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Full Length Research Paper

Immobilization of catalase via adsorption into natural and modified active carbon obtained from walnut in various methods

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In the present work, the immobilization of catalase into natural active carbon and active carbon modified by hydrochloric acid was carried out. In the experimental section, the effects of pH, ionic strength and reaction temperature were chosen as parameters, with experiments performed in batch system. For the optimization of immobilization procedure, values of kinetic parameters were evaluated. It was observed that storage and operational stabilities of the enzyme increased with immobilization. The results obtained from experiments showed that active carbon is a valuable support for the adsorption of enzymes.

Key words: Immobilization, active carbon, catalase.

INTRODUCTION

There are many advantages of using immobilized enzymes and some of them have a special relevance in the area of food technology. In this industrial area, the control of the expenses must be rigid because of the low added value of the products (Guisan et al., 1993). Both organic and inorganic materials such as porous glass, silica gels, hydro gels, and cellulose are used for preparation of immobilized enzymes. Immobilization of enzyme through physical methods is still most commonly used (Baileey and Ollis, 1986; Kennedy et al., 1990).

Enzyme immobilization by physical adsorption has the benefit of a wide applicability, and may provide a practical convenience of simple regeneration of support by removing the deactivated enzyme and reloading the support with fresh batch of active catalyst (Khan et al., 2006). Different procedures have been developed for enzyme immobilization; these include adsorption to insoluble materials, entrapment in polymeric matrix, encapsulation, crosslinking with a bifunctional reagent, or covalent linking to an insoluble carrier. Among these, adsorption to a solid support material is the most general, easiest to perform and oldest protocol of physical immobilization methods. The most important advantages of this method are the stability of enzyme activity after immobilization and reuse of the enzyme and support material for different purposes because of reversibility of the method (Akgol et al., 2005).

The goals of this work were to immobilize catlase with a significantly higher activity, more stable than in other earlier studies (Alkan et al., 2005), and to determine optimum conditions of immobilization process such as pH, ionic strength and reaction temperature.

MATERIALS AND METHODS

Materials

Catalase (CAT) (hydrogen peroxide oxidoreductase; EC.1.11.1.6), from bovine liver (250.000 U mg^{-1}), was obtained from Sigma (St. Louis, MO, USA), and active carbon was provided from region of Eskisehir in Turkey. All other chemicals and solvents used in this study were purhased fom Merck AG (Darmstadt, Germany) and were of analytical grade. Walnut used in this study was obtained from region of Bahcesaray (Van-Turkey).

Modification of active carbon surface by hydrochloric acid

To prepare acid-active carbon, 50 g natural active carbon was mixed with 1 M 250 ml HCI in mechanically stirring for 1 h and boiled

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for 5 h. All active carbon samples were dried in an oven at 80°C for 12 h, ground and passed through 270 mesh sieve.

Immobilization of catalase with adsorption

Catalase adsorption on the natural active carbon and active carbon modified by hydrochloric acid were carried out at different pH in phosphate buffer (pH 4.0 – 9.0). The initial catalase concentration was 0.2 mg cm^{-3} in the corresponding buffer. The adsorption experiments were carried out at different temperatures (20 - 70°C) for natural and modified active carbon respectively, with continuous stirring for 1 h. After this period, catalase immobilized membrane was removed from the enzyme solution, washed with same buffer 4 times and stored at 4°C in fresh buffer.

In order to determine the adsorption capacity of natural and modified active carbon, the amount of catalase in the medium was made to be 0.01 g. The adsorption tests were performed at different pH, temperatures and ionic strength.

The amount of the enzyme activity adsorbed on the clay was calculated as the difference between the values obtained in the original preparation and the supernatants (Alkan et al., 2005).

Protein estimation

The amount of protein in the crystalline enzyme preparation and in the wash solution was determined by the method of Bradford, using a Shimadzu (Model 1601) spectrophotometer. A calibration curve constructed with the bovin serum albumin (BSA) solution was used as a standard (Bradford, 1976).

Activity assay of free and immobilized catalase

Catalase activity was determined spectrophotometrically by direct measurement of the decrease in the absorbance of hydrogen peroxide at 240 nm due to its decomposition by the enzyme. Hydrogen peroxide solutions (50 mM) were used to determine the activity of both the free and immobilize enzyme. A 5 cm^3 of reaction mixture was preincubated at 30°C for 60 min and the reaction was started by adding 5 ml of buffer solution. The rate of change in the adsorbance (ΔA_{240} min^{-1}) was calculated from the initial liner portion with the help of the calibration curve (the adsorbance of hydrogen peroxide solutions of various concentrations (50 mM)) at 240 nm.

