Reactive Green 19 Modified poly(HEMA) Nanostructures for Alcohol Dehydrogenase Immobilization

Deniz Aktaş Uygun*[1], Nazife Kaya[2], Murat Uygun[3]

[1,2,3]Chemistry Department, Adnan Menderes University, Aydın, Turkey, +90 256 2128498
daktasuygun@gmail.com
*Corresponding author / İletişimden sorumlu yazar

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Abstract

In this presented work, poly(HEMA) nanostructures were prepared by using surfactant free emulsion polymerization technique. Magnetic character was introduced to the polymer by simple adding of Fe_3O_4 to the polymerization medium while polymerization taken place. These magnetic nanostructures were modified with Reactive Green 19 dye by nucleophilic substitution reaction. Synthesized dye attached poly(HEMA) nanostructures were characterized by FTIR, ESR, AFM and EDX analysis. These nanostructures were used for immobilization of alcohol dehydrogenase by the help of dye affinity interaction. Effects of medium pH and temperature, and substrate concentration on the activity of the alcohol dehydrogenase were investigated. Optimum pHs for free and immobilized alcohol dehydrogenase were found to be 8.0 (in phosphate buffer), while optimum temperature of both enzyme types was found to be 25 °C. Kinetic parameters of free and immobilized enzyme were also determined. Both K_m ad V_{max} values of the immobilized alcohol dehydrogenase were found to be higher than that of free enzyme. Also, thermal, operational and storage stability of immobilized enzyme were investigated and it was found that immobilized preparation showed more stability than free one. Immobilized preparation worked 10 successive reuse with a little decrease in its activity.

Keywords— Alcohol dehydrogenase; Reactive Green 19; Nanostructure; Dye affinity

1 Introduction

Magnetic nanostructures have been developed and studied in various biotechnological studies because of their unique advantageous properties. For example, their size can be easily controlled, they are able to direct by external magnetic field, and they can be used in magnetic resonance imaging applications because of their unique contrast properties. These nanostructures have found various applications such as detection of bacteria, purification of biomolecules, immobilization of enzymes, decorporation of contaminants, drug delivery, hyperthermia and so on [1]. Their usage for immobilization of different kinds of enzyme has been very popular in biotechnological studies due to their unique advantages: 1) high enzyme loading capacity because of large specific surface area, 2) reduced mass transfer resistant properties and lower fouling behaviors, and 3) easy and fast separation of immobilized enzyme from a reaction mixture by using external magnetic field [1-6].

Alcohol dehydrogenase (EC 1.1.1.1) is widespread enzyme found in bacteria, yeasts, plants and animals. The main function of alcohol dehydrogenase in metabolism is the catalyzing the reversible oxidation of alcohols to their carbonyl compounds [7]. Its affinity decreases with the carbon number of the aliphatic alcohol structure [8]. Overall structure of the enzyme is a tetramer which comprise of four identical subunits. Each subunit has a molecular mass of 36 kDa which contains a single protein chain with 347 amino acids. Each subunit also has a binding site for a coenzyme (NAD^+) and one tightly bound zinc atom which are very important for catalysis [9]. Handicaps of the

alcohol dehydrogenase for industrial applications are their high sensitivity and poor stability. Because of these, immobilization of alcohol dehydrogenase onto different type of support materials had been carried out in order to overcome these drawbacks [10-19]. Because alcohol dehydrogenase requires NAD^+ as a cofactor, its specific interaction with reactive dyes which are act as a NAD analog has been investigated intensively [20]. Reactive dyes have been used deeply for protein separation or adsorption as dye affinity ligands due to their specific interactions with various proteins and enzymes. General structure of reactive dyes has a chromophore (either azo dyes, phthalocyanine or anthroquinone), a reactive group (generally a mono- or dichlorotriazine ring) and sulfonic acid groups which enhance the solubility of the dye in aqueous media. Dye ligands are very cheap materials and commercially available. Their immobilization onto hydroxyl group bearing support materials is very easy [21-24]. In this paper Reactive Green 19 dye ligand has chosen as an affinity ligand of alcohol dehydrogenase. Reactive Green 19 is a dichlorotriazine dye which has six sulfonic acid groups and five amino groups in their structure [25].

