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ORIGINAL PAPER

Immobilization horseradish peroxidase on amine-functionalized glycidyl methacrylate-*g*-poly(ethylene terephthalate) fibers for use in azo dye decolorization

Metin Arslan

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Abstract Activated fibers were used as a new support material for the immobilization of horseradish peroxidase (HRP). Poly(ethylene terephthalate) (PET) fibers were grafted with glycidyl methacrylate (GMA) using benzoyl peroxide (Bz_2O_2) as initiator. 1,6-diaminohexane (HMDA) was then covalently attached to this GMA grafted PET fibers. HMDA-GMA-*g*-PET fibers were activated with glutaraldehyde and HRP was successfully immobilized. Both on the free HRP and the immobilized HRP activities, pH, temperature, thermal stability, and reusability were investigated. Both free enzyme and immobilized enzyme were used in a batch process for the degradation of azo dye. About 98% of azo dye removal was observed with immobilized HRP, while 79% of azo dye removal was found with the free HRP. 45 min of the contact time is sufficient for the maximum azo dye removal. The HRP immobilized on modified PET fibers were very effective for removal of azo dye from aqueous solutions.

Keywords Poly(ethylene terephthalate) fibers · Graft copolymerization · Glycidyl methacrylate · Immobilization · Horseradish peroxidase · Dye decolorization

Introduction

Azo dyes are a major class of pollutions in wastewater from a number of industries such as food, paper, carpets, rubbers plastics, dyeing, leather, and textiles. Azo dyes are known to be toxic and also, some of them, hazardous carcinogenic. Azo dyes

M. Arslan (✉)
Department of Chemistry, Faculty of Science and Art, Çankırı Karatekin University,
18100 Cankiri, Turkey
e-mail: marslan@hotmail.com

must be removed from wastewater before they are discharged into the environmental [1, 2].

Many physical and chemical methods for azo dye removal from industrial wastewaters such as ozonation, adsorption, precipitation, chemical degradation, electrochemical, and photochemical are currently used [3]. However, these methods are expensive, low efficiency, highly toxic by-products, and intensive energy requirement [4, 5]. Therefore, research has been focused on the development of a biodegradation process in which enzymes are used to remove azo dye from polluted effluents. Microbial decolorization has been reported to be less expensive, lower energy requirements, easier to control, and less environmentally intrusive alternative [6, 7]. Horseradish peroxidase (HRP) is known to be effective in the removal of a wide spectrum of aromatic compounds in the presence of H_2O_2 [8] and in the degradation of important industrial dyes [9, 10].

Different procedures have been developed for enzyme immobilization such as adsorption [11], entrapment [12], encapsulation [13], cross-linking [14], and chemical binding [15]. Covalent bindings are preferred to physical or ionic bindings because non-covalent binding processes are simple, but they are weak in enzyme binding. Covalent attachments require active groups on the support surface [16]. The most important advantages of covalent bindings are that they are effective and long-lasting as well as being used in batch and continuous processes. They also can be removed easily from the reaction medium and are used to facilitate controlled production. The polymeric supports used in covalent bindings are mechanically durable and reusable [16–18].

The properties of immobilized enzymes are governed by the properties of both the enzyme and the support material [19, 20]. Immobilized enzymes exhibit higher stability and enable a continuous conversion process with good product recovery and minimal loss of enzyme activity [21]. Enzymes have been immobilized on various natural and synthetic supports [12–25]. A large of polymer matrices have been used to enzyme immobilization, such as on polyaniline [26], poly(urethane methacrylate-*co*-glycidyl methacrylate) [27], poly(*N*-vinyl-2-pyrrolidone-*co*-styrene) [28], and poly(methyl metacrylate) [29].

One of the new developments in recent years to immobilization enzyme is the use of Poly(ethylene terephthalate) (PET) fibers as support material [30, 31]. This is mainly attributed to the relatively large external specific surface areas, low cost and has good resistance to weak mineral acids, even at boiling temperature, and to most strong acids at room temperature, oxidizing agents, sunlight, and microorganisms [32–34]. PET fiber can be modified by grafting with different vinyl monomers such as 4-vinylpyridine [35], 2-hydroxyethylmethacrylate [36], acrylic acid [37], methacrylic acid [38], and acrylamide [39]. Graft polymerization of GMA onto PET fibers is advantageous for the immobilization of enzymes because the epoxy group of GMA is modified easily [40].