Catalases immobilized on natural and modified active carbon were introduced to the assay mixture to initate reaction above. After 60 min, the reaction was terminated by removal of active carbon from the reaction mixture. The absorbance of the reaction mixture was determined and the immobilized catalase activity was calculated. The activity assay was conducted over the pH of 4.0 - 9.0 and temperature range of 20 - 70°C to determine the pH and temperature profiles for the free and immobilized enzymes. The effect of the substrate concentration was tested in the 50 mM H_2O_2 concentration. The results of pH and temperature are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

Stability of immobilized catalase during repeated reuses

The retention of immobilized catalse activity was tested in a batch system as described in section 2.5. After each reaction period, the enzyme-active carbon disks were removed from reaction medium and washed with phosphate buffer (0.05 M, pH 7) at 30°C for 15 min to remove any residual substrate on the membrane disks. They were reintroduced into fresh reaction medium containing 50 mM H_2O_2.

RESULTS AND DISCUSSION

In our study, the immobilized enzyme gave a K_m value of 7.1 mM as compared with 3.57 mM for the free enzyme. V_{max} values were found to be 39.40 IU/ml/min and 37.03 IU/ml/min for the free enzyme, respectively. The Vmax value of immobilized catalase was found lower than that of free catalase. The loss in activity might be attributed to the interaction of enzyme and the functional groups on the surface of beads or large areas of contact between enzyme and support, causing large deformations of enzymatic conformation (Çetinus and Öztop, 2000; Li et al., 2004). The difference in Km values between free and the other immobilized catalase can be attributted to the the limit of accesibility of substrate molecules to the active sites of the immobilized catalase. The decrease in V_{max} value, a result of immobilization, is considered to be associated with Km value, since the lower the value of Km, the greater the affinity between the enzyme and the substrate (Demir et al., 2008; Akgöl et al., 2001).

It was found as Km 7.1 mM, Vmax 39.40 mmole/min mg protein for natural active carbon and; Km 3.84 mM, Vmax 36.80 mmole/min/mg protein for acid-active carbon im-mobilized catalase. That results were found different from the other findings (Çetinus and Öztop, 2003, 2000a,b; Li et al., 2004; Gupta and Ravikumar, 2000). Similar results involving change in Km and Vmax values of enzyme after immobilization have been reported in literature (Çetinus and Öztop, 2000; Li et al., 2004; Gupta and Ravikumar, 2000).

The PPO activity of immobilized clay was determined, and then the effects of reaction optimum temperature, thermostability, optimum pH, ionic effect and kinetic parameters were investigated (Demir et al., 2008).

Effect of temperature on catalytic activity

At the determination of the effect of temperature on free, natural active carbon and active carbon modified by acid, enzyme activity was investigated in phosphate buffer in a temperature range of 20 - 70°C. The results show that the optimum temperature of the highest activity of catalase was 30°C for all (Figure 1). The optimum temperature of immobilized mushroom catalase was given as 40°C and its activity was found similar to our findings (Estrada et al., 1991). The optimum reaction temperature for the free enzyme has been found to be 40°C; and for the immobilized enzyme, 45°C (Yakup Arica, 2000). The optimum temperature of immobilized catalase was given as 35°C and its activity was found similar to our findings. This is the optimum temperature of free enzyme given in literature and enzyme handbooks. So, immobilization method did not show an effect on the optimum temperature of the enzyme (Yildiz et al., 2004). A maximum activity for free and immobilized enzymes has been found at about 25°C in literature (Savran et al., 2006). In literature optimum temperature was found at about 35°C for

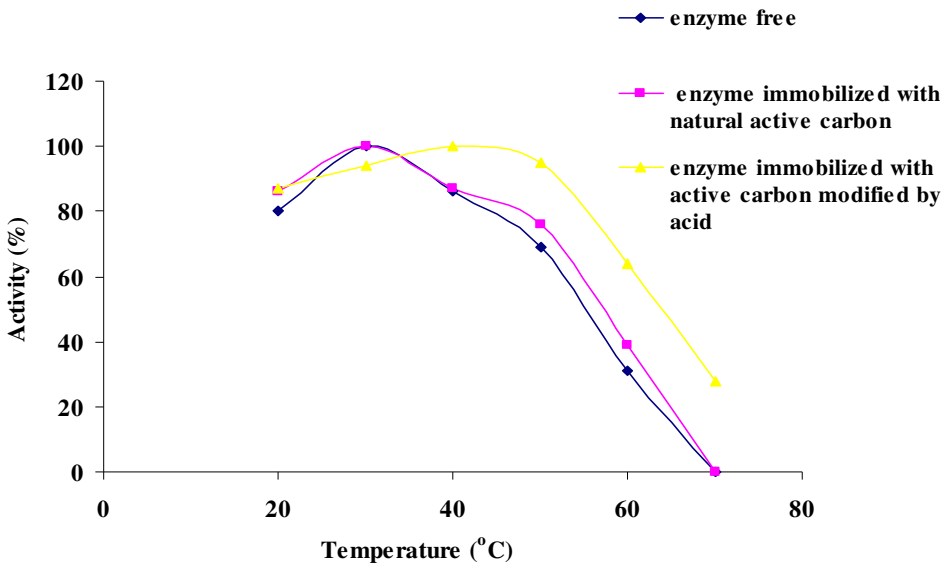

Figure 1. The effect of temperature on catalytic activity of free and immobilized catalase.