This article reports on the immobilization of alcohol dehydrogenase by using dye ligand affinity technique with magnetic nanostructures. The poly(HEMA) magnetic nanostructures were synthesized by surfactant free emulsion polymerization in the presence of Fe_3O_4. Then the hydroxyl groups on the mag-poly(HEMA) nanostructures were derivatized by reaction with Reactive Green 19 and these synthesize nanostructures were characterized. Dye attached magnetic nanostructures were used for alcohol dehydrogenase immobilization. The effects of some factors such as pH, temperature and substrate concentration to activity of immobilized and soluble alcohol dehydrogenase were investigated. Additionally, thermal, operational and storage stability of immobilized alcohol dehydrogenase were studied.

2 Materials and Methods

2.1 Materials

2-hydroxyethyl methacrylate (HEMA), N,N,N',N'-tetramethylene diamine (TEMED), ethylene glycol dimethacrylate (EGDMA), ammonium persulfate (APS), magnetite powder (Fe_3O_4, average diameter, 20-50 nm), Reactive Green 19, alcohol dehydrogenase (*S. cerevisiae*, EC 1.1.1.1), phenylglyoxylic acid, nicotinamide adenine dinucleotide (NADH) and poly(vinyl alcohol) (molecular weight, 100,00; 98 % hydrolyzed) were purchased from Sigma (St. Louis, USA). All other chemicals were of the highest purity and used without further purification. Deionized ultrapure Millipore simplicity® (18.2 mΩcm) water was used for all solutions.

2.2 Preparation and characterization of dye-attached magnetic nanostructures

Surfactant free emulsion polymerization technique was used for the synthesis of magnetic poly(HEMA) nanoparticles. Then Reactive Green 19 was attached to the magnetic nanostructures with nucleophilic substitution reaction. Characterization of magnetic nanostructures was performed by using FTIR, ESR, AFM and EDX analysis. Details of preparation and characterization of Reactive Green 19 attached poly(HEMA) nanostructures were given in our previous article [26].

2.3 Immobilization of alcohol dehydrogenase on the dye attached magnetic nanostructures

Immobilization of alcohol dehydrogenase was performed by adsorption technique in a batch experimental set-up. For this, dye attached magnetic nanostructures were mixed with alcohol dehydrogenase solution (in 100 mM of phosphate buffer; pH 7.0) and magnetically stirred (120 rpm) for 24 h at 25 °C. The alcohol dehydrogenase concentration was measured at 280 nm by using a double beam UV/Vis spectrophotometer (Model 1601, Shimadzu, Japan). The amount of immobilized alcohol dehydrogenase on the dye attached magnetic nanostructures was determined by measuring the initial and final concentrations of alcohol dehydrogenase solution.

2.4 Activity of alcohol dehydrogenase

Activity studies of alcohol dehydrogenase were carried out by the method described by Li et al. [27]. Briefly, 1.0 mL of phosphate buffer (0.1 M, pH 7.0), 0.25 mL of 1.0 mM NADH and 0.25 mL of phenylglyoxylic acid were mixed in a cuvette. The enzymatic reaction was started with the addition of 0.25 mL of enzyme solution at room temperature and absorbance of NADH at 340 nm was followed for 3 min by using a UV-Vis spectrophotometer. One alcohol dehydrogenase activity unit was defined as the alcohol dehydrogenase amount which used up 1.0 μmol NADH/min

under the specified conditions [19]. Bradford method was used for protein concentration determinations [28].

2.5 Characterization and stability of immobilized alcohol dehydrogenase

In order to characterize the free and immobilized alcohol dehydrogenase, effects of some factors such as pH, temperature and substrate concentration to the activity were determined. The optimum pH of free and immobilized alcohol dehydrogenase was determined by using 0.1 M phenylglyoxylic acid as substrate in acetate buffer (100 mM, for pH 4.0-5.0), phosphate buffer (100 mM, for 6.0-8.0) and carbonate buffer (100 mM, for pH 9.0). In order to determine the optimum temperature, the activities of free and immobilized alcohol dehydrogenase were performed with increasing temperatures in the range of 4-55 °C. The Lineweaver-Burk plots were evaluated for the determination of kinetic constants (K_m, V_{max} and k_{cat}). For this purpose, series measurements of the reaction rate were carried out in the substrate (phenylglyoxylic acid) concentration range of 0.0-0.10 mol/L for both free and immobilized alcohol dehydrogenase. In order to determine the thermal stability of free and immobilized alcohol dehydrogenase, the free and immobilized enzymes were incubated at temperatures of 35 °C and 45 °C for 240 min. The specific activities of the free and immobilized alcohol dehydrogenase were determined at apparent intervals by the activity method. To test the operational stability of immobilized enzyme, the immobilized alcohol dehydrogenase was used ten times and enzymatic activities were measured after each use. Alcohol dehydrogenase immobilized dye attached magnetic nanostructures was washed with phosphate buffer (100 mM, pH 7.0) after each use. The enzyme activities of the freshly prepared dye attached magnetic nanostructures measured in the first run were determined as 100 %. For the storage stability, free and immobilized alcohol dehydrogenases were stored in a 100 mM phosphate buffer (pH 7.0) at 4 °C for 15 days. Activities of the samples taken from the store were assayed periodically at 25 °C.