In this study, PET fibers were grafted with GMA using Bz_2O_2 as initiator. HMDA was then covalently attached to this GMA grafted PET fibers. Aminated GMA-*g*-PET (HMDA-GMA-*g*-PET) fibers were activated by glutaraldehyde. Activated PET fibers were used as a new support material for the immobilization of HRP. The activity and stability of the immobilized enzyme and the free enzyme

were investigated under different experimental conditions. Both free HRP and immobilized HRP were used to decolorize a methyl orange from aqueous solutions through batch method.

Experimental

Materials

The PET fibers (122 dTex, middle drawing) used in these experiments were provided by SASA Co. (Adana, Turkey). The fibers samples were Soxhlet-extracted until constant weight (for 6 h) with acetone and dried in a vacuum oven at 50 °C. GMA was used without further purification. Bz_2O_2 was recrystallized twice from chloroform and dried in a vacuum oven for 2 days. HRP (EC 1.11.1.7) and glutaraldehyde were obtained from Sigma-Aldrich. Pyrogallol was purchased from Fluka. Sodium hydroxide, hydrogen peroxide, benzoyl peroxide, and acetic acid were purchased from Merck. Ethanol, methanol, acetone, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Riedel-deHaen. Solutions were prepared with deionized water (Millipore, Elix 3 water purification system).

Apparatus

Pharmacia Biotech Ultrospec 2000 model UV–Visible spectrophotometer was used for the determination of optical density of solutions in the visible region. The infrared spectrums were obtained by Thermo-Nicolet 6700 FT-IR spectrometer attached to an attenuated total reflection (ATR) apparatus, using diamond prism with an incident angle 45°. All pH measurements were performed with HANNA 221 model digital pH meter.

Polymerization procedure

The PET fiber samples (0.3 ± 0.01 g) were dipped into the polymerization medium. Polymerization was carried out in a thermostated 50 mL tube under reflux. The mixture containing the PET fiber sample (0.3 ± 0.01 g), appropriate amount of GMA and Bz_2O_2 at required concentration in 2 mL acetone was made up to 20 mL with deionized water. The mixture was immediately placed into the water bath adjusted to the polymerization temperature. At the end of the predetermined polymerization time, the grafted fibers were taken out. Residual solvent, monomers and free homopolymers were removed by Soxhlet-extracting the PET fibers in acetone for 24 h. The grafted fibers were then vacuum-dried at 50 °C for 72 h and weighed. The graft yield (GY) was calculated from the weight increase in grafted fibers as follows:

$$\mathrm{GY}(\%) = \left[\left(w_{\mathrm{g}} - w_{\mathrm{i}}\right)/w_{\mathrm{i}}\right] \times 100 \quad (1)$$

where w_i and w_g denote the weights of the original (ungrafted) and grafted PET fibers, respectively.

Activation of the GMA-*g*-PET fibers

Grafted PET fibers were placed in 30 mL of 100% HMDA in a 50 mL Erlenmeyer. The Erlenmeyer was shaken at 125 rpm for 30 min at 50 °C using orbital shaker (Medline BS-21). Then, HMDA-GMA-*g*-PET fibers were separated from the HMDA, washed with hot water. The PET fibers were shaken in 5 mL of 5% glutaraldehyde solution at 125 rpm for 2 h [41]. Activated fibers were washed with deionized water three times to remove the unreacted glutaraldehyde.

Immobilization of HRP onto the activated support

The activated PET fiber was shaken in solution of the HRP (0.2 mg mL^{-1}) in 50 mM buffer solution at 125 rpm for 2 h. Then the PET fiber was washed with phosphate buffer solution for 5 min by shaking at 125 rpm three times.

