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Application of Immobilized *Ipomoea batata* β Amylase in the Saccharification of Starch

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Abstract

Present study demonstrates the immobilization of acetone fractionated *Ipomoea batata* (sweet potato) β amylase on an inorganic support, Celite-545 by simple adsorption mechanism. The adsorbed enzyme exhibited an activity yield of 244 U g^{-1} of the matrix with effectiveness factor 'η' 0.83. Interaction between Celite-545 and enzyme was confirmed by fourier transform infrared spectroscopy and atomic force microscopy. The binding efficiency of enzyme to the support was analyzed by eluting it with 1.0 M NaCl. Both soluble and immobilized β amylase exhibited same pH optima while temperature optimum of immobilized enzyme was shifted from 50°C to 60°C. The immobilized β amylase preparation was superior to the free enzyme in hydrolyzing starch in a batch process: it hydrolyzed starch to 88% and 96% at 40°C and 50°C, respectively whereas soluble enzyme hydrolyzed only 83% and 80% of starch under similar experimental conditions. The immobilized β amylase retained 84% of its original activity after 30 days storage at 4°C, while the soluble enzyme showed only 41% of the initial activity under identical conditions. Immobilized β amylase retained 79% activity even after its 7th repeated use.

Keywords: β amylase; Celite-545; immobilization; sweet potato; batch process

Abbreviations: AFM, atomic force microscopy; DNS, 3,5 dinitro-salicylic acid; FT-IR, fourier transform infrared spectroscopy

INTRODUCTION

β Amylase (E.C. 3.2.1.2) is an exoamylase which is widely distributed in higher plants and microorganisms. Among plant sources sweet potato is thought to be a promising source of β amylase with fair thermostability [1]. It catalyzes the hydrolysis of α-1, 4 glycosidic linkages at non reducing end of starch and related carbohydrates [2]. The biotechnological application of β amylase includes the production of maltose and high maltose syrups. Maltose is widely applied in food and pharmaceutical industries, since its properties are represented by mild sweetness, good thermal stability and low viscosity in solution [3,4].

An effective application of free enzymes is hindered due to several drawbacks like thermal instability, rapid loss of catalytic activity during operation and storage period and sensitivity to numerous denaturing agents [5,6]. Such drawbacks can be circumvented by using enzymes in their immobilized form [7-9].

Several methods including entrapment, adsorption, encapsulation in membranes, chemical cross linking by using bifunctional or multifunctional reagents and bioaffinity based procedures have been employed previously for enzyme immobilization [10,11]. Among these methods, adsorption of enzymes on particulate carriers is one of the simplest and cost effective immobilization techniques [12].

Celite-545 has recently been used as an immobilization matrix owing its inexpensive nature and other desirable physical properties like large surface area with high enzyme loading and enormous porosity which increase enzyme accessibility to the substrate. Moreover, it shows higher thermal and chemical stabilities with greater resistance to microbial degradation [13,14].

In this study, *I. batata* β amylase has been immobilized on an inexpensive support, Celite-545. The immobilized enzyme preparations were characterized by fourier transform infrared spectroscopy (FT-IR) and atomic force microscopy (AFM) in order to monitor the functional groups and surface topography, respectively. The stability of soluble and immobilized β amylase has been investigated against several physical and chemical denaturants. Hydrolysis of starch in batch processes at varying temperatures by soluble and immobilized enzyme was also evaluated.

MATERIALS AND METHODS

Materials

Celite-545 (20-45 μ mesh) was obtained from Serva Labs (Heidelberg, Germany). Starch, maltose, glucose and DNS were purchased from SRL Chemicals (Mumbai, India). Acetone was obtained from Merck (Darmstadt, Germany). *I. batata* was purchased from local market. Other chemicals and reagents employed were of analytical grade and used without any further purification.

Extraction and partial purification of β amylase from sweet potato

Mature, healthy *Ipomoea batata* roots (200 g) were washed thoroughly with distilled H_2O, cut into small pieces and homogenized in 100 ml of 0.1 M sodium phosphate buffer, pH 6.0. The suspension was filtered through 4 layers of cheesecloth. Filtrate thus obtained was centrifuged at 10 000 x *g* for 30 min at 4°C. The crude extract was initially fractioned by 50% (v/v) chilled acetone saturation. This solution was continuously stirred overnight at 4°C for complete precipitation of proteins. After centrifugation at 10 000 x *g* for 30 min, precipitated pellets were collected and resuspended in two-pellet volume of cold buffer. The solution was dialyzed against 0.1 M phosphate buffer of pH 6.0 for overnight. Undissolved particles were removed by centrifugation and the clear solution was stored in assay buffer at 4°C for further use.

