Immobilization of Yeast Alcohol Dehydrogenase by Entrapment and Covalent Binding to Polymeric Supports

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Received 17 July 2000; accepted 29 December 2000

ABSTRACT: Yeast alcohol dehydrogenase (YADH), which catalyzes oxidoreductions of a broad spectrum of substrates, was immobilized by entrapping it into a network of a poly(acrylamide-*co*-hydroxyethyl methacrylate) copolymer and was also covalently bound onto porous chitosan beads activated through glutaraldehyde. Maximum retention of YADH activity achieved was 90 and 24% for entrapment and covalent binding, respectively. The results obtained for thermal, storage, and operational stability of entrapped and covalently bound YADH were compared with free YADH. The immobilized enzyme showed improved thermal and storage stability. The immobilized enzymes also retained 50% activity after six and eight cycles. Enzyme-catalyzed oxidation of ethanol was observed to be diffusion-controlled through Lineweaver–Burk plots. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 82: 1299–1305, 2001

Key words: copolymerization; chitosan; poly(acrylamide-*co*-HEMA); immobilization; entrapment

INTRODUCTION

Immobilization of biologically active species is industrially important due to the ease of product separation, reuse of the enzyme, and no unfavorable side reactions. The choice of the support and the method of immobilization play a crucial role in governing enzymatic reactions. Naturally occurring polymers such as chitin and chitosan offer certain advantages due to low bulk density, coarse porous structure, no toxicity, biocompatibility, and possibility of immobilization either by adsorption or by a chemical reaction. However, they lack conformational stability and resistance to internal mass transfer, whereas synthetic polymers such as polyacrylamide, poly(hydroxyethyl methacrylate), graft polymers, and crosslinked copolymers offer stiffness as well as internal mass-transfer resistance.

Alcohol dehydrogenase (ADH), a complex redox enzyme, contains four subunits bound to zinc and a sensitive sulfhydryl group essential for its activity.[1] The immobilization of ADH onto numerous supports was reported in the literature.[2–5] ADH from yeast and horse liver has been coupled to natural[6,7] and synthetic[8] carriers. Many studies have been reported on the stability of immobilized ADH toward heat,[9] continuous operation,[10] storage,[11] and different substrates.[12] Various techniques like covalent binding,[13] crosslinking,[14] and entrapment of ADH were used for immobilization.

Ooshima et al.[12] reported only 10.4 U/g loading of yeast alcohol dehydrogenase (YADH) onto agarose gel through covalent coupling. Sepharose, an expensive and mechanically inferior support, was

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Journal of Applied Polymer Science, Vol. 82, 1299–1305 (2001)

used by Helmut and Schneider[15] for the coupling of YADH using cyanogen bromide activation. However, it could retain only 50% of enzyme activity at 60°C. Stabilization of immobilized YADH was attempted using different additives such as glycerol, sorbitol, and sucrose. Quantitative retention of enzyme activity to 30 days at 4°C was reported by Brougham and Johnson.[16] Lactase and glucoamylase were immobilized on chitin through glutaraldehyde activation.[17] Porous beads of chitosan were used as a support for the immobilization of ∝-galactosidase[18] and β-galactosidase.[19,20] Covalent binding of enzymes involves the chemical conversion of a functional group of the polymer through a multifunctional group reagent, prior to the coupling of the enzyme, while entrapment of an enzyme is performed by polymerizing an aqueous solution containing monomers and the enzyme in the presence of a crosslinking agent.

Studies of the stability[21,22] or activity[23–25] of immobilized enzymes showed that immobilization leads to deviation from their native form. Hence, we undertook the immobilization of YADH through covalent coupling (CB-YADH) and entrapment (ENT-YADH) on natural and synthetic polymers to study their behavior toward the oxidation of ethanol.

EXPERIMENTAL

Materials

YADH (450 U/mg) and *N*-*N*′-methylene bisacrylamide were from the Sigma Chemical Co. (St. Louis, MO), and nicotinamide adenine dinucleotide (NAD) in oxidized and reduced form, hydrogen peroxide 30% (w/w), and 2-hydroxyethyl methacrylate (HEMA), from E. Merk India, Ltd. (Mumbai, India) were used as received. Ascorbic acid and acrylamide (AAm) were purchased from Spectrochem Pvt. Co. (Mumbai, India). Chitosan was received as a gift material from the Central Institute of Fisheries Technology (CIFT; Cochin, India). Glutaraldehyde from S.D. Fine Chemicals (Mumbai, India) was distilled before use. All other reagents and chemicals used were of analytical grade. Double-distilled deionized water was used throughout the work.

