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A Comparative Study of Activity and Stability of the Free and the Immobilized Endoglucanase from *Alicyclobacillus Acidocaldarius*

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ABSTRACT

AaCel9A [β-1,4-endoglucanase, (E.C:3.2.1.4)], was immobilized onto glutaraldehyde activated chitosan macrosphere by covalent attachment. The properties of the immobilized AaCel9A were investigated by determining the optimum pH and optimum temperature for activity, thermal stability, and kinetic parameters. The immobilization process shifted the enzyme's optimum temperature from 65 °C for the free enzyme towards a wider temperature range from 60-80 °C by the immobilized enzyme. The optimum pH of immobilized AaCel9A shifted to basic pH (pH 8) relative to free AaCel9A (pH 6.5). The immobilization on chitosan macrosphere enhanced half-life of AaCel9A enzyme. After 60 min, the immobilized and the free enzyme retained 75% and 40% their activity at 65 °C, respectively. The immobilized enzyme showed higher thermal stability than the free form. Km value of immobilized AaCel9A (17.05 mg ml^{-1}) was higher than free AaCel9A (7.75 mg ml^{-1}). Also, CMC hydrolysis by immobilized and free AaCel9A in the presence of SDS detergent was investigated. The results showed that the immobilized enzyme maintained its activity more than the free form in different concentrations of SDS.

Abbreviations: AaCel9A, *Alicyclobacillus acidocaldarius* endoglucanase Cel9A; CMC, Carboxymethyl cellulose; DNS, 3,5-Dinitrosalicylic acid; Ig-like, Immunoglobulin-like

Keywords: AaCel9A, Immobilization, Chitosan, Thermal stability

INTRODUCTION

Cellulase is the third industrial enzyme in the world [1,2]. Cellulases are multicomponent enzymes that consist of three different enzymes [3,4]. Endoglucanases (β-1,4-glucanase) have been received considerable attention among cellulases family, because they cut at random at internal amorphous sites in the cellulose polysaccharide firstly and generate substrates for cellobiohydrolase and β-glucosidase enzymes to complete the hydrolysis of cellulose [5]. Cel9A from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* (AaCel9A) is a β-1,4-endoglucanase which can degrade cellulose polymers to shorter fragments and belongs to the E1 subfamily of family 9 glycoside hydrolases [6,7]. Many members of this family have an N-terminal Ig-like domain followed by its catalytic domain [8,9].

Cellulases (especially thermophilic endoglucanases such as AaCel9A) possess several desirable qualities for a wide range of applications, ranging from pulp and paper, textile, laundry, food and feed industry, agriculture and production of biofuels from renewable sources [10-12]. Because of diverse practical applications, cellulase should be stable over the wide pH and temperature ranges. Enzymes that can resist higher temperatures and a range of pHs are required since heat and/or chemical pretreatment processes have been used to remove lignin to expose liberated cellulose fiber to cellulases for hydrolyzing into monomeric fermentable sugars [3,13-15].There are different approaches for enzyme stabilization such as protein engineering, immobilization and use of additives. Immobilization of bioactive materials onto solid supports provides a way to improve stability [16-18]. Although immobilized enzymes usually show lower catalytic activity than the free ones, they are stable and reusable. Therefore the immobilized enzymes are cost effective and more efficient for large scale applications [19,20].

Among different methods for immobilization, covalent

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immobilization of an enzyme to a support is the strongest attachment that permanently (irreversibly) restricts enzyme detachment [21]. In covalent immobilization, bifunctional reagents such as glutaraldehyde were employed for immobilization of enzymes to different surfaces.

Chitosan is a natural polyamino-saccharide obtained by N-deacetylation of chitin. Chitosan's availability and its unique chemical and biological properties like nontoxicity, biocompability and biodegradability make it a very attractive biomaterial for enzyme immobilization [22-24].

In the present study, AaCel9A endoglucanase was immobilized onto chitosan macrospheres. Then the activity and stability of the immobilized enzyme were investigated in comparison with the free enzyme (wild-type enzyme).