3 Results and Discussion

3.1 Properties of the dye attached magnetic structures

Intensive works have been carried out for the preparation of nanosized materials with various shapes and sizes, recently. Nano-structured materials have been deeply used in diverse biotechnological studies such as sensors and separation applications due to the unique properties of the nanostructures. Magnetic nanostructures have also been intensively used in different biotechnological applications such as protein or enzyme immobilization, diagnostic, therapeutics, bioseparations, immunoassays, drug delivery, biosensors, magnetic imaging [29-35] and so on [22, 36-38].

In this presented work, nano sized mag-poly(HEMA) structures were synthesized by using surfactant free emulsion polymerization technique. Then, functional dye molecule Reactive Green 19 was attached covalently to these magnetic nanostructures by simple nucleophilic substitution reaction. Preparation of Reactive Green 19 attached magnetic nanostructures are schematically demonstrated in Fig 1. Table 1 shows the physicochemical properties of the Reactive Green 19 attached mag-poly(HEMA) nanostructures. General structure and morphology of the synthesized nanostructures were demonstrated by AFM (Fig 2). As seen here, synthesized nanostructures are monosized and have spherical shape. Their diameter was found to be 70 nm and their magnetic character was also showed.

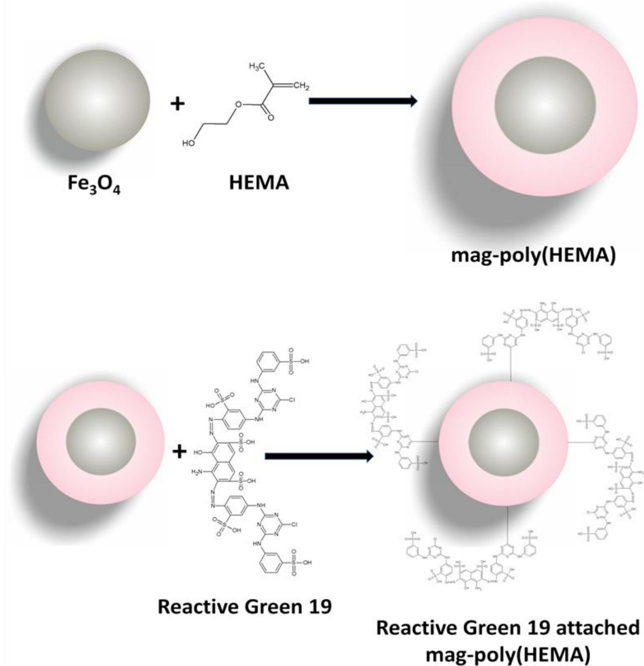

Figure 1. Schematic presentation for the preparation of Reactive Green 19 attached poly(HEMA) nanostructures.

Table 1. Some properties of Reactive Green 19 attached mag-poly(HEMA) nanostructures

Particle diameter	70 nm
Magnetic resonance point	2844 Gauss
g factor	2.299
Attached amount of Reactive Green 19	63 μmol/g

3.2 Characterization of the free and immobilized alcohol dehydrogenase

There are three main methods for enzyme immobilization have been studied intensively; binding to a support (carrier), entrapment (encapsulation) and cross-linking. Using an enzyme in immobilized form has several advantages. For example, they can easily be removed from the reaction medium by using simple filtration or magnetic separation; contamination of the product can be eliminated by immobilization of enzyme onto a solid supports. Easy recovery and reusability properties of the immobilized systems decrease the cost of an enzymatic process. Their stabilities often increase upon immobilization, and their storage and operational stabilities also develop. By immobilization, their denaturation by heat and organic solvents can be inhibited [39].

