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Characteristics of Immobilized Urease on Grafted Alginate Bead Systems

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ABSTRACT

This study evaluated the biological importance of immobilized urease enzyme over the free urease. The support material used for urease immobilization was alginate. Generally, the immobilization of urease in alginate gel showed a marked increase in K_m and V_{max}. However, the immobilized urease showed higher thermal stability than that of free enzyme. The rate of thermal inactivation of the immobilized enzyme decreased due to entrapment in gel matrix. Also, the activity of the immobilized urease was more stable in retention than that of the free enzyme during the storage in solution, although the activity of the immobilized enzyme was lower in comparison with the free enzyme. A stable immobilized system and long storage life are convenient for applications that would not be feasible with a soluble enzyme system. These results highlighted the technical and biochemical benefits of immobilized urease over the free enzyme.

Key words: Urease, immobilization, Kinetic, reusability, storage stability

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel-containing enzyme that catalyzes urea to yield ammonia and carbamat, the latter degrades to generate second molecule of ammonia and carbon dioxide (Lubbers et al. 1996 and Khan et al. 2013) as shown in eq 1 and 2.

$NH_2\text{-}CO\text{-}NH_2 + H_2O \longrightarrow NH_3 + NH_2\text{-}CO\text{-}OH$-- eq (1)
$NH_2\text{-}CO\text{-}OH + H_2O \longrightarrow NH_3 + H_2CO_3$ -------- eq (2)

Urease has been used in several applications, e.g., in alcoholic beverages as a urea reducing agent (Fujinawa and Dela 1990), in diagnostic kits for measuring urea (Fumuyiwa and Ouch 1991), and in biosensors of haemodialysis systems for determining blood urea (Smith et al. 1993). Urease is found in the plants, yeasts, algae and filamentous fungi (Mirbod et al. 2002; Liu et al. 2012). The urease from jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized and it remained the best-characterized urease (Andrews et al. 1986). There are many microbial sources for this enzyme, including bacteria such as *Corynebacterium lillium*, *Lactobacillus fermentum*, *Lactobacillus reuteri* and *Lactobacillus ruminis*, (Kakimoto et al. 1989; Kakimoto and Suzuki1992) and fungi such as *Aspergillus niger* (Smith et al. 1993; Island and Hausinger 1995), *A. nidulans* (Mackay and Pateman 1980; Mackey and Pateman 1982; Creaser and Porter 1983) and *Rhizopus oryzae.*

Immobilized enzymes find several biotechnological applications due to their inherent properties, such as stability, reusability and specificity. The storage stability of free enzymes in solution is poor. Recovery from the solution

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with their activity retained is not possible. Amongst the supports used for immobilization, biological macromolecules (such as cellulose, chitin and chitosan) and their derivatives have been studied most extensively through physical adsorption or chemical linkages (Khan et al. 2013).

The immobilized enzymes have been used in the construction of artificial organ systems, bioreactors or biosensors. The advantageous of immobilization include increase in the stability of the bioactive species by protecting the active material from deactivation; reuse significant low cost operations, easy separation and speedy recovery of the bioactive agent, etc (Andrews et al. 1986; Island et al. 1995; Liu et al. 2012; Khan et al. 2013). Many methods exist for the immobilization of enzymes but usually one of four methods is used, co-polymerization, covalent attachment, physical adsorption, and entrapment, (Arica et al. 2001). The methods and supports employed for enzyme immobilization are chosen to ensure the highest retention of enzyme activity and its stability (Arıca et al. 1998).

The objective of this study was to characterize the urease entrapped in calcium alginate beads for its thermal stability, Michaelis-Menten kinetic constants (K_m and V_{max}), optimum pH and T°. The storage stability of the immobilized and free urease was also studied with repeated use.

MATERIALS AND METHODS

Reagents

Urease (EC 3.5.1.5 from jack beans) was purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Sodium alienate was obtained from Fluka (Buchs, Switzerland) Germany. All other chemicals were of analytical grade and were used without further purification.