Enzyme Immobilization

Covalent Binding of YADH

Chitosan beads prepared as per the procedure given by Sun and Payne[26] were incubated with various concentrations (0.01–0.2% v/v) of glutaraldehyde for the activation of free amino groups. The activated support was further used for the immobilization of YADH (1000 U/g) in 50 m*M* phosphate buffer of different pH at 10°C. Julabo SW1, a low-temperature shaker, was used for the reaction. The immobilized protein content was estimated by determining the protein from the supernant liquid by Lowrys' method.[27]

Entrapment of YADH During Copolymerization

Entrapment of YADH (400 U/g) into poly(AAm-*co*-HEMA) gel was done by the method reported by us[28] for α-chymotrypsin.

Assay of YADH Activity

The activity of the free and immobilized enzyme was determined spectroscopically using the standard method[29] and ethanol as the substrate. The enzyme-catalyzed reaction rate was monitored in 100 m*M* glycine buffer of pH 9.2 using 6 m*M* NAD and 200 m*M* ethanol at 27°C. The absorbance of NADH was measured at 340 nm. The amount of aldehyde formed was measured from the calibration plot.

pH Activity Profile

The activity of the free and immobilized YADH was measured by incubating the free and immobilized enzyme at 27°C for 30 min in the buffers of different pH and using ethanol as a substrate. The absorbance of the reaction mixture was measured at 340 nm and correlated to the concentration of the enzyme. From the calibration plot, the activity of the enzyme was determined.

Thermal Stability

Free and immobilized enzymes were placed in the optimum pH buffer and incubated at different temperatures (40–70°C) for different time intervals. The activity of the enzyme was then determined as described earlier. The thermodeactivation constant (K_d) was calculated using the following equation[30]:

$$\ln A_t = \ln A_0 - K_d(t)$$

where A_0 is the initial activity, and A_t, the activity after heat treatment for t minutes.

Storage Stability

The residual activities of the free and immobilized enzymes stored at room temperature (35°C) were determined and the activities were expressed as the percentage retention of their residual activities at different times.

Reusability of Immobilized YADH

To evaluate the reusability of the immobilized YADH, it was washed with water and the buffer after use and then suspended again in a fresh reaction mixture to measure the enzymatic activity. This procedure was repeated until the enzyme lost 50% of its activity. The reusability of immobilized YADH was examined using ethanol, propanol, butanol, and pentanol as substrates. The oxidation product formed was measured spectrophotometrically at 340 nm using a 100 m*M* glycine buffer of pH 9.2 and 6 m*M* NAD and a 200-m*M* substrate concentration at 27°C. Leakage of the enzyme, if any, was determined by measuring the enzyme activity in the washings.

Determination of Kinetic Constants

The Michaelis constant (K_m) and the maximum reaction velocity constant ($V_{\max}$) for the free and immobilized YADH were determined by measuring the velocity of the reaction at varying substrate concentrations from 50 to 500 m*M*. Free and immobilized enzymes in the optimum pH buffer were incubated with substrates for 30 min at 27°C. From the activity of the enzymes, K_m and $V_{\max}$ were calculated using the Lineweaver–Burk plot of $1/S$ versus $1/V$.

Oxidation of Ethanol Using Fixed-Bed Reactor

A fixed-bed reactor study of the immobilized YADH was carried out using ethanol as a substrate. Immobilized YADH [2 g each of chitosan and poly(AAm-*co*-HEMA)] under optimum conditions was packed in the column of 1.2 × 20-cm dimension. The column was maintained at 27°C and aliquots of 25 mL of ethanol were passed through the reactor at 1–4 mL min^{-1} using a peristaltic pump. The efficiency of the reactor containing immobilized YADH was determined by operating the reactor continuously and measuring the absorbance at 340 nm at fixed time intervals, for different concentrations of ethanol (100–400 m*M*).