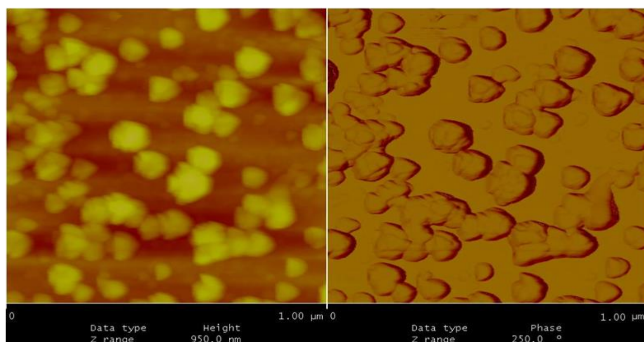

Figure 2. AFM picture of Reactive Green 19 attached magnetic poly(HEMA) nanostructures.

In this study, alcohol dehydrogenase was immobilized onto Reactive Green 19 attached magnetic nanostructures via adsorption. Immobilized amount of alcohol dehydrogenase was found to be 176.09 mg/g polymer in pH 7.0 phosphate buffer. The effects of pH on the activities of the free and immobilized alcohol dehydrogenase were investigated at the pH range of 4.0-9.0. Phenylglyoxilic acid was used as a substrate in the activity studies. Optimum pH of the alcohol dehydrogenase is variable depends on the source of the alcohol dehydrogenase and substrate used in activity studies. Zhou et al. [19] reported that, optimum pH of the *S. cerevisiae* alcohol dehydrogenase was found to be 6.8 by using phenylglyoxylic acid as a substrate. In this work, optimum pH was found to be 8.0 both for free and immobilized alcohol dehydrogenase (Fig 3). Although the optimum pH was not change upon immobilization, immobilized enzyme was more active than the free one within the studied pH range.

Activities of the free and immobilized alcohol dehydrogenase were studied at six different temperatures between 4 °C and 50 °C. Fig 4 demonstrates that, immobilized enzyme showed more activity than free enzyme at all tested temperatures. Optimum temperature of free and immobilized alcohol dehydrogenase was found to be 25 °C. Optimum temperature of the enzymes generally increases upon immobilization onto a solid support, and this serves some advantages for industrial applications. But optimum temperature was not change in this study. A similar optimum temperature value for *S. cerevisiae* was reported by Li et al. [18]. These researchers reported that, optimum temperature value for both free and immobilized alcohol dehydrogenase was 30 °C.

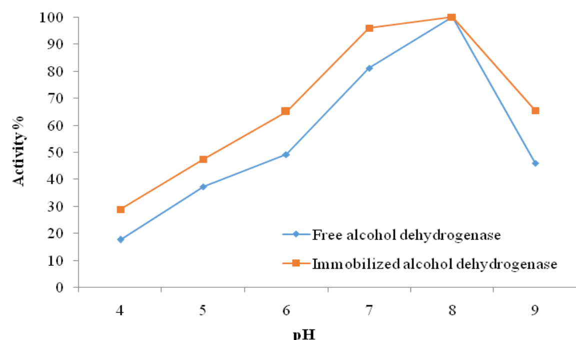

Figure 3. Effect of pH on the activity of free and immobilized alcohol dehydrogenase.

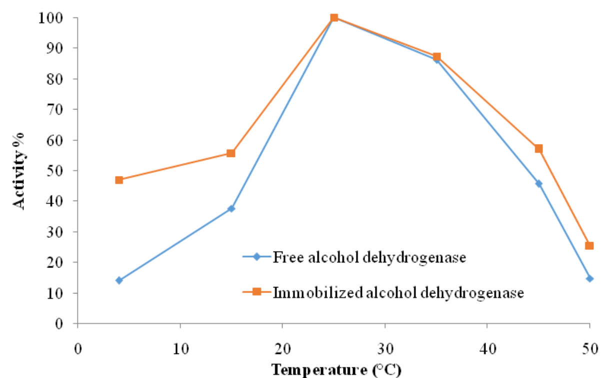

Figure 4. Effect of medium temperature on the activity of free and immobilized alcohol dehydrogenase.