Preparation of urease immobilized in calcium alginate gel spheres

Sodium alginate (200 mg) with 10 mg of urease in 10 mL aqueous medium was thoroughly mixed until a honey-like consistency was attained. Then the mixture was filled in a syringe and allowed to drop into 50 mL of $CaCl_2$ solution (2%, w/v) from a constant height to form 12 beads. The beads were then stirred slowly for 20 min, removed from the solution, and washed subsequently with buffer before use (Danial et al. 2010).

Enzyme assay

Assay of native urease

For determining the urease activity, Nessler's reagent was used to estimate the ammonia liberated after enzyme incubation with urea for 10 min (Srinivasa et al. 1995). For this, 400 µg urease was taken in a test tube and 1.0 ml of a phosphate buffer (100 mM, pH 7.5) containing 150 mM urea, was added and incubated for 30 min. A volume of 0.3 ml of 0.3% sodium tungstate and 0.3 ml of 0.68 M sulfuric acid was added and the volume was made up to 5.0 ml. Then, 1.0 ml of Nessler's reagent was added to the previously amnestied solution and the total volume was completed to 10 ml with distilled water. The absorbance of the solution was measured using spectrophotometer (Hitachi U-2001, model 121-0032) at 480 nm. The amount of ammonia released was calculated using a standard curve for ammonium sulfate. One unit of urease activity liberated 1.0 mol of ammonia per min from 0.10 M urea under the assay conditions (Srinivasa et al. 1995).

Assay of immobilized urease

The alginate beads (12 beads) with urease entrapped were taken in 1.0 ml phosphate buffer (0.10 M, pH 7.5) solution and reacted with 1.0 mL of a 3% urea solution for 15 min. The same procedure was used to follow the reaction and the measurements were performed in the same manner as for the native enzymes studies. Here, the beads were removed before chilling the solution and H_2SO_4 was added to stop the reaction (Busto et al. 1987).

Properties of free and immobilized enzymes

Optimum pH and temperature

The optimum pH for the free and immobilized urease was studied by incubating at 40°C for 20 min into 1.0 mL of 3% (w/v) urea at pH 4.0-10.0 using acetate buffer (0.1 M) in the pH range of 4.0 to 6.0 and in phosphate buffer (0.1 M) in the pH range of 7.0 to 9.0 by using the standard activity assay. The data were normalized to 100% activity. The highest enzyme activity was expressed as 100%, and each pH was expressed relatively as a percentage of the 100% activity (Danial et al. 2010). To study the optimum temperature, the enzymes were incubated for 30 min into 1.0 mL of 3% (w/v) urea at pH 7.5 and temperatures from 30 to 70°C. The optimum temperature was taken as 100% activity and the relative activity at each

temperature was calculated as a percentage of the 100% activity.

Thermal stability
The enzymes in free and immobilized form were incubated in the buffer solution for 6 h at 30 and 70°C and examined for activity as above.

Kinetic parameters
The Line-Weaver-Burk plot (double reciprocal) method was used to obtain the Michaelis-Menten kinetic models adequate for the description of the hydrolysis of urea by the free and the immobilized enzyme, apparent K_m and V_{max} of free and immobilized urease where 1/[*S*] was plotted against 1/[*V*]. Here, [*S*] is the substrate concentration (urea), V^o is the initial enzyme velocity, V_{max} is the maximum enzyme velocity, and K_m is the Michaelis constant and is defined only in experimental terms and equals the value of [*S*] at which V^o equals 1/2 V_{max}) (Elnashar 2010).

$$\frac{[S]}{V^\circ} = \frac{1}{V\max} * [S] + \frac{Km}{V\max} \quad (1)$$

For the determination of the kinetic parameters, the substrate concentration was varied and the optimum pH for native and immobilized enzyme was determined by varying the pH of the assay buffer. The relative enzyme activity was determined for each pH according to the method described above. The rate of the reaction was measured as mM of ammonia produced/min/µg enzyme. The assay mixture comprised 10 U of free and immobilized enzyme, substrate concentration of 100-1000 mM at 40°C and pH 7.5 for 20 min.

Operational stability
The reusability of the immobilized urease was tested under standard assay conditions. After each activity, buffer was used to wash the samples and stored until the next assay in buffer solution.