MATERIAL AND METHODS

Chemicals

Chitosan (medium molecular weight, degree of deacetylation is range from 75-85%) was purchased from Sigma-Aldrich, USA. Glutaraldehyde (25%), Carboxy methyl cellulose (CMC), dinitrosalcylic acid (DNS) and all other chemicals were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Enzyme Production

The DNA sequence that encodes wild-type AaCel9A was amplified by PCR reaction using pDEST17-AaCel9A as a template (which was gifted to this work by Professor S. More´ra from Laboratoired'Enzymologie et BiochimieStructurales (LEBS), CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France). The amplified fragment was cloned to pET28(+) expression vector as previously described [25]. Then, *E. coli* BL21 (DE3) cells were transformed by constructed vector and expression induction was done with 1 mM of IPTG (as an inducer). Protein purification did by Ni-NTA column. Finally, SDS-PAGE analysis and enzymatic activity assay were done for verification (Fig. 1).

Determination of Endoglucanase Enzyme Activity

Activity assay of AaCel9A endoglucanase was determined by incubating the enzyme for 3 min with 1% (CMC) in phosphate buffer (0.05 M, pH 7.0) at 65 °C using DNS reagent as color developing agent [26]. The reducing ends of sugars were measured spectrophotometrically at 540 nm, with glucose as a standard. One unit (U) of activity was defined as the amount of enzyme that produces 1 μmol of glucose equivalent per minute under above conditions.

IMMOBILIZATION OF ENDOGLUCANASE ONTO CHITOSAN MACROSPHERE

Macrosphere Preparation

Chitosan macrospheres were prepared with precipitation method. 0.2 g of chitosan powder was dissolved in 10 ml acetic acid (0.2% V/V) by constantly stirring. This solution was taken in a syringe and was allowed to fall drop to an aqueous solution of sodium hydroxide (10% w/v) and ethanol in a volume ratio of 4:1 under continuous magnetic stirring. Beads of uniform size and shape were formed (Fig. 2). The beads were then washed and stored in distilled water [27].

Enzyme Immobilization

The AaCel9A endoglucanase was immobilized onto chitosan macrospheres by using glutaraldehyde as linker. After Addition of 1 g chitosan beads into 10 ml of 0.7% glutaraldehyde and cross-linking at 28 °C for 4 h, the chitosan beads washed with distilled water for complete removal of unreacted glutaraldehyde until the absorbance was lower than 0.01 at 280 nm. The enzyme immobilization reaction was carried out for 16 h at 4 °C in a shaking incubator. Then, beads were washed gently for 2-3 times with Na-phosphate buffer (pH 6.5) in order to remove unbound enzyme molecules from the bead surface. The immobilized enzymes were stored at 4 °C until use.

Immobilization Efficiency Calculation

Immobilization efficiency was determined from differences in enzyme activity in the solution before and after the immobilization that represented in Eq. (1) [28]

Immobilization efficiency (%) = Activity of immobilized enzyme (unit)/Activity of soluble enzyme (unit) (1)

The activity of immobilized enzyme equals to activity of soluble enzyme minus activity of unbound enzyme.