Adsorption of β amylase on Celite-545

Celite-545 (1.0 g) was suspended in 50 ml of 0.1 M phosphate buffer and stirred for 1 h at room temperature. The fine particles present in suspension were removed by decantation and similar procedure was repeated thrice [15]. The binding of β amylase on support was carried out by incubating 1232 U of enzyme g^{-1} of Celite-545 and this mixture was stirred overnight in 0.1 M sodium phosphate buffer, pH 6.0 at 4°C. The enzyme bound on Celite-545 was collected by centrifugation at 3 000 x *g* for 15 min at 4°C. Unbound enzyme was removed by washing thrice with buffer and immobilized enzyme was stored in assay buffer at 4°C for further use.

FT-IR spectra of Celite-545 and Celite-545 adsorbed β amylase

FT-IR spectra of Celite-545 and Celite-545 adsorbed β amylase were recorded by the potassium bromide pellet method on INTERSPEC 2020 (USA) in the range of 400-4000 cm^{-1}. The calibration was done by polystyrene film. The samples were injected by Hamiet 100 µl syringe in ATR box. The syringe was first washed with acetone followed by distilled water. FT-IR analysis was done to examine the functional groups present in enzyme and support.

AFM analysis

Tapping mode AFM experiments of Celit-545 and Celite-545 adsorbed β amylase were performed using commercial etched silicon tips as AFM probes with typical resonance frequency of ca. 300 Hz (RTESP, Veeco). The samples were placed drop wise on a mica wafer, air dried at room temperature for 12 h and the images were recorded with a Veeco Innova nanoscope II AFM. AFM scans were carried out on several surface positions to check the surface uniformity.

Effect of NaCl on immobilized enzyme

The Celite-545 adsorbed β amylase (1.0 U) was incubated with 1.0 M NaCl in 0.1 M sodium phosphate buffer, pH 6.0 at 50 °C for varying times. Activity of untreated enzyme was considered as control (100%) for the calculation of remaining percent activity.

Effect of pH

Soluble and immobilized β amylase (1.0 U) was assayed in the buffers of different pH (pH 2.0-8.0). The buffers used were glycine-HCl (pH 2.0), sodium acetate (pH 3.0, 4.0), sodium phosphate (pH 5.0-7.0) and tris-HCl (pH 8.0). The molarity of each buffer was 0.1 M. Maximum activity obtained at pH 6.0 was taken as control (100%) for the calculation of remaining percent activity.

Effect of temperature

Effect of temperature on soluble and immobilized β amylase (1.0 U) was studied by measuring activity of enzyme preparations at various temperatures (20-80°C) in 0.1 M sodium phosphate buffer, pH 6.0.

In another set of experiment, soluble and immobilized β amylase (1.0 U) was independently incubated at 60°C in 0.1 M sodium phosphate buffer, pH 6.0, for varying times. Aliquots of each preparation were taken at indicated time intervals, chilled quickly in ice for 5 min and activity was measured. The activity obtained without incubation at 60°C was taken as control (100%) for the calculation of remaining percent activity.

Storage stability

Soluble and the immobilized β amylase were stored at 4°C in 0.1 M sodium phosphate buffer, pH 6.0 for over 30 days. The aliquots from each preparation (1.0 U) were taken in triplicates at the gap of 5 days and were then analyzed for the remaining enzyme activity. The activity determined on the first day was taken as control (100%) for the calculation of remaining percent activity.

Reusability of immobilized β amylase

Immobilized enzyme was taken in triplicates for assaying β amylase activity. After each assay the immobilized enzyme preparation was taken out, washed, and stored overnight in 0.1 M sodium phosphate buffer, pH 6.0, at 4°C. The activity was assayed for seven successive days. The activity determined for the first day was considered as control (100%) for the calculation of remaining percent activity after each use.

Starch hydrolysis in batch process

Starch solution (1% w/v) was independently incubated with soluble and immobilized β amylase (500 U) at 50°C and 60°C respectively under stirring condition for 6 h. Aliquots were taken out at different time intervals and assayed for the formation of maltose by DNS method [16].

Measurement of β amylase activity

Activity of β amylase was assayed by DNS method with slight modifications [16]. 250 µl of enzyme in buffer was added to 250 µl substrate (1% w/v) and the resulting mixture was incubated for 30 min at 50°C. Reaction was stopped by adding 1.5 ml of DNS solution and then heated in a boiling water bath for 5 min. After cooling, reaction mixture was diluted with distilled water. Absorbance was measured spectrophotometrically at 540 nm with maltose as standard.

