in solution is not stable during storage and the activity is gradually decreased by the time. In fact, immobilized enzymes display a good storage stability compared to free counterparts. Hence, storage stability of both free and immobilized esterase was investigated at 4 and 25 °C for 3-month of period and the activity of the esterase was determined as a function of time. The results are given in Fig. 9. Under storage conditions, free enzyme lost about 29% and

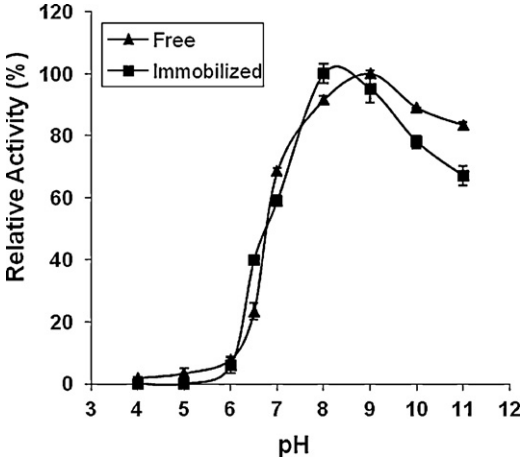


Fig. 7. Effect of pH on the activity of free and immobilized thermophilic esterase.

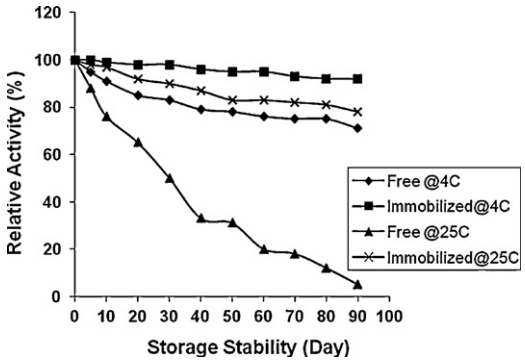


Fig. 9. Storage stability of immobilized esterase at 4 °C and 25 °C.

95% of its initial activity over the time at 4 and 25 °C, respectively. The immobilized esterase lost only about 8% and 28% of its activity over the same period of time at 4 and 25 °C, respectively. By this period, no enzyme release was observed therefore the decrease in activity can be explained as time dependent natural loss. These results indicated that storage stability of immobilized esterase was improved compared to free esterase. Similar observations have also been reported by other researchers [24,25]. The results indicated that the immobilized enzyme had good storage stability.

The kinetic parameters Km and kcat for immobilized esterase were determined by varying the *p*NPC2 concentration from 0.05 to 1 mM in the reaction mixture. The immobilized esterase exhibited a simple Michaelis–Menten kinetics and Lineweaver–Burk plot showed a linear response over the tested concentration range. The Km and kcat values of immobilized esterase were estimated to be 0.11 mM and 12,857 s^{-1}. The Km value for immobilized esterase was lower than that of the free enzyme (0.17 mM). The structural changes in the enzyme and/or steric limitations introduced by the immobilization may be attributed to this change in the enzyme affinity for its substrate. A decrease in the kcat value during the immobilization process is commonly observed because of limitations on diffusion [16].

4. Conclusion

For industrial applications, the immobilization of enzymes may offer several benefits over free enzymes. Through this study, due to special interest of esterase in a variety of biotechnological applications, for the first time we have used recombinant thermoalkalophilic esterase enzyme from Balçova Geothermal region for immobilization studies. Enzyme was immobilized effectively via a simple and cost-effective protocol using Ca-alginate by entrapment and optimal conditions were obtained with 2% alginate using 0.5 mg/ml enzyme and 0.7 M $CaCl_2$ solution. In order to eliminate leakage problem of entrapped esterase in alginate beads, alginate beads were coated with hexane and TEOS. The results have demonstrated that silicate coated alginate beads enhanced immobilization and permitted reusability of esterase in continuous processes. The silica coating of alginate beads was characterized by several methods including FT-IR, SEM and EDX. Using the optimum immobilization conditions of esterase enzyme in silicate coated Ca-alginate beads, the hydrolytic properties of free and immobilized enzyme in terms of storage and thermal stability as well as the effects of the temperature and pH were determined and compared.

Acknowledgments

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