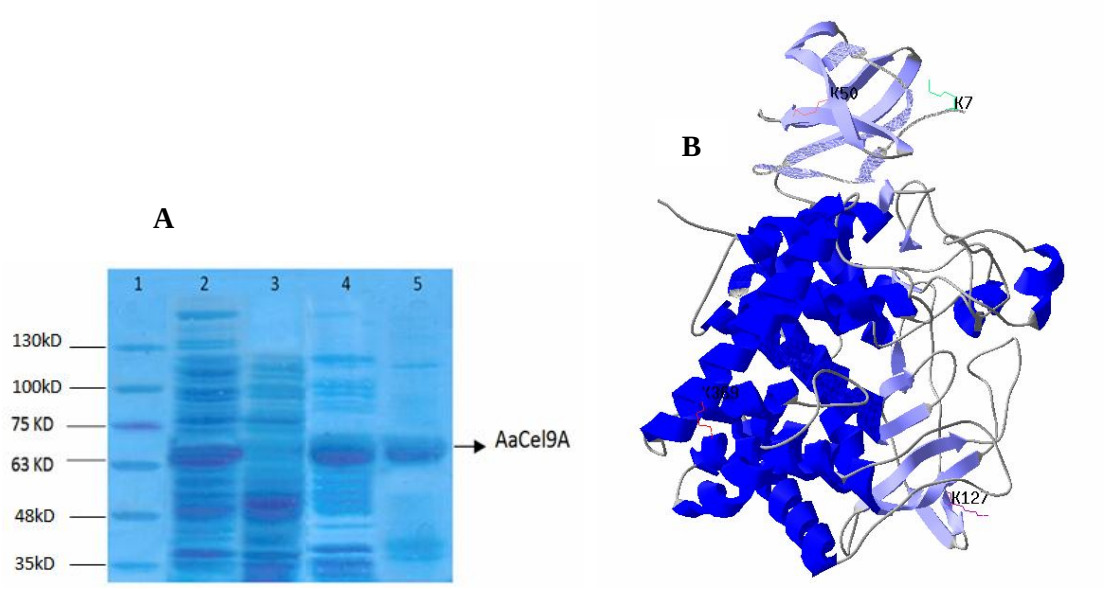

Fig. 1. A. SDS-PAGE analysis of enzyme production. Lanes 1: Marker, 2: Extract of *E. coli* BL21 induced by 1 mM IPTG, 3: empty pET28(+) induced by IPTG, 4: product of enzyme heat shock (semi purified), 5: purified AaCel9A (by Ni-NTA column) B. Tertiary structure of AaCel9A. The structure of the enzyme obtained by using PDB file with ID of 3H3K. The lysine residues 7, 50, 127 and 369 are accessible for covalent attachment.

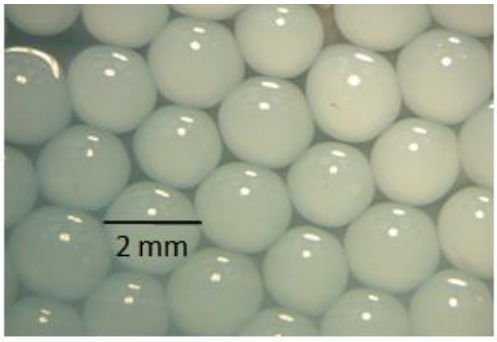

Fig. 2. Micrograph of Chitosan macrosphere. The micrograph was obtained by optical microscope. The diameter of macrospheres (beads) is 2 mm.

Effect of pH and Temperature on Free and Immobilized Endoglucanase Activity

The optimum pH for endoglucanase activity was studied over a pH range of 3-10 (50 mM citrate buffer for pH 3-6, 50 mM phosphate buffer for pH 7-8 and 50 mM glycine-NaOH buffer for pH 9-10) in order to determine pH optima of free and immobilized enzyme. The optimum temperature for endoglucanase activity was determined by incubating the reaction mixture containing free and immobilized enzyme over the temperature range of 30-75 ºC at the optimum pH. All experiments were carried out in triplicates.

Kinetic Characterization of Free and Immobilized Endoglucanase

The kinetic constants of Michaelis values (K_m and V_{max}) for the free and immobilized enzyme preparations were

determined using Lineweaver-Burk plot by measuring the enzymatic activity at different substrate concentrations (1-30 mg ml^{-1}).

Thermal Stability of Free and Immobilized Endoglucanase

Irreversible thermal inactivation of the free and immobilized enzymes was measured by incubating of the enzymes at different temperatures for 10 min. After incubating the enzymes on ice for 30 min, their residual activities were determined (as described above) the activity of the enzymes which incubated at 0 °C was considered as control (100%). For studying the time course thermal stability (or irreversible thermal inactivation), the enzymes were incubated at 65 °C for different times (0, 10, 20, 30, 40, 50 and 60 min). After cooling on ice for 30 min, the residual activity of the enzymes was measured. Residual activity of the enzymes which incubated at 65 °C for 0 min considered as control (with 100% activity).