One unit (1.0 U) of β amylase activity is defined as the amount of enzyme that liberating 1 mg of maltose min^{-1} under the standard assays conditions. A standard curve of absorbance against amount of maltose was constructed to calculate the amount of maltose released during assay.

Estimation of protein

Protein concentration was estimated according to the procedure described by Lowry et al [17]. BSA was used as a standard protein.

Statistical analysis

Each value represents the mean for three independent experiments performed in duplicates, with average SDs, <5%. Data were analyzed by one-way ANOVA. *P*-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Immobilization efficiency of β amylase on Celite-545

The present study involves direct immobilization of partially purified β amylase from *I. batata* on an inexpensive support, Celite-545. Thus, the cost of enzyme purification is minimized. Celite-545 is an inorganic mechanically stable, non-toxic and non-biodegradable diatomaceous earth which has been used widely to immobilize various enzymes and proteins [15,18]. The binding of β amylase on support was significantly affected by change in pH. Enzyme was maximally adsorbed at pH 6.0 and retained 244 U β amylase activity g^{-1} of Celite-545 with 83% preserved activities (Table 1). In the literature, for immobilized β amylase, various values for binding capacities and preserved activities are given. For example, when immobilization of sweet potato β amylase was achieved on chitosan beads and spheron based support, preserved activities were reported as 59% and 76%, respectively[19,20]. Furthermore 49% immobilized efficiency was observed on immobilizing barley β amylase on polyacrylamide polymer [21].

Table 1. β Amylase Immobilized on Celite-545

Enzyme Loaded (X) (U)	Enzyme activity in washes (Y) (U)	Activity bound/g of celite 545 (U)			Activity yield % (B/A×100)
		Theoretical (X-Y=A)	Real (B)	Effectiveness factor (B/A) = (η)	
1232	939	292	244	0.83	83%

Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, < 5%.

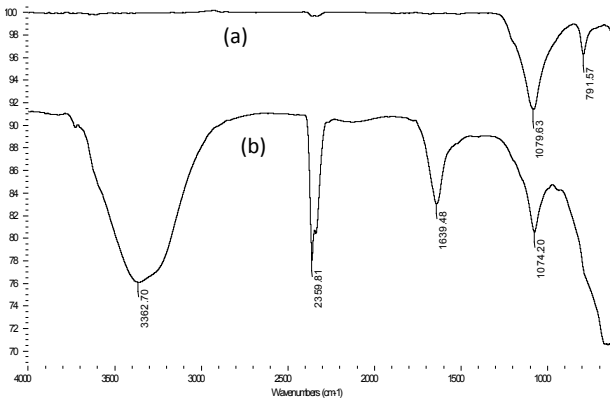

Figure. 1. FT-IR spectra of Celite-545 (a) and Celite-545 adsorbed β amylase (b)

FT-IR analysis

The FT-IR spectra of Celite-545 and Celite-545 adsorbed β amylase are used to investigate the interaction between enzyme and support (Fig. 1). The premier intensity peak at 1079.63 cm^{-1} is due to asymmetric Si-O-Si stretching vibration of Celite-545. The band at 791.57 cm^{-1} is attributed to the symmetric stretching of the ring structure of $(SiO)_4$ tetrahedra [22]. The vibration of H_2O caused by the hydrogen bonds of protein with silanol groups is presented at 1639.48 cm^{-1} [23]. Furthermore, peak at 3362.70 cm^{-1} due to C-H stretching indicated strong interactions of enzyme with Celite-545 [24]. Intensity peak of Si-O-Si stretching vibration at 1079.63 cm^{-1} was decreased to 1074.20 cm^{-1} when enzyme was adsorbed to support surface.

AFM analysis

Visualization of surface topography of support and enzyme adsorbed on it with AFM revealed a significant amount of enzyme molecules immobilized on the support (Fig. 2). The functional groups existing on Celite-545 surface were also verified by FT-IR spectroscopy. We used the peak-to-valley distance in these images as an indicator of the surface roughness. The support surface, before β amylase immobilization, was smooth (Fig. 2a), compared with the enzyme immobilized surfaces. It is evident that as the immobilization progressed the roughness of support surface increases, which could be seen from the increase of peak-to-valley value (Fig. 2b). The roughness of the sample surface is an important feature and should play an important role in affecting the enzyme activity. This observation is consistent with those in previous reports [25].