It is very important to determine the kinetic parameters of an enzyme for express its efficiency. In this work, Michaelis constant (K_m) and maximum velocity (V_{max}) of free and immobilized alcohol dehydrogenase were determined by using phenylgyloxylic acid as a substrate. Table 2 summarizes the kinetic values of the both free and immobilized enzymes. Kinetics of an enzyme change when it is immobilized onto a support material due to the some important factors such as the character of the support, heterogeneity of system and the accessibility of substrate to the enzyme. In this presented work, K_m values of free and immobilized alcohol dehydrogenase were found to be 0.040 and 0.073 mol/L, respectively. K_m is known as the affinity of an enzyme to its substrate [40] and it is expected that K_m value of an enzyme generally increase upon immobilization. In this work, K_m value of the alcohol dehydrogenase also increased. It means that, the affinity of the immobilized alcohol dehydrogenase towards its substrate decreased. V_{max} is the maximum enzymatic velocity when all enzymes are saturated with their substrate and this parameter reflects the real properties of the immobilized enzyme, but it is affected highly with diffusional limitations [41]. In this presented study, V_{max} of the immobilized alcohol dehydrogenase (0.460 μmol/min) was found to be higher than that of free enzyme (0.249 μmol/min). It was shown by Pahujani et al. [42] with the lipase immobilization study that maximum velocity of immobilized enzymes could increase. However, in order to estimate the behaviors of immobilized enzyme, k_{cat} (turnover number) is much more beneficial than V_{max} and K_m. When considering the k_{cat} values of the free and immobilized alcohol dehydrogenase, k_{cat} of free alcohol dehydrogenase was found to be 1402 min^{-1}, while k_{cat} of immobilized enzyme was calculated as 736 min^{-1}. This decrease in the k_{cat} showed that molecule number which converted by the enzyme per unit of time decreased upon immobilization. This expected case generally occurs when enzymes are immobilized onto solid support materials.

Table 2. Kinetic parameters for free and immobilized alcohol dehydrogenase (ADH) onto Reactive Green 19 attached mag-p(HEMA) nanostructures.

Enzyme type	K_m (mol/L)	V_{max} (μmol/min)	k_{cat} (min^{-1})
Free ADH	0.040	0.249	1402
Immobilized ADH	0.073	0.460	736

3.3 Thermal, storage and operational stability

Effects of temperature on the stability of the free and immobilized alcohol dehydrogenase are demonstrated in Fig 5 (A) and (B), respectively. As seen in Fig 5 (A), free enzyme protected 31 % of its initial activity at the end of the 240 min at 35 °C, while immobilized alcohol dehydrogenase protected 52 % of its initial activity with same conditions. In case of 45 °C, while free alcohol dehydrogenase protected 34 % of its initial activity, immobilized preparation protected % 56 of its initial activity. These findings demonstrate that, thermal stability of immobilized alcohol dehydrogenase was higher than that of free alcohol dehydrogenase. Zhou et al. [19] also demonstrated in their work that, thermal stability of alcohol dehydrogenase from *S. cerevisiae* increased after immobilization. Industrial processes are carried out at room or higher temperature and increased thermal stability enhance the usability of the immobilized enzyme for industrial applications. As known, immobilization generally increases the stability of enzyme at high temperatures and this develops the immobilized enzymes for more beneficial and economic in industrial applications.