Calculation of Half-life and k_{in} of the Enzymes

For measuring of the half-life and rate constant of enzyme inactivation (k_{in}), the time course of the decrease in the enzyme residual activity needs to be evaluated. In this work, the decrease in the residual activity with respect to the enzyme incubation incubation time at 65 °C was linear, which indicates first order reaction for enzyme inactivation. Therefore, for determining of the rate constant of enzyme inactivation (k_{in}) and the half-lives the following equations were used, respectively:

$$\ln(\text{Activity}) = \ln(\text{Activity})_0 - k_{in}t \quad (2)$$

and

$$Halflife = \frac{0.693}{k_{in}} \quad (3)$$

Effect of Detergent on the Free and Immobilized Enzyme Activity

This experiment was carried out to determine the effect of SDS on the activity of free and immobilized AaCel9A. Activity of Both the free and the immobilized enzyme determined with SDS (an anionic detergent) in concentration ranging 0.025-5% mixed with CMC. The enzymes activities were determined at the optimum pH value and incubation temperature for each enzyme.

RESULTS

Immobilization Efficiency

The percent immobilization of enzyme on chitosan macrosphere was determined from equation 1 and was evaluated 85%, and amount of enzyme that immobilized on support as Bradford test estimated 2.79 mg of enzyme per 1 g of chitosan support. It should be noted that the enzyme immobilization was done at different concentration of the enzyme. The results showed that the enzyme immobilization in 2.79 mg of enzyme per 1 g of chitosan support had most immobilization efficiency (Data not shown).

Effect of pH and Temperature on the Enzymes Activity

To evaluate the optimum pH for activity of the free and immobilized forms of the enzyme, the enzyme activity was assayed in different range of pH (pH 3-9.5). As shown in Fig. 3a, the optimum pH of the AaCel9A was 6.5 (the data for free enzyme was obtained from reference [25]). On the other hand, the immobilized enzyme has an optimum activity in pH 8 (Fig. 3a). Results indicated the enzyme immobilization was increased the optimum pH for activity from 6.5-8.

Effect of temperature on the enzymatic activity of free and immobilized forms of the enzyme determined at different temperatures (30-90 °C). As shown in Fig. 3b, free enzyme has an optimum temperature for activity at 65 °C (the data for free enzyme was obtained from reference [25]), whereas the optimum temperature of the immobilized enzyme has broadened from 60-80 °C.

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Thermal Stability of the Free and Immobilized Enzyme

Thermal stability (or irreversible inactivation) of the free and immobilized enzymes was determined by incubating the enzyme solution for 10 min at different temperatures. As shown in Fig. 4a, the stability of free and immobilized enzymes has decreased after 30 °C. The free enzyme completely loses its activity (stability) at 90 °C, whereas the