Storage stability
For determining the storage stability of the free and immobilized enzyme, both enzymes were kept at room temperature (25ºC) and the activity was measured after one month. Sample of the free enzyme (0.1 mL) or the immobilized enzyme (12 beads) were withdrawn every week and assayed for enzyme activity. Fresh preparations of immobilized enzymes were taken as controls for each assay. The immobilized enzyme was preserved in $CaCl_2$ solution because wet beads gave better result than dry beads (Pithawala et al. 2010).

RESULTS AND DISCUSSION

Preparation of immobilized enzyme
The structural integrity and activity of the urease were retained due to mild and precise physical condition during the immobilization procedure. The sodium alginate beads were chemically inert to the entrapped enzyme. The porosity of the gel was such that it allowed easy movement of the substrate molecules through the beads. The beads were quite stable in phosphate buffer with pH > 7.5. However, at higher pH values, the beads showed softening and stickiness of the surface; the immobilized enzyme retained 76% of its activity. Immobilization of urease by entrapment technique into gel matrix under mild conditions using sodium alginate involves ionotropic gelation. When urease sodium alginate mixture was dropped into $CaCl_2$ solution, Na^{+2} ions of Na-alginate were replaced by the Ca^{+2} ions of $CaCl_2$ forming Ca-alginate beads and ionic cross linking of carboxylate group in uronate block of alginate occurs, giving it a gel-like character (Le-Tein et al. 2004). The urease remained entrapped within the gel matrix of calcium alginate beads as shown in Figure 1.

Figure 1 - Optical images of alginate beads immobilized by urease.

Optimum pH

The optimum pH values for the free and the immobilized enzyme were very close as they were at pH 7.0 and pH 8.0, respectively. However, the immobilized enzyme showed a higher relative activity of 69% as compared to 51% for the free enzyme by increasing the pH to 9.0 as shown in Figure 2. The shift to more basic optimal pH upon immobilized could be explained as a result of the diffiusional constraint of the support retaining a higher concentration of enzyme product, ammonia, in the vicinity of the pore space of the support that adsorbed enzyme present (Elnashar 2010). Thus, the microenvironment around the enzyme was more basic than that of the bulk solution. Similar observations upon the immobilization of urease and other enzymes have been reported by Arica et al. (2001).

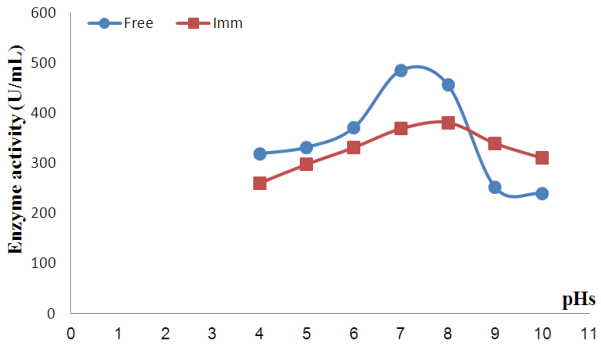

Figure 2 - pH profile of free and immobilized urease.

Optimum temperature

The optimum temperature of the free enzyme was 40°C, but the immobilized enzyme was still at the optimums activity from 40 to 50°C (Fig. 3). This was supported by Danial (2010) who reported that only polyamines substantially improved hydrogel's thermal stability. This shift of the enzyme's optimum temperature after immobilization could be due to the possible protection of immobilized enzyme from the bulk temperature through the formation of a molecular cage around the enzyme protein.

Thermal Stability of the Free and Immobilized urease

One of the main goals of this study was to improve the enzyme's thermal stability to be suitable for industrial use. The immobilized enzyme revealed a higher thermal stability over the free enzyme as shown in Figure 4, which showed the heat stability profile of free and immobilized urease. Evidently the activity of the immobilized urease was 87% while the free enzyme 51% at 50°C after 120 min for the same incubation period. The activities of the immobilized and free enzymes were retained at 73 and 23% at 60°C for 60 min, respectively. The thermal stability of immobilized urease increased considerably as a result of immobilization in alginate gel. These results could be explained by the existence of a local environment for the immobilized enzymes, which was less damaging than bulk solution conditions. These results were in accordance with Bailey and Ollis (1986).