Activity measurement of free and immobilized HRP

The activities of the free and the immobilized enzymes were determined by using pyrogallol and H_2O_2 as substrates as described by Halpin et al. [42]. The reaction mixture containing 2 mL of the pyrogallol solution (20 mmol L^{-1}) and 1.5 mL of buffer solution (0.1 mol L^{-1}) was incubated with 0.1 mL the free HRP (0.01 mg mL^{-1}) and the immobilized HRP in shaking water bath at 125 rpm. The reactions were started with the addition of 0.5 mL of 10 mmol L^{-1} H_2O_2 solution. The color densities of product solutions were measured at 420 nm, 1 min after H_2O_2 addition for the free HRP and 5 min after H_2O_2 addition for the immobilized HRP. The values obtained in the blank reactions, performed in the presence of H_2O_2 without enzyme, were discounted from all readings. One unit (U) of enzyme activity was defined as the amount of enzyme which produced 1 μmol purpurogallin per min under assay conditions [30]. The relative activity was calculated as follows:

$$\text{Relative activity } (\%) = (\text{Activity}/\text{ Maximum activity}) \times 100 \tag{2}$$

Thus the highest activity is regarded as 100% for free and immobilized studies separately.

Degradation of azo dye by free and immobilized HRP

The reaction mixture containing 2 mL of the methyl orange (Azo dye, Orange III, C.I. 13025, procured from Sigma, Fig. 1) solution (100 mg L^{-1}) and 1.7 mL of phosphate buffer solution (pH 6, 0.1 mol L^{-1}) was incubated with 0.1 mL the free HRP (0.01 mg mL^{-1}) and the 0.04 g immobilized HRP. The contents were shaken at 125 rpm for a predetermined period of time at 40 °C using orbital shaker. The

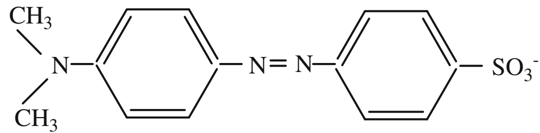


Fig. 1 Chemical structure of methyl orange

reactions were started with the addition of 0.3 mL of 10 mmol L^{-1} H_2O_2 solution. After a predetermined time period, the decrease in the absorbance of the reaction medium was measured by using UV/Visible spectrophotometer ($\lambda = 465$ nm, Pharmacia Biotech Ultrospec 2000). Calibration curves were plotted between absorbance and concentration of the standard dye solutions. Percent dye removal was evaluated by using the following expression:

$$\text{Removal dye\%} = ((C_o - C)/C_o) \times 100 \tag{3}$$

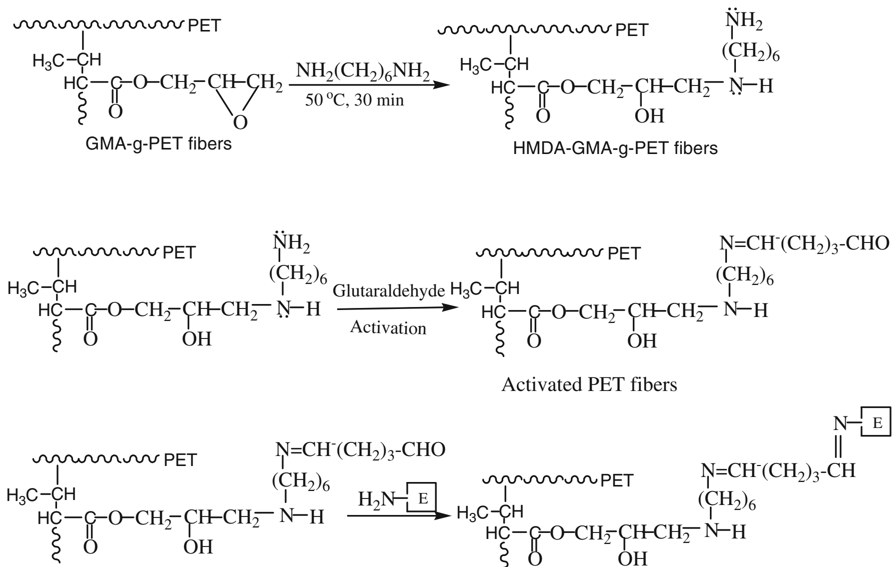
where C_o and C are the concentration of the azo dye in the initial solution and in the final solution for a certain period of time (mg L^{-1}).