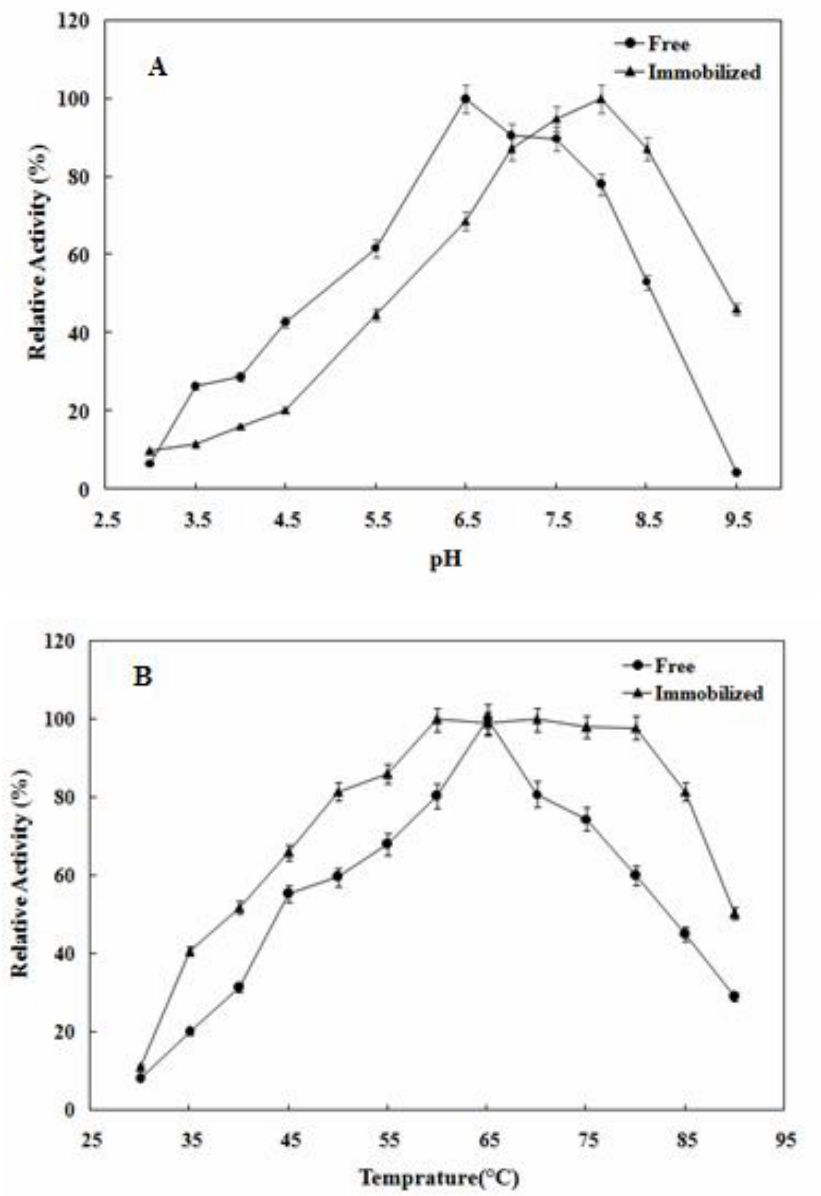

Fig. 3. The effect of pH (A) and temperature (B) on the activity of free and immobilized AaCel9A.

immobilized enzyme maintained 40% of its activity at 90 °C (Fig. 4a). For determination of the time course irreversible thermal inactivation of the enzymes, the free and immobilized enzymes were incubated at 65 °C for different times (10-60 min) (Fig. 4b). Then the results exhibited that the half-life of the enzyme increased from 49.5 min (in free enzyme) to 102 minutes (in the immobilized enzyme) by immobilization (Fig. 4b, Table 1).

The Effect of Immobilization on the Kinetic Properties of AaCel9A

Immobilization can influence the kinetic parameters of enzymes. To determine the effect of immobilization on the kinetic parameters of AaCel9A, the activities of the free and immobilized forms of the enzyme were determined in different concentration of CMC and the Michaelis-Menten and Linweaver-Burk curves for both enzymes were