Effect of 1.0 M NaCl on immobilized β amylase

The activity of immobilized enzyme was evaluated after incubating it with 1.0 M NaCl for 4 h at 50°C (Fig. 3). The adsorbed enzyme exhibited retention of signifcant enzyme activity even in presence of 1.0 M NaCl. Result showed that binding of β amylase with Celite-545 was quite strong and such type of immobilized enzyme preparations could be easily exploited for industrial applications. Ashraf and Husain, showed similar results with radish peroxidase when immobilized on DEAE cellulose[26].

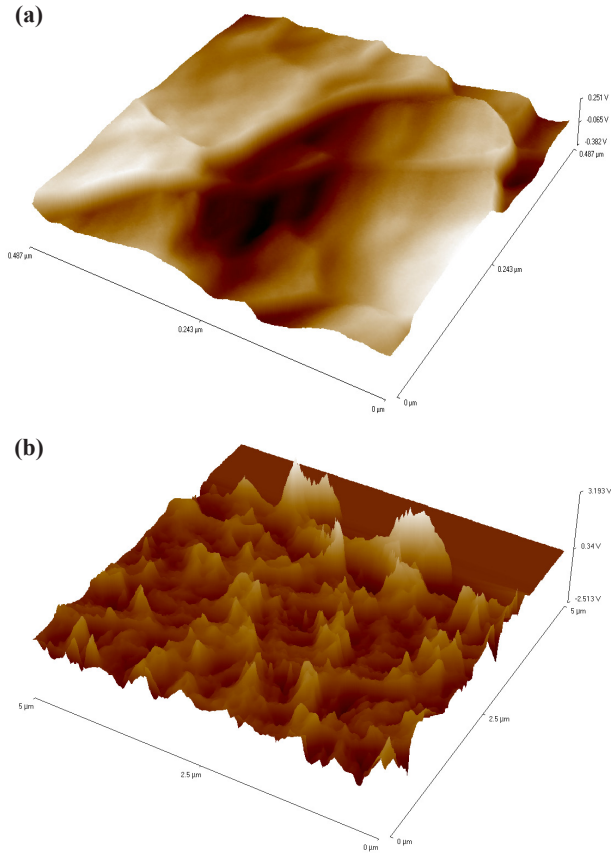

Figure 2. 3D AFM images of Celite-545 (a) and Celite-545 β amylase (b)

Effect of pH

A shift in enzyme activity upon immobilization towards acidic or basic directions is natural since the microenvironment of the free and immobilized enzyme is quite different. Charge and structure of support material, along with nature of the activators impart a significant effect on enzyme activity. Therefore, comparing the activity of soluble and immobilized enzyme as a function of pH forms an important part of the study [27].

Fig. 4 demonstrates the pH-activity profiles for soluble and immobilized β amylase. Both soluble and immobilized enzyme preparations showed the same pH-optima, pH 6.0. However, immobilized β amylase retained significantly higher enzyme activity at alkaline sides as compared to soluble under similar experimental conditions. Several earlier investigators have previously reported the broadening in pH optima for the immobilized amylases [18,28].

Effect of temperature

The inability to enhance the thermal stability of a native enzyme is one of the most important limitations for their application in continuous reactors. Studies showed an increase in temperature-optimum from 50°C to 60°C for immobilized enzyme (Fig. 5). At 70°C the activity retained by immobilized enzyme was significantly higher (83%) as compared to soluble enzyme (28%). Thermal inactivation studies showed 88% of the initial activity retained by immobilized enzyme after 2 h exposure at 60°C whereas the free enzyme exhibited 62% activity under identical thermal exposure (Fig 5). Further incubation of soluble enzyme at 60°C for 4 h resulted in a loss of 64% activity whereas immobilized enzyme retained significantly higher activity, 66%.

Noda et al in their studies observed higher temperature optima for immobilized β amylase in comparison to its soluble countepart[18]. Similarly an increase in temperature-optima was noticed when soybean β amylase was immobilized on chitosan beads. The shift in temperature optima of immobilized enzyme might be due to conformational changes at higher temperatures that might expose active sites more appropriate for substrate interaction thereby increasing its apparent enzymatic activity [29,30].

Reusability of immobilized β amylase

Enzymes are quite expensive products. Immobilization as a technique ensures the recycling and reusability of the enzyme. The most important advantage of immobilization is its repeated uses. Reusability pattern of immobilized β amylase showed about 79% of initial activity retention even after its 7th repeated use (Fig. 7). The trivial activity loss upon reuse could be due to frequent encountering of substrate into the same active site which might distort it and this distortion could dwindle the catalytic efficiency either partially or fully[31].