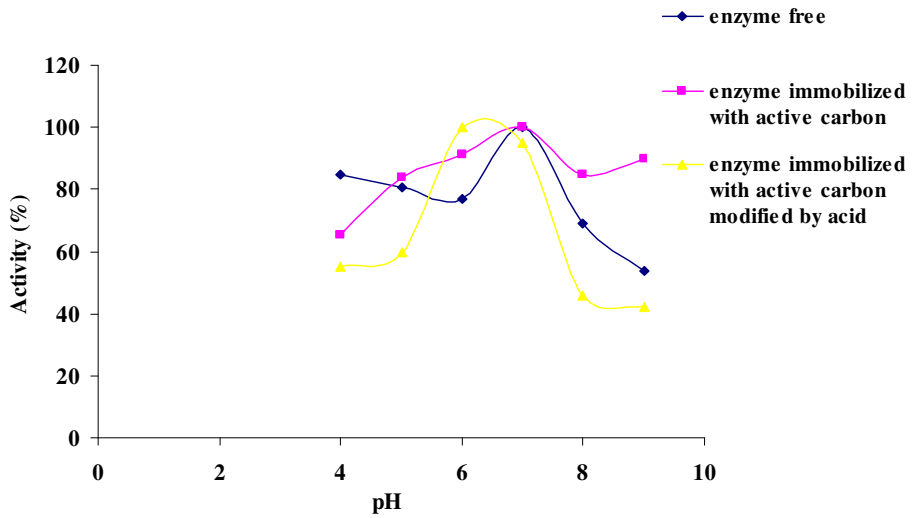

Figure 2. The effect of pH on catalytic activity of free and immobilized catalase.

free and immobilized catalase shows that the loss of the activity of immobilized catalase was lower than that of the free catalase for high temperatures. The support has a protecting effect on the high temperatures at which enzyme deactivation takes place (Akgöl et al., 2001). It seems that active carbon may be protecting the enzyme against denaturation at higher temperatures.

Effect of pH on catalytic activity

For the determination of the effect of pH on the enzyme free, enzyme immobilized by natural active carbon and enzyme immobilized with active carbon modified by acid, phosphate buffers were used within the pH range of 4 - 9. The optimum medium pH values for enzyme free, immobilized by natural active carbon, immobilized by mixing acid-active carbon were obtained as 7, 7 and 6, respectively. The optimum pH of immobilized bovine liver catalase was given as 7 and its activity was found similar to our findings (Yildiz et al., 2004). Immobilized enzyme was less sensitive to pH changes at pH, 7 and slightly more sensitive at pH 7 than free catalase. In our studies, the effect of pH on the activity of free and immobilized catalase preparations for hydrogen peroxide degradation has been studied at various pH values at 35°C. The reactions were carried out in phosphate buffers and the results are presented in Figure 2. Both enzymes showed

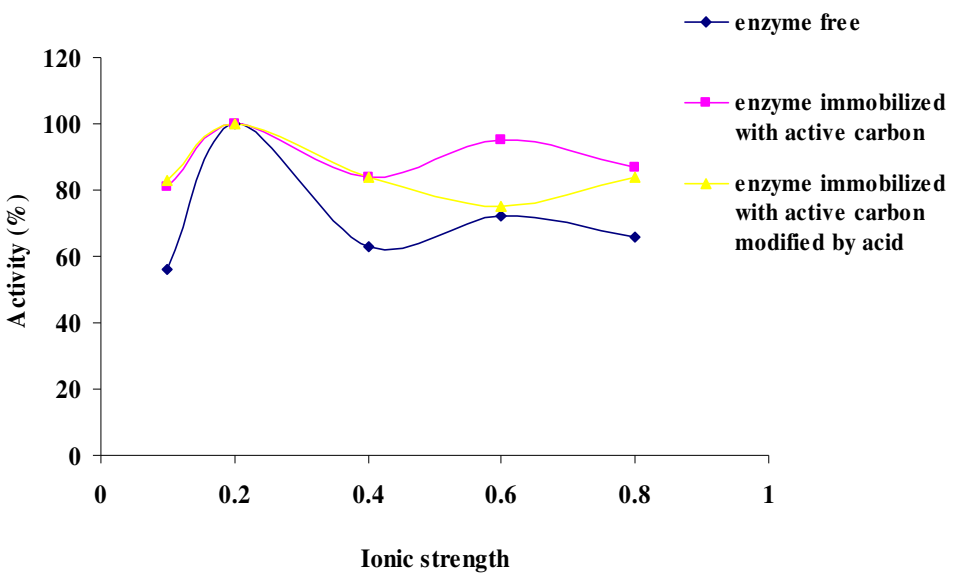

Figure 3. The effect of ionic strength on catalytic activity of free and immobilized catalase.