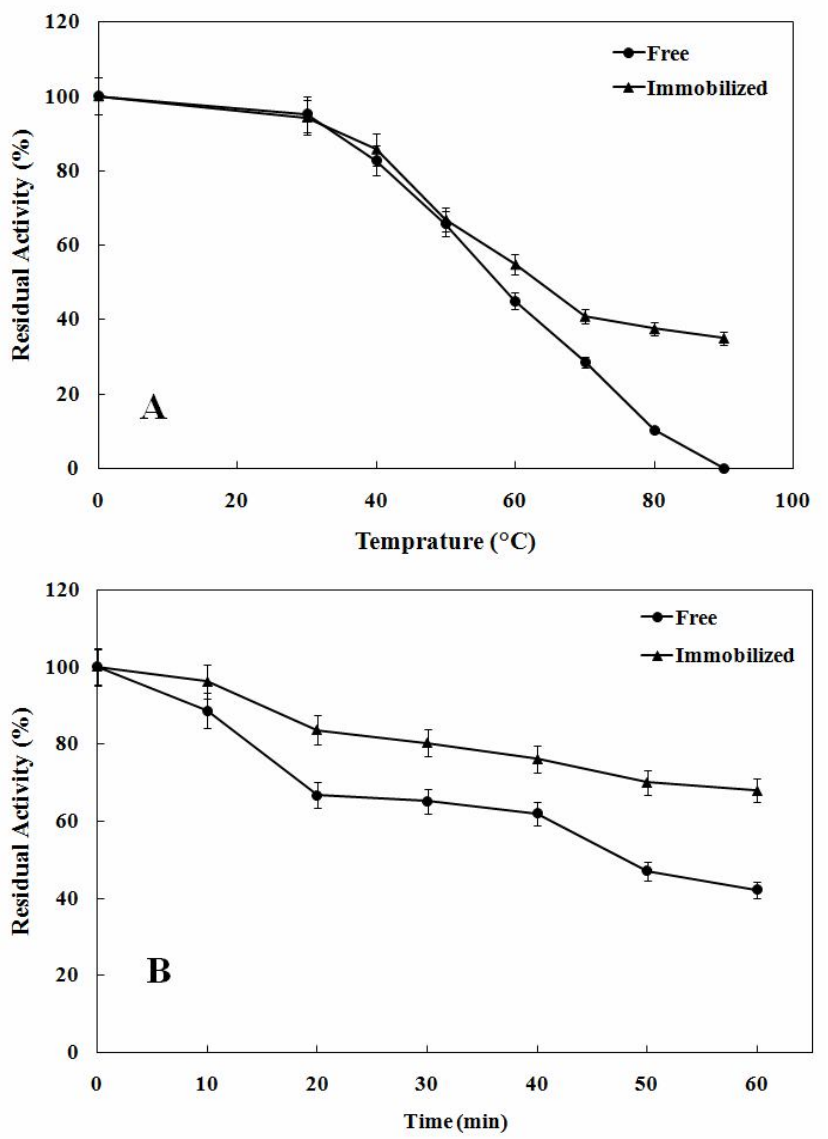

Fig. 4. Thermal stability of free and immobilized AaCel9A. (A) Thermal stability of the enzymes in different temperature. (B) Time course thermal stability of the enzymes at 65 °C.

determined. Results exhibited that K_m of the free and immobilized enzymes were 7.75 and 17.05 mg ml^{-1}, respectively (Figs. 5a and 5b and Table 2). However, k_{cat} of the enzyme was decreased from 0.75 (s^{-1}) (in the free enzyme) to 0.14 (s^{-1}) (in the immobilized enzyme) (Table 2). Also, the results indicated that the catalytic efficiency of the enzyme was decreased up to 11 fold by immobilization (Table 2). It should be noted that, the kinetic data for the free enzyme which obtained in this study were similar to the our previous work [25].

The Eeffect of Detergent on the Activity of Free and Immobilized AaCel9A

To study the effect of detergent on the activity of the free enzyme and immobilized form, the enzymes activities were determined in different concentration of SDS (Fig. 6). Results showed that SDS decreased activity of the free enzyme (Fig. 6), but immobilization increased the enzyme