RESULTS AND DISCUSSION

The immobilization of a water-soluble enzyme through covalent coupling to a water-insoluble polymer support should preferably involve functional groups of the enzyme that are not essential for enzymatic catalysis and, if reacted, would not alter any chemical or physical properties of the enzyme. Hence, covalent binding of YADH to natural polymer chitosan involving free amino groups activated through glutaraldehyde was examined. Quantitative coupling of YADH is achieved at a 0.05% concentration of glutaraldehyde in 50 m*M* phosphate buffer of pH 7 at 10°C for a 12-h coupling time. Although the protein coupled to the support was observed to be 450 U/g of the dry polymer, only 25% of the enzyme activity was retained. This indicates that either the coupling has taken place through active sites, decreasing the enzyme activity, or denaturation of the enzyme is taking place during the immobilization process. However, only 3.6 U/g of the active enzyme on immobilization using a glutaraldehyde-activated glycidyl methacrylate–ethylene dimethacrylate copolymer was reported by Kovar et al.[31]

Ooshima et al.[12] carried out immobilization of YADH by covalent coupling to glass using a glutaraldehyde coupling agent as well by entrapping YADH in polyacrylamide gel. The retention of the enzyme activity was observed to be 8.25 and 1.07 mg/g of the wet support. To increase the retention activity of immobilized YADH, attempts were made by Carrea et al.[32] and Godbole et al.[33] using a very high concentration of YADH using cynogen-activated Sepharose 4B support.

Use of poly(AAm) or poly(HEMA) gels for enzyme entrapment is known. However, crosslinked poly(AAm) does not have dimensional stability, whereas crosslinked poly(HEMA) imparts stiffness and also contributes to the internal mass-transfer resistance. Hence, to achieve balanced properties, AAm/HEMA copolymers with a 1 : 1 composition (w/w) was used for the entrapment of YADH. Free-radical polymerization was initiated using 2.4 m*M* hydrogen peroxide (30%, w/w) and 1 m*M* ascorbic acid with a 2% *N*-*N*′methylene bisacrylamide crosslinker. Approximately 90% of active enzyme was observed to be entrapped in the polymer network.

Optimum pH

Figure 1 illustrates the effect of immobilization on the optimum pH for the enzyme activity. The

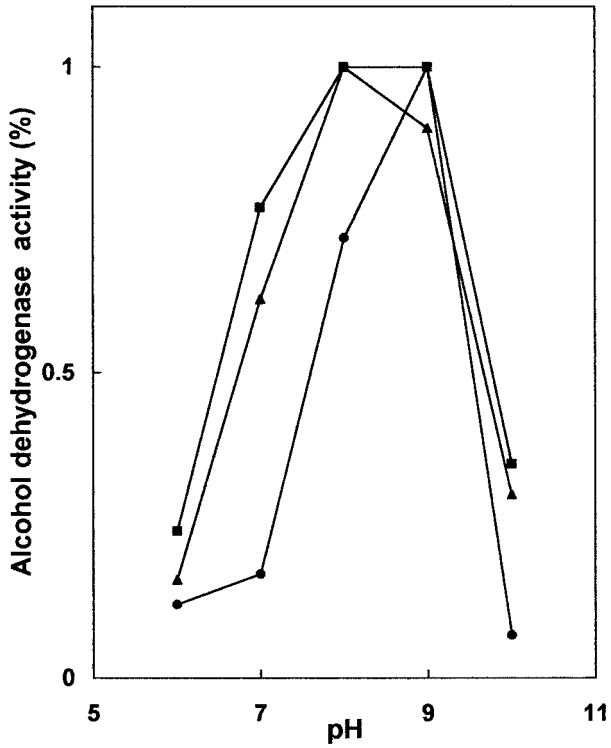

Figure 1 pH activity profile of free and immobilized enzymes at 27°C: (●) free YADH; (▲) CB-YADH; (■) ENT-YADH.

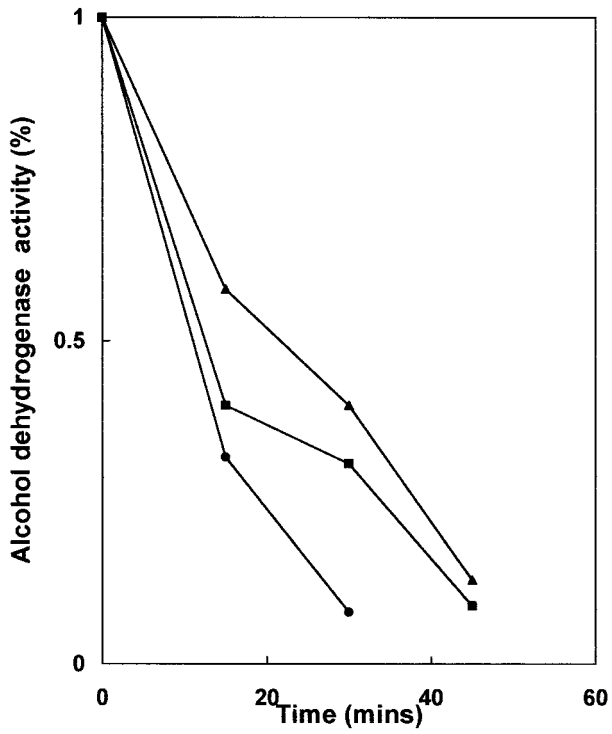

Figure 2 Thermal stability of free and immobilized enzymes at 70°C: (●) free YADH; (▲) CB-YADH; (■) ENT-YADH.