an optimum pH 7.0 but the immobilized enzyme has a broader pH range of high activity (Çetinus and Öztop, 2003). The stability of immobilized catalase at low pH could be attributed to the amino/imine groups, because they can bind to proton in the matrix, resulting in a decrease of local concentration of proton near the enzyme molecules (Gupta and Ravikumar, 2000). Results obtained from the studies showed that phosphate buffer system gave the most suitable results for both free and all the immobilized catalase.

Effect of ionic strength on catalytic activity

Effect of ionic strength on catalase immobilized mixing acid-active carbon, catalase-natural active carbon and free was investigated. Direct immobilization of enzyme on celite 545 from ammonium sulphate fractionated proteins potato has been shown (Kennedy et al., 1990). Several researchers have already shown the immobilization of potato enzyme via adsorption on chitin, chitosani and celite 545 supports resulted in the stabilization of enzyme activity against water-miscible organic solvents (Bradford, 1976; Estrada et al., 1991; Savran et al., 2006). The results show that the highest activity of free, immobilized by natural active carbon and immobilized by mixing acid-active carbon was at 0.20 M (Figure 3).

Effect of time

Catalase activity for immobilized ezyme found that 76.8% of the enzyme activity was retained during storage at +20°C for 60 days. The results indicated that immobilized enzyme was more stable than free enzyme (Figure 4). In

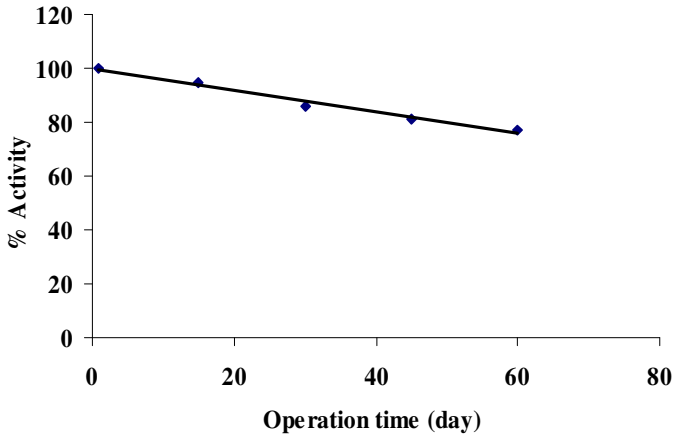

Figure 4. Stability of of immobilized catalase.

another work, free and immobilized catalases have been stored in a phosphate buffer (50 mM, pH 7.0) at 4°C and the activity measurements were carried out for a period of 100 days. Free catalase lost about 50% of its activity within 20 - 25 days and lost its remaining activity within 70 days, whereas Ch-CAT and CBCh- CAT lost about 50% of their activity within 60 and 70 days, respectively. These two immobilized catalases protected 50% of their activity after 70 days. It was observed that immobilized catalase retained about 50% of its activity after 7 - 8 cycles. It was found that Ch-CAT and CB-Ch-CAT have higher operational stability (Çetinus and Öztop, 2000). In a work, although the free enzyme retained about 50% of its activity for 18 days, immobilized catalase that was stored wet remained about 50% of its activity level for 25 days, and the one stored dry remained about 50% of its

activity level for 5 days (Çetinus and Öztop, 2000). These results revealed that the thermostability of immobilized catalase becomes significantly higher than that of free catalase at higher temperature (Çetinus and Öztop, 2003).

Conclusions

The development of versatile supports constitutes one of the aspects which deserve more attention among workers in the field of enzyme immobilization. The nature of the carrier selected influences for immobilization methods. Consequently, experimental data obtained from this investigation reveal that physical adsorption is suitable for the attachment of enzyme into active carbon as support. Adsorption of enzyme on active carbon can reduce a large amount of enzymatic activity. Immobilized catalases showed better storage stabilities and it also can be useful for support applications. Owing to its availability, active carbon could be substituted for the other expensive adsorption materials. Morever, from catalase activity it was found that 76.80% of the enzyme activity was retained during storage at +20°C for 60 days. The results show that the highest catalase activities free immobilized by natural clay and immobilized with clay modified by acid were determined as 0.20 M. The results indicated that immobilized enzyme was more stable than free enzyme.

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