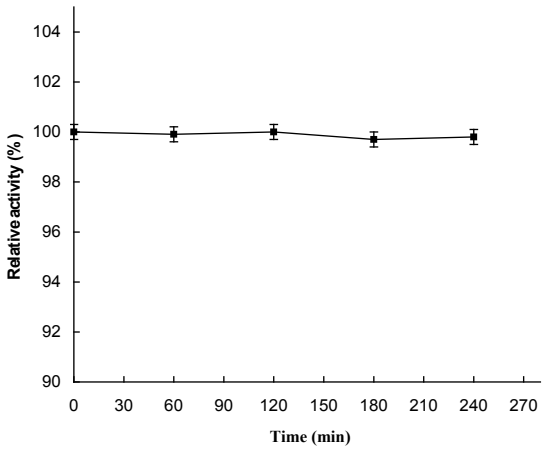

Figure 3. Effect of ions (NaCl) on immobilized β amylase

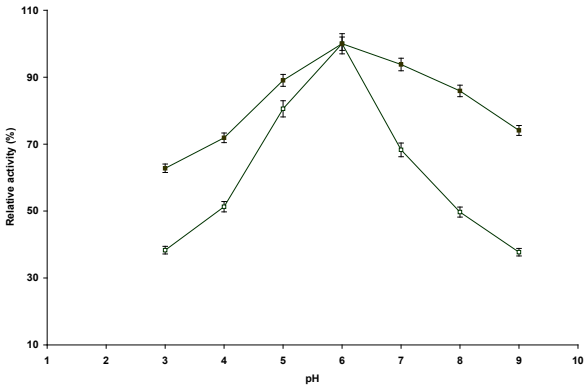

Figure 4. pH-activity profiles of soluble and immobilized β amylase

Storage stability

Table 2 depicts the storage stability of soluble and immobilized β amylase. Immobilized enzyme retained 84% of its initial activity after 30 days of storage while its soluble counterpart exhibited only 43% of its original enzyme activity. The greater stability offered by this inexpensive immobilized β amylase preparation for longer duration and at higher temperatures might bring about its use in the continuous production of novel products of industrial importance at large scale.