free enzyme shows maximum activity at pH 9, whereas the CB-YADH enzyme, at pH 8, indicating that the polymer matrix behaves as a polycation to some extent. The behavior is of considerable interest for the use of the enzyme in the food-processing industries.[34] However, ENT-YADH shows an optimum pH between 8–9.

Thermal Stability

Knowledge of thermal stability of an immobilized enzyme is very useful in the investigation of potential applications of enzymes. Figure 2 shows a comparison of free and immobilized YADH. It was observed that an immobilized enzyme has a higher thermal stability than that of the free enzyme. Free enzyme loses its 90% activity, whereas ENT-YADH retained 30% and CB-YADH retained 40% activity over a 30-min incubation at 70°C. From the study, the thermodeactivation constants (K_d), calculated as discussed earlier, are given in Table I. From the data, it is observed that rate of deactivation increases with the temperature for both free and immobilized YADH. However, it can be seen that the rate of deactivation is higher for the ENT-YADH system in comparison with CB-YADH, indicating the improved thermal stability of immobilized systems other than free YADH.

Storage Stability

Destabilization is considered to be caused by autolysis or microbial growth on the enzyme. Immobilization reduces autolysis and/or prevents microbial growth. The storage stability of free and immobilized YADH was investigated and results

Table I Effect of Temperature on the Deactivation of YADH

Temperature (°C)	Deactivation Rate Constant ($K_d \times 10^2$)		
	Free YADH	CB-YADH	ENT-YADH
40	0.08	0.05	0.05
50	0.31	0.22	0.24
60	1.46	1.11	1.39
70	7.65	3.70	5.20

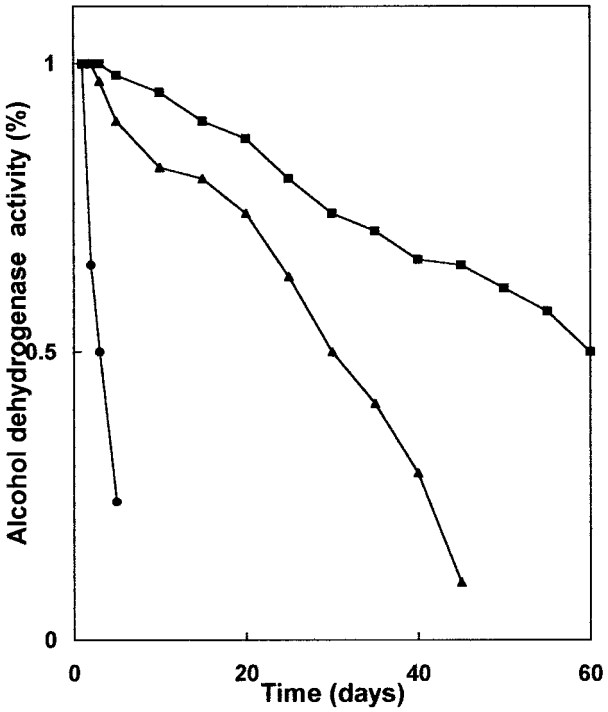

Figure 3 Storage stability of free and immobilized enzymes at 35°C: (●) free YADH; (▲) CB-YADH; (■) ENT-YADH.

are given in Figure 3. At room temperature (35°C), the free enzyme loses 50% of its activity after 3 days, whereas ENT-YADH and CB-YADH retain 50% of their activity after 60 and 30 days, respectively. The stabilization may be due to multipoint attachment of the enzyme to the support and/or it acts as semipermeable membrane, creating a more rigid enzyme molecule.[7] Hence, disruption of the active center becomes less likely to occur. Similar results were also observed for YADH immobilized on a cyanogen bromide-activated Sepharose system by Li et al.[35] However, Millis and Wingard[36] observed retention of only 10% of YADH activity on storage at pH 8.8 at 30°C after 2-days storage.