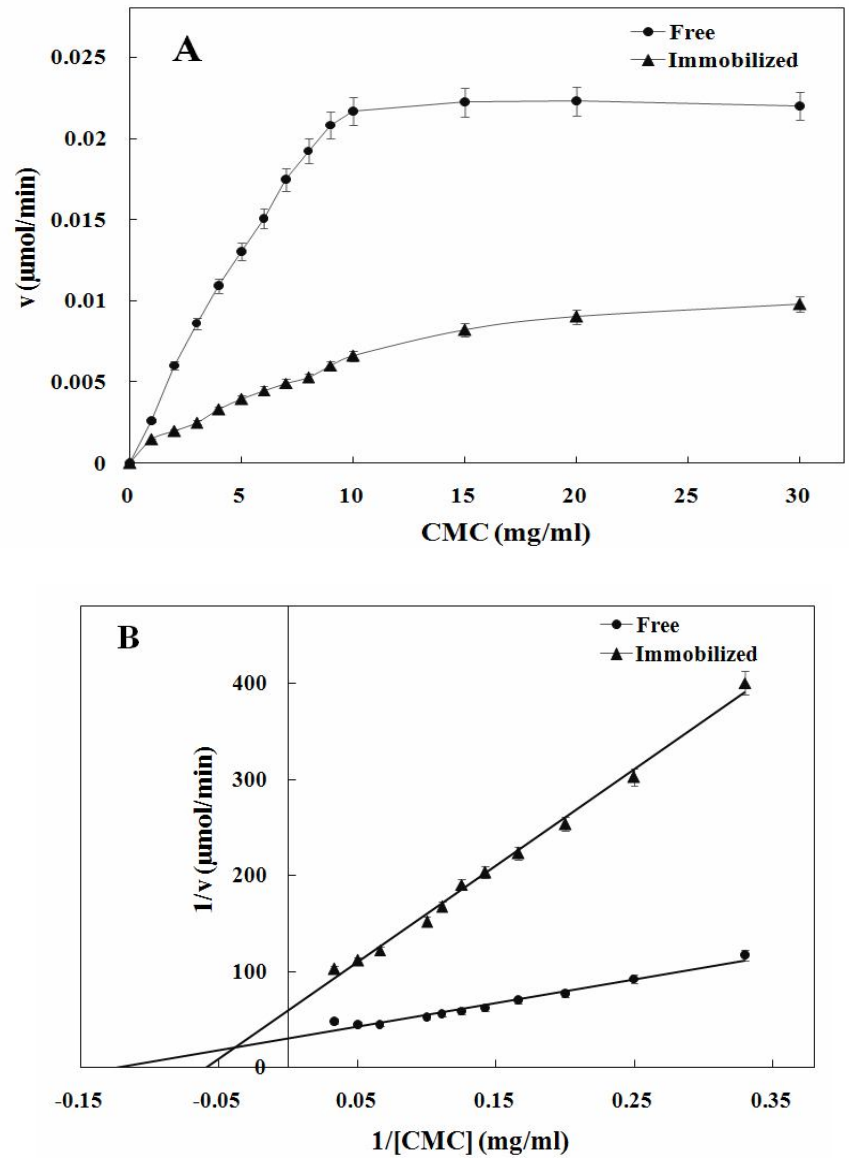

Fig. 5. Kinetic behavior of free and immobilized enzyme. For measuring the kinetic parameters of the enzymes, the Michaelis-Menten (A) and Linweaver-Burk (B) curves of the enzymes were determined for the immobilized enzyme. The curves for free enzyme were obtained from Ref. [25].

activity relative to free form of enzyme in the presence of SDS, slightly.

DISCUSSIONS

Enzymes are undergo a variety of denaturation reactions during production, storage and application in industry. One of the challenges in industrial use of enzymes is their low stability. Therefor enzyme stabilization has industrial importance [30,31]. For enzyme stabilization, several approches such as immobilization were developed [32,33]. In this study we used immobillization of AaCel9Ato improve the enzymatic properties for industrial applications.

The results showed that immobilization efficiency of

Table 1. Inactivation Rate Constant (k_{in}) and Half-life of the Free and the Immobilized AaCel9A at 65 °C

Enzyme form	k_{in} (min^{-1})	Half life (min)
Free	0.0140	49.5
Immobilized	0.0068	102

Table 1. Kinetic Parameters of the Free and the Immobilized AaCel9A

Enzyme form	K_m (mg ml^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (ml/m s)
Free	7.75	0.75	0.0903
Immobilized	17.05	0.14	0.0082