Results and discussion

The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization, although it is difficult to predict in advance which support will be most suitable for a particular enzyme. The most important requirements for a support material are that it must be insoluble in water, have a high capacity to bind enzyme, be chemically inert and be mechanically stable [43]. Therefore, activated PET fibers were used as a new good solid support material for the immobilization of HRP. PET fibers were grafted with GMA using Bz_2O_2 as initiator. HMDA was then covalently attached to this GMA grafted PET fibers. Finally aminated GMA-*g*-PET (HMDA-GMA-*g*-PET) fibers were activated by glutaraldehyde. HRP was covalently bound to the activated PET fiber. The schematic representation for overall processes was illustrated in Scheme 1.

The scanning electron micrographs of GMA-*g*-PET fiber (140%) are shown in Fig. 2. It is clear from the SEM results that the ungrafted PET fiber surface (Fig. 2a) has a smooth and relatively homogeneous appearance. The grafted side chain GMA seems to form microphages attached to the PET back-bone and causes a heterogeneous appearance in the graft copolymer (Fig. 2b), showing proof of grafting [44].

The FTIR spectra of ungrafted (a), 40% GMA-*g*-PET (b), HMDA-GMA-*g*-PET (c), glutaraldehyde loaded HMDA-GMA-*g*-PET (d), and HRP bonding activated PET fiber (e) is given in Fig. 3. It is seen that the peaks of the ungrafted PET (Fig. 3a) can be assigned as follows: 3428 cm^{-1} (OH), 2953 cm^{-1} and 2874 cm^{-1} (C–H asymmetric and symmetric in C–H, CH_2 groups), 1708 cm^{-1} (C=0), 1406 cm^{-1} (CH_2). After the grafting with GMA, the spectrum of the GMA grafted PET fiber (Fig. 3b) changed. The characteristic peak at 904 cm^{-1} corresponds to the asymmetric stretching vibration of the epoxy group. Hence these results provided proof for the grafting of GMA onto PET fiber. After the attaching of HMDA on



Scheme 1 Immobilization of enzyme on activated PET fibers

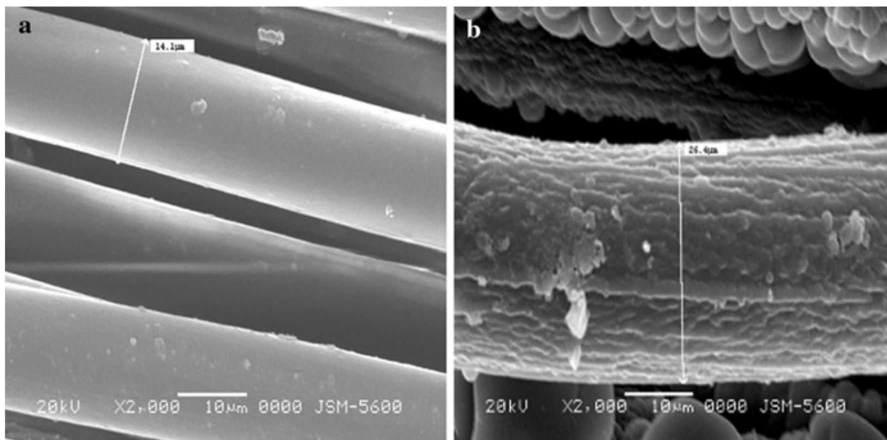


Fig. 2 **a** SEM micrograph of ungrafted PET fiber. **b** SEM micrograph of GMA-*g*-PET fiber (140%)

GMA-*g*-PET fiber (Fig. 3c), a strong broad band appeared at 3294 cm^{-1}, attributed to the N–H stretching vibration. The new peaks at 1630 and 1533 cm^{-1} for the N–H bending vibration and the new peak at 1151 cm^{-1}, for the C–N stretching vibration have all supported the existence of amine groups from HMDA on GMA-*g*-PET fiber. The characteristic peak at 904 cm^{-1} for the epoxy group of GMA-*g*-PET fiber, however, disappeared, indicating that HMDA reacted with the epoxy groups and changed their structure during the attaching process [44]. After the loading with