Reusability

Free enzymes suffer from a major drawback of nonreusability. This is an advantage for immobilized enzymes. The activity of the immobilized system after successive uses is given in Figure 4. ENT-YADH retains 50% activity after five cycles, whereas CB-YADH retains 50% activity after eight cycles for ethanol oxidation. Limited experiments were also carried out for the oxidation of higher alcohols like propanol, butanol, and pentanol for the reusability of immobilized YADH. It was observed that as the number of carbon atoms in alcohol increases the number of cycles decreases. This is because YADH is a very specific enzyme, which can accept only a hydrogen atom or a smaller methyl group of the substrate. Therefore, it is less active as the chain length of corresponding alcohol increases.[37] These results are promising for the application of immobilized YADH in the industry.

Determination of Kinetic Constant

The effect of the substrate concentration on the reaction rate catalyzed by free and immobilized YADH was studied using an ethanol substrate. As shown in Figure 5, reciprocal Lineweaver–Burk plots were used for calculation of the Michaelis constant constant (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized YADH, which are presented in Table II. The values of K_m and V_{max} of the free YADH were found to be 8.3 $\times$ 10^{-2} mM and 9.1 mM min^{-1}, respectively.

When YADH was immobilized by covalent binding onto chitosan and entrapped into poly(AAm-*co*-HEMA), K_m values were observed to

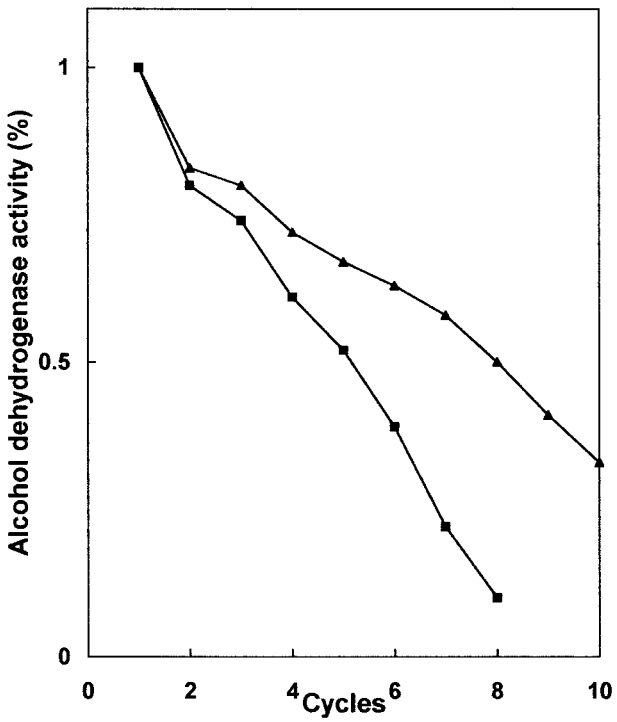

Figure 4 Reusability of immobilized enzyme at 30°C: (▲) CB-YADH; (■) ENT-YADH.

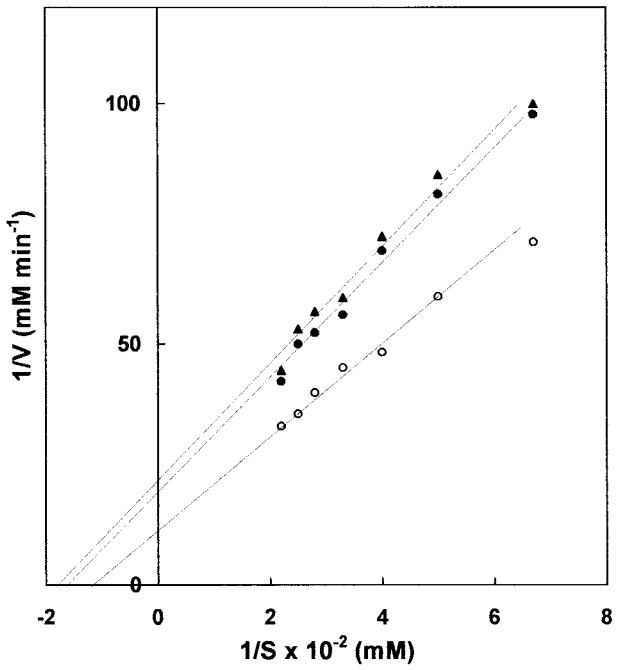

Figure 5 Lineweaver–Burk plots for ethanol oxidation at 27°C, pH 9.2, for 30 min by (○) free YADH, (●) CB-YADH, and (▲) ENT-YADH.