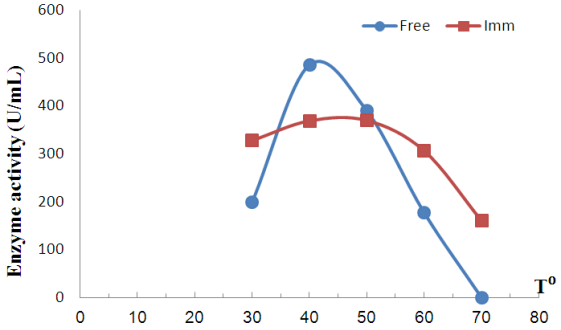

Figure 3 - Optimum temperature profile of free and immobilized urease.

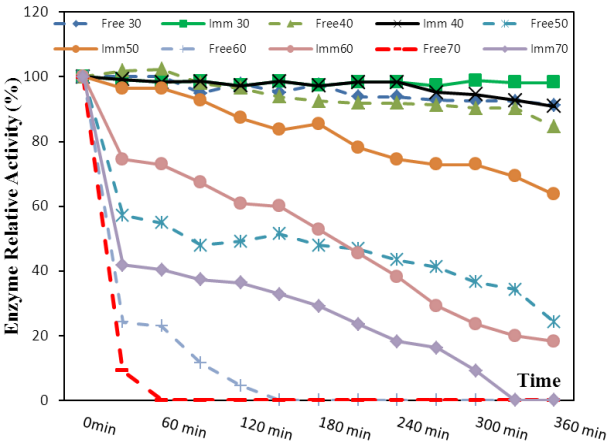

Figure 4 - Temperature-stability profile of free and immobilized urease.

Kinetic Parameters

Kinetic parameters of the hydrolytic reaction of urea using the free and immobilized urease were investigated. Figure 5 represents the relation between initial rate and substrate concentration for the free and immobilized urease. From the Lineweaver-Burk plot of $1/[V]$ versus $1/[S]$

Michaelis-Menten constants (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized enzyme were calculated. The V_{max} value free urease (0.07 μM min^{-1} mg^{-1}enzyme) decreased comparing with the immobilized urease (0.1 μM min^{-1} mg^{-1}enzyme). The calculated *Km* values of free and immobilized urease were 1.8 and 3.8 mM, respectively. Therefore, K_m of the immobilized urease was approximately 2-folds higher than that of the free urease, while V_{max} was 1.5-fold. When an enzyme was immobilized in gel matrix such as alginate gel, the K_m and V_{max} of immobilized enzyme increased. The increase in K_m after immobilization clearly indicates a clear low affinity of the enzyme to its substrate compared with the free enzyme; this may be attributed to the tendency of enzyme to leave substrate within short time without giving a product. An increase in K_m was also reported when urease was immobilized onto collagenpoly (GMA) copolymer (Raghunath et al. 1984) and chitosan (Krajewska 2009).

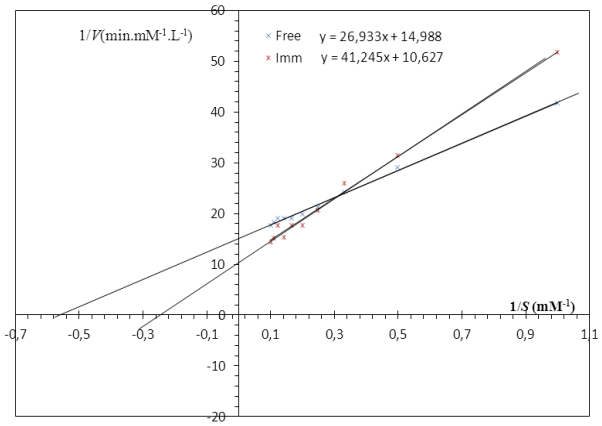

Figure 5 - Kinetic constants of free and immobilized inulinase using Lineweaver-Burk plot method.