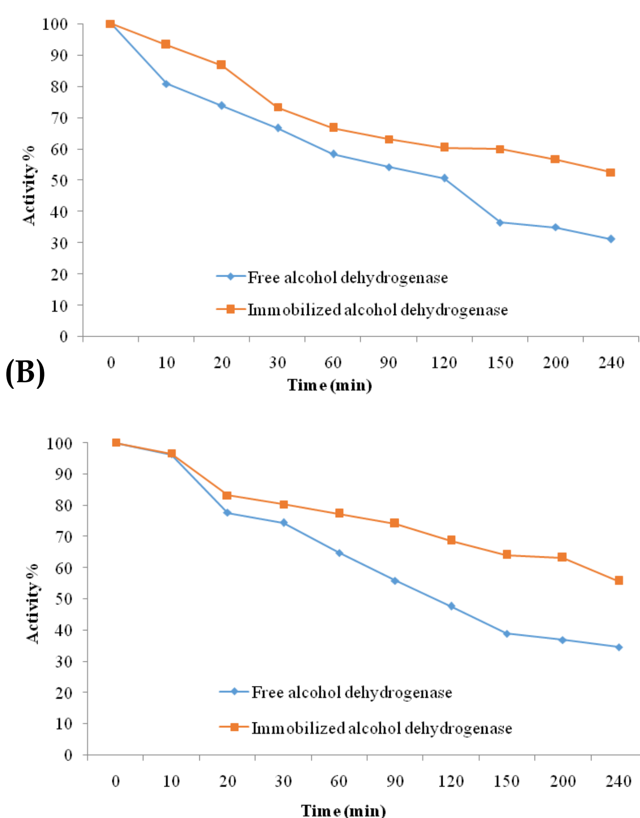

Figure 5. Thermal stability profile of free and immobilized form of alcohol dehydrogenase at 35 °C (A) and 45 °C (B).

Free and immobilized alcohol dehydrogenases were incubated at 4°C for 15 days and storage stabilities were investigated by activity tests. Free alcohol dehydrogenase protected % 16 of initial activity at the end of 15 days, while immobilized alcohol dehydrogenase showed % 34 of its initial activity after the same time period (Fig 6). Time depended activity lost of the immobilized *S. cerevisiae* alcohol dehydrogenase onto dye attached nanostructures was very slow when compared with free enzyme. With these results it can concluded that, the immobilized enzyme have much better storage stability than free enzyme.

Operational stability of immobilized enzymes is very important in order to evaluate the efficiency of the support material and immobilization method. In this work, operational stability of the immobilized alcohol dehydrogenase investigated by sequential activity studies at 25 °C. Immobilized alcohol dehydrogenase protected 79 % of its initial activity after the 10 successive uses. This slightly decrease in activity might be carried out by the inactivation of alcohol dehydrogenase which happens when used repeatedly. Results taken from the 10 recycle use showed that operational stability of the immobilized alcohol dehydrogenase was very high. Zhou et al. [19] immobilized the *S. cerevisiae* alcohol dehydrogenase onto magnetic nanostructures and they found that, activity of the immobilized alcohol dehydrogenase was 39 % of its initial activity at the end of the 6 reuse.

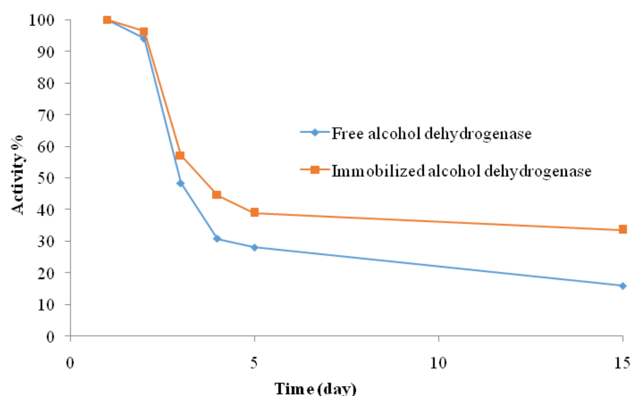

Figure 6. Storage stability of free and immobilized alcohol dehydrogenase for 15 days.

4 Conclusion

Nanoparticulated polymeric materials present unique advantages such as highly increased specific surface area, and enhanced physical and chemical properties. Because of their high surface area, they can bind huge amount of adsorbent for per mass of polymeric material and therefore, they have been intensively used for the adsorption or separation of biomolecules for biotechnological applications. Because of their coenzyme mimicking structures, dye molecules demonstrates high specificity towards the dehydrogenase type enzymes and they have been used as an affinity ligand for certain proteins and enzymes. Reactive Green 19 is a dichlorotriazine dye and has been used for binding of biomolecules. In this presented work, magnetic poly(HEMA) nanostructures were synthesized and modified with Reactive Green 19 dye, in order to bring an affinity towards the alcohol dehydrogenase. Magnetic properties of the nanostructures allow them to control their mobility with external magnetic fields. Optimum activity conditions for free and immobilized were determined and their stability profile was also investigated. It was shown by this study that, stability of the immobilized alcohol dehydrogenase increased and immobilized alcohol dehydrogenase could be used several times without significant decrease in their activity. In the light of this study it can be concluded that, these immobilized preparation of alcohol dehydrogenase can be used for biotechnological studies and industrial applications because of their increased stability and good operational properties.

5 References

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