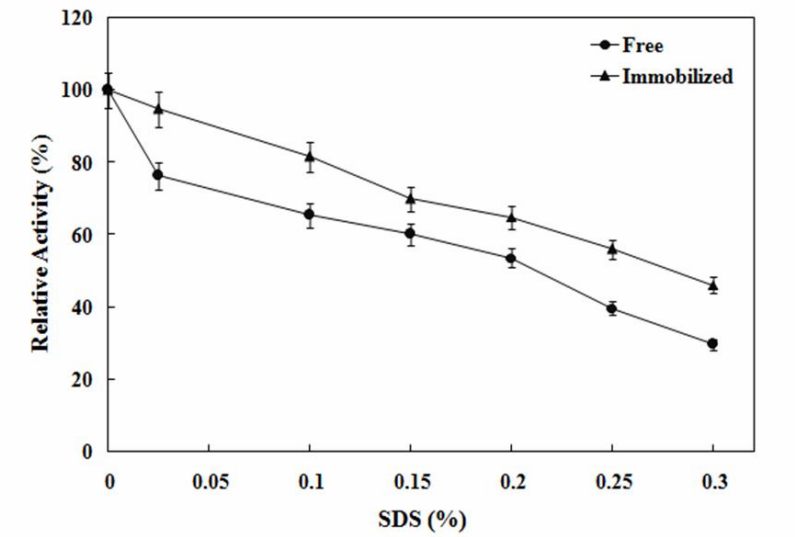

Fig. 6. Effect of detergent on activity of free and immobilized enzyme. For study of the effect of detergent on the enzyme activity, the enzymes activities were determined in different concentration of SDS.

AaCel9A was 85%. This is similar to the previous works of enzyme immobilization onto chitosan support [28,34]. The effect of pH on the enzymes Activity, exhibited that the optimum activity of immobilized AaCel9A was shifted from pH 6.5 (in free enzyme) to pH 8. In previous studies it has been shown that immobilization can alter the pH optimum of enzymes [34-37]. pH has an important role on enzyme activity in reaction mixture. Depending upon the surface and ionic groups on the solid matrix as well as nature of bound enzyme, the local pH value in near vicinity of immobilized enzyme could be different from pH of bulk solution which measured by pH meter [35,38]. This can alter the pH optimum for activity in immobilized enzymes [35]. Therefore, one can conclude that the pH optimum for

activity of immobilized AaCel9A was changed probably due to the changing in the pH close the enzyme by ionic groups on chitosan support which affect the enzyme active site properties.

The effect of temperature on activity of the enzyme exhibited that the immobilized enzyme had an extended optimum temperature for activity (60-80 °C) relative to the free enzyme (which had optimum temperature for activity at 65 °C). A similar increase have been showed for cellulase immobilization on chitosan support by El-Ghaffar *et al*. [39]. Covalent bond formation between enzyme and support and following thatrelative rigidity of enzyme structure without loss of activity could be the reason for maintain the activity of immobilized enzyme at high temperatures.

Thermal stability results showed that immobilization of AaCel9A increased the thermal stability of the enzyme. Stability incerasing of the immobilized enzyme can refered to strong binding between enzyme and support [40].

kinetic parameters of enzymes were changed during the process of immobilization [25,29]. The results of this study revealed that immobilized enzyme has lower affinity than that the free enzyme. It has been shown that the immobilization increased the K_m of trypsin [41]. The decreseing in enzyme affinity (or increasing in K_m) to the substrate has a correlation with enzymes conformational changes and substrate diffusion limitation [42,43].

The effect of chemical detergent on the activity of free and immobilized enzyme was tested and was shown in Fig. 6. In the presence of SDS with 0.2% concentration, the free enzyme lost 50% of its activity, that in comparision with immobilized enzyme this amount was 36%. SDS is an ionicdetergent and can denature proteins. In the first stage,inhibitoryeffect of SDS caused by conformational changes and protein unfolding. Then, SDS (in high concentration) prevent substrate binding. Inhibitory effects of SDS on immobilized enzyme is lower due to the relative rigidity of the enzyme structure. But in highconcentration of detergent, SDS can cover enzyme active site and effectonactivityeven in the immobilized form [44,45].

Finally, Cel9A from *Alicyclobacillus acidocaldarius* was immobilized on chitosan activated macrospheres. Properties of the free and immobilized enzyme were compared. The results showed that immobilization was decreased catalytic efficiency of the enzyme drastically. However, the broader optimum temperature for activity, better enzymatic activity in basic pH, increased thermal stability and better enzymatic activity in the presence of SDS, make the immobilized enzyme desirable for industrial application. Moreover availability, nontoxic nature of chitosan and ease of chitosan macrosphere synthesis make it suitable support for enzyme immobilization.

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