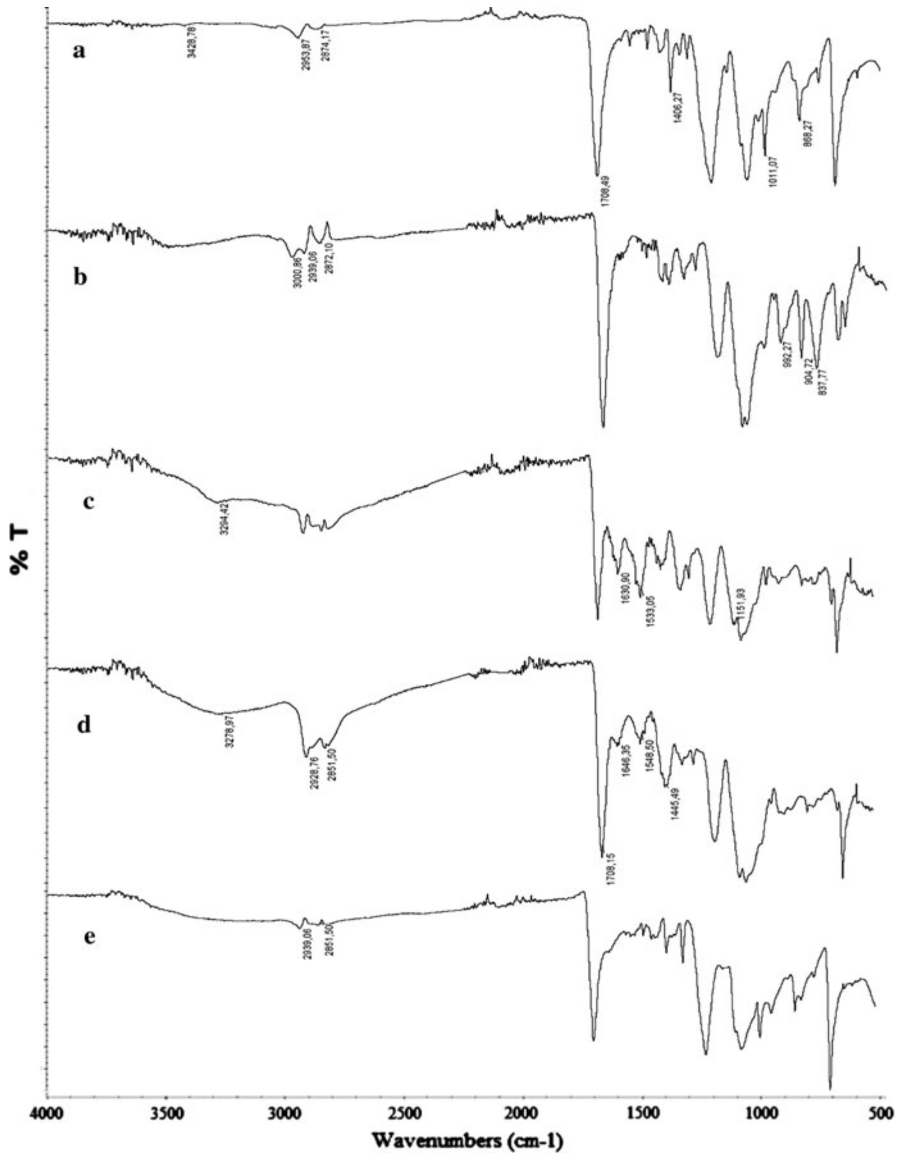


Fig. 3 FTIR spectra of PET (*a*), 40% GMA-*g*-PET (*b*), HMDA-GMA-*g*-PET (*c*), the glutaraldehyde loading PET (*d*), and HRP binding activated PET fiber (*e*)

glutaraldehyde, the spectrum of the glutaraldehyde loaded HMDA-GMA-*g*-PET fiber (Fig. 3d) changed. $–NH_2$ groups of HMDA bind to -CHO groups of glutaraldehyde. The peak at 1646 cm^{-1} for the R–CH=N bending vibration. Because of the presence of glutaraldehyde, intensity of C–H band (2928 cm^{-1}) was observed to increase. After the attaching of HRP on activated PET fiber (Fig. 3e), a

strong broad band decreased at 2939 cm^{-1}, attributed to the NH_2 groups at HRP are covalently bonded to free CHO groups of glutaraldehyde [45].