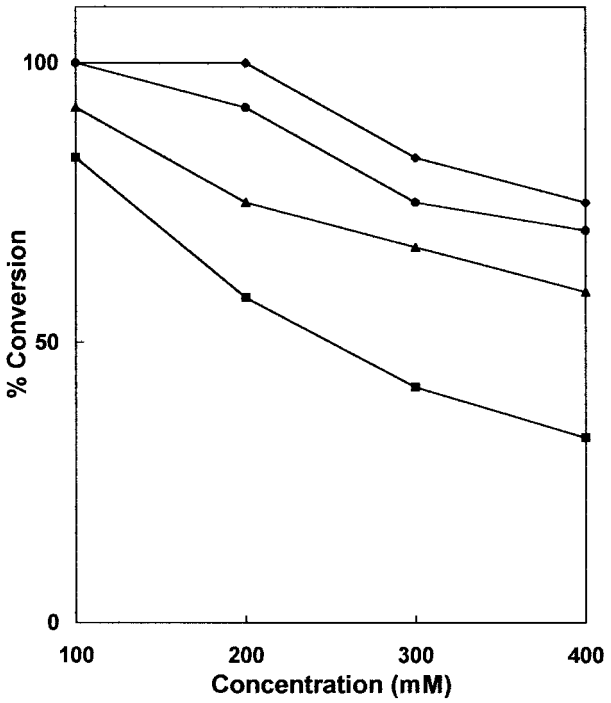

Figure 6 Oxidation of ethanol using fixed-bed reactor (CB-YADH) at 27°C, pH 9.2, for 300 min: (◆) 1 mL min^{-1}; (●) 2 mL min^{-1}; (▲) 3 mL min^{-1}; and (■) 4 mL min^{-1}.

decrease about 1.2- and 5.2-fold, respectively. The lower values of for the K_m immobilized enzyme may be due to strong electrostatic attractions between the polymeric support and the protein chains as well as to protein substrate interaction.

On the other hand, the values of V_{max} of CB-YADH and ENT-YADH was 1.7- and 2.5-fold smaller, respectively, than that of the free YADH. This may be due to greater rigidity of the gel which limits the substrate diffusion in the matrix toward the enzyme reaction sites and due to a possible deactivation of the enzyme which occurred during gel formation.

Oxidation of Alcohol Using Fixed-Bed Reactor

The activity of CB-YADH in a packed bed reactor is shown in Figure 6. The activity decreased to 80% of its initial value after four cycles operated at flow rate (1 mL min^{-1}) and at a low concentration (100 m*M*) of ethanol, while at a higher flow rate (4 mL min^{-1}) and higher concentration (400 m*M*) of ethanol, the extent of oxidation decreased to 35% after five cycles.

Similar results were obtained (Table III) when ENT-YADH was used in the reactor. However, the values obtained are smaller than are those of CB-YADH. This can be attributed to the difference in the nature and surface area of the polymeric supports used for the immobilization. This confirms that there is a stronger bond formation between chitosan and YADH than for entrapped YADH into poly(AAm-*co*-HEMA) in which leakage of enzyme is likely to take place.

CONCLUSIONS

YADH was successfully immobilized on poly(AAm-*co*-HEMA) and onto the chitosan matrix by

Table II Kinetic Parameters for Free and Immobilized YADH

	Free YADH	CB-YADH	ENT-YADH
K_m (m*M*)	8.3×10^{-2}	6.7×10^{-2}	1.6×10^{-2}
V_{max} (m*M*/min)	9.1	5.3	3.6

Table III Effect of Concentration and Flow Rate on the Oxidation of Ethanol Using ENT–YADH

Concentration (m*M*)	% Conversion at Flow Rate/Min			
	1 mL	2 mL	3 mL	4 mL
100	100	92	50	25
200	90	83	42	17
300	83	70	33	11
400	75	58	23	8

the entrapment and the covalent binding technique, respectively. Under all conditions of the stabilities tested, immobilized YADH was observed to be better than was free YADH. However, CB-YADH has more potential than has ENT-YADH. These differences between the two methods are dependent on the nature and surface area of the polymeric supports used for the immobilization. The stability of YADH was improved on immobilization. Using a continuous packed bed reactor with an immobilized enzyme, 80% oxidation of ethanol could be achieved.

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