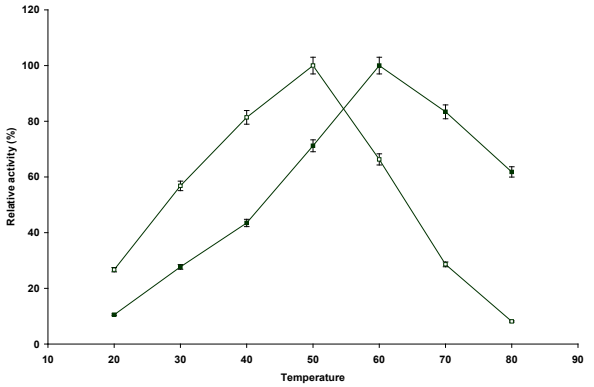

Figure 5. Temperature activity profiles of soluble and immobilized β amylase

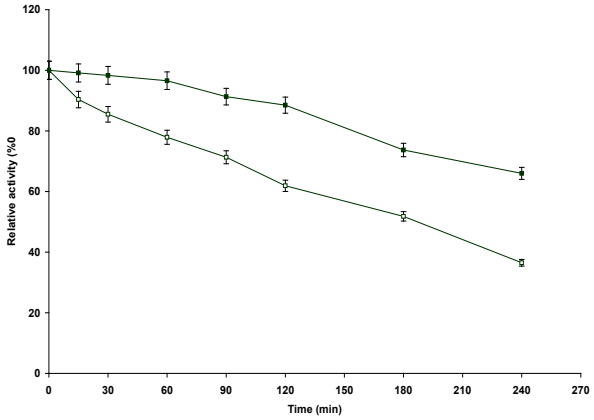

Figure 6. Thermal denaturation of soluble and immobilized β amylase

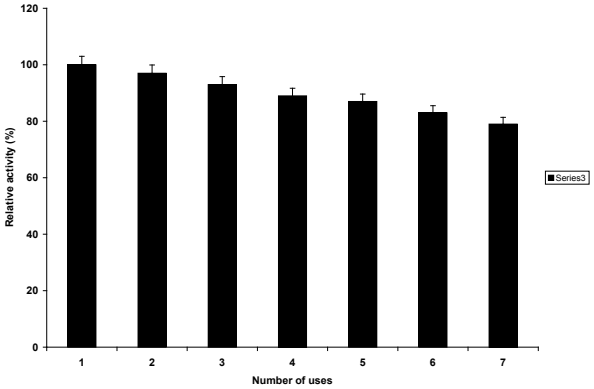

Figure 7. Reusability of immobilized β amylase

Starch hydrolysis in batch processes

Starch is a very important raw material for the production of sweeteners, adhesives, thickening and binding agents. Table 3 illustrates the hydrolysis of starch in batch process by soluble and immobilized β amylase at 40°C and 50°C, respectively. It was noticed that the hydrolysis of starch by soluble enzyme was 82% after 5 h at 40°C whereas immobilized enzyme exhibited 88% of the hydrolytic activity under identical conditions.

Hydrolysis of starch with free and immobilized β amylase at 50°C after 3 h incubation was 74% and 85% respectively. It was observed that only 80% starch was hydrolyzed by the free enzyme after 4 h at 50°C. However, the hydrolysis of starch by soluble enzyme beyond this limit did not exhibit any significant increase whereas the maximum starch hydrolysis achieved by immobilized enzyme reached to 96% in 6 h (Table 3). The results showed that the rate of hydrolysis was more in case of soluble enzyme for the first few hours as compared to immobilized enzyme. It was due to the fact that soluble enzyme was more accessible to starch for first few hours but after prolonged incubation, the rate of hydrolysis decreased. It might be due to enzyme unfolding or inhibition of enzyme activity by its own product [32,33]. Satish et al have reported similar results where starch was hydrolyzed by immobilized *α*-amylase on super porous CELBEADS [34].

Table 2. Storage stability of soluble and immobilized β amylase at 4°C

Number of days	Remaining activity (%)	
	SβA	IβA
Control	100	100
05	91 ± 1.41	97 ± 1.68
10	77 ± 1.25	94 ± 1.57
15	60 ± 1.13	93 ± 0.97
20	52 ± 2.31	88 ± 1.22
25	47 ± 1.88	85 ± 2.10
30	43 ± 1.09	84 ± 1.32

SβA; Soluble β amylase
IβA; Immobilized β amylase

Soluble and the immobilized β amylase were stored at 4 °C in 0.1 M sodium acetate buffer, pH 6.0 for over 30 days. The aliquots from each preparation were taken in triplicates at the gap of 5 d and were then analyzed for the remaining enzyme activity. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation, < 5%.

Table 3. Hydrolysis of starch by soluble and immobilized β amylase in batch process at different temperatures

Time (min)	RA% (at 40°C)		RA% (at 50°C)	
	SβA	IβA	SβA	IβA
0	ND	ND	ND	ND
30	59 ± 1.21	46 ± 1.71	52 ± 2.20	49 ± 1.20
60	64 ± 1.53	53 ± 2.44	56 ± 2.63	54 ± 1.74
90	67 ± 1.33	59 ± 1.22	63 ± 1.87	60 ± 1.09
120	67 ± 1.88	64 ± 0.79	64 ± 1.24	72 ± 2.15
150	72 ± 2.34	70 ± 1.57	69 ± 1.88	78 ± 1.99
180	81 ± 1.09	82 ± 1.63	74 ± 1.51	85 ± 1.53
240	82 ± 0.97	85 ± 2.09	80 ± 0.99	92 ± 1.41
300	82 ± 1.86	88 ± 1.11	80 ± 0.83	96 ± 2.00
360	82 ± 1.14	88 ± 2.34	80 ± 1.29	96 ± 1.57

RA; Remaining activity
ND; Not determined

Starch hydrolysis was performed as described in text. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, < 5%.

CONCLUSION

Amylases are among the most important enzymes used for industrial purposes, and now in the light of biotechnology they are considered useful for biopharmaceutical applications. Here, an attempt has been made to obtain a simple, inexpensive and stable immobilized β amylase. The proteins were directly immobilized by adsorption from the crude homogenate, thus avoiding the high cost of enzyme purification. The immobilized β amylase exhibited better thermostability than the free enzyme which resulted in several benefits including low viscosity of substrates and products, minimized bacterial contaminations, increased reaction rates and decrease of operation time. Furthermore, immobilized enzyme significantly hydrolyzed starch in batch processes at high temperatures. Thus the reactors containing such types of inexpensive immobilized enzyme preparations could be exploited for the continuous hydrolysis of starch at large scale.

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