Optimization of the HRP immobilization process

The HRP was covalently bonded on activated PET fibers with various graft yields (%). The results are shown in Fig. 4. PET fibers do not contain suitable functional groups and thus cannot interact with HRP. The activity of immobilized HRP increases significantly by increasing the graft yield up to 25%, and then falls down upon further increase in graft yield. Increasing graft yield increases the number of functional groups and thus, increases the number of enzyme bonded surface of activated PET fibers. The results showed that the enzyme activity is greater at smaller graft yield. This can be explained by sterical impediment at higher graft yield [30].

Because the pH value influences the surface structure of activated PET fibers, pH is one of the most important factors influencing the efficiency of enzyme immobilization on the fibers. The effect of pH on the immobilization of HRP onto activated PET fibers was studied and presented in Fig. 5. The maximum HRP immobilization was observed pH 8 on the activated PET fibers. The decrease in the activation of immobilization enzyme in more acidic and more alkaline district can be attributed to electrostatic repulsion effect between the opposite charged groups.

The effect of the protein concentration on the efficiency enzyme immobilization was investigated, while keeping all other conditions constant. The results are shown in Fig. 6. The activity of the enzyme increases significantly by increasing the concentration of the protein up to 0.1 mg mL^{-1}, and beyond that point it almost remained unchanged.

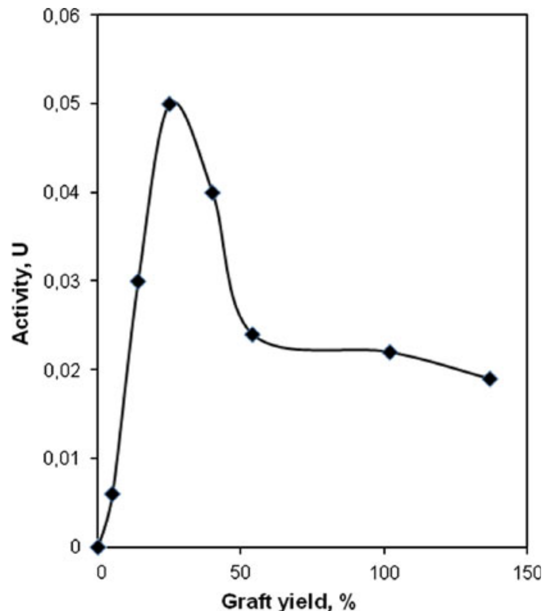


Fig. 4 The effect of graft yield on enzyme activity ($T = 25$ °C, pyrogallol concentration = 10 mmol L^{-1})

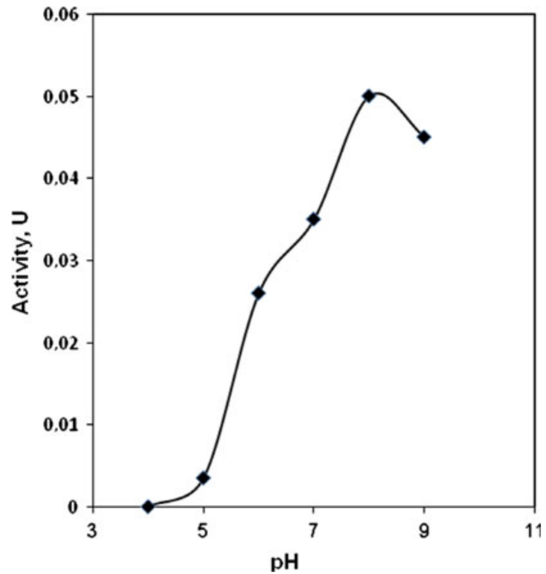


Fig. 5 The effect of pH of immobilization media on activity ($T = 25$ °C, pyrogallol concentration = 10 mmol L^{-1})