Reusability

One of the main advantages of enzyme immobilization is the easy of separation and reusability. The data shown in Figure 6 showed that the immobilized urease using sodium alginate retained over 85% of its activity after five cycles, while free enzyme, being water soluble, could not be recovered and reused. A complete loss in the activity of entrapped enzyme was observed after 10 cycles. The decrease in enzyme activity could be due to the leakage of enzyme from the beads, occurring due to the washing of beads at the end of each cycle (Elnashar 2010). In 2007 Selvamurugan reported that urease entrapped in Ca-alginate beads was reused for six cycles with ~ 30% loss in activity. The immobilized urease using the alginate could be reused more times and retained its enzyme activity without loss, making it more economical as it saved time, carriers, and enzymes (Abida et al. 2009). Kespi et al. (2010) used calcium alginate to entrap the urease into alginate and obtained 60% retention of the enzyme activity after the third cycle, whereas the present work showed retaintion of 92% of the immobilized enzyme activity. The loss of enzyme from the alginate pores by reuses could due to using the non-covalent technique (entrapment). The decrease in enzyme activity after the 10th cycle could be explained by the inactivation of enzyme due to continuous use (Danial et al. 2010).

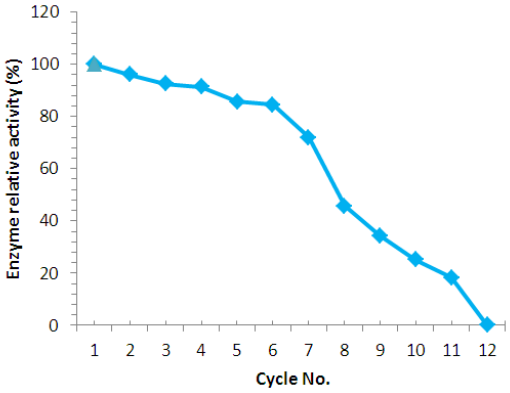

Figure 6 - Repeated use of immobilized urease.

Storage Stability of Immobilized Enzyme:

The shelf stability for the free and immobilized enzymes are presented in Figure 7. The free and immobilized enzymes were stored at room temperatures and the activity was measured for 60 days to determine the storage stability of entrapped enzymes. In general, the enzymes are not stable during storage in solution and their activities are gradually reduced or lost through time. The immobilized enzyme showed 83% loss of activity after 21 days. Major loss in enzyme activity of immobilized enzyme was observed after 50 days. This observation distinctly indicated that the immobilized urease exhibited greater stability than the free enzyme. These results could be explained by the fact that immobilization reduced the interaction between enzyme molecules, which led to the deactivation of the enzyme activity; the aggregation and autolysis by proteolytic enzymes could be other factors. A dramatic stability

enhancement have been reported based on this strategy in which gel entrapment was applied to form a local support microstructure complementary to enzyme surface (Danial et al. 2010).

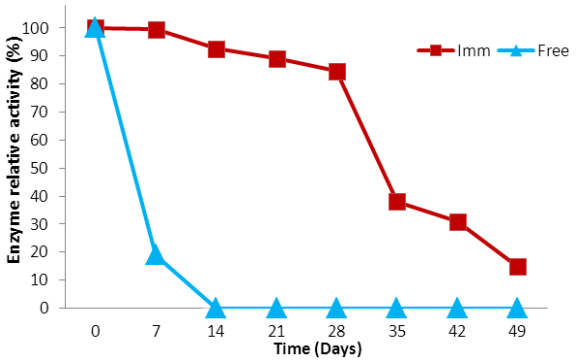

Figure 7 - Shelf stability of free and immobilized urease at room temperature.

In general, the present results revealed that enzyme immobilization in alginate gel could reduce enzyme deactivation, provided a stable environment and prevented the loss of activity during the storage of the enzyme in solution. The beneficial effect of immobilized enzyme over the free enzyme is summarized in Table 1.

Table 1 - Properties distinguishing the immobilized and free urease.

Kinetics property	**Free Enzyme**	**Immobilized Enzyme**
Optimum pH	7	7-9
Optimum T°	40°C	40-50°C
Thermal T (min)	120	>360
K_m	1.8 mM	3.8 mM
V_{max}	0.07 µM $min^{-1}mg^{-1}$	0.1 µM $min^{-1}mg^{-1}$
Storage at Room T(day)	<7	>30
Reusability (Cycle)	1	11

CONCLUSION

Based on the results, it could be concluded that the immobilized urease showed many benefits over the free enzyme, which could make its usage and application in industries more valuable. This was clear through the increase in thermal stability, pH level and reusability of the immobilized enzyme over the free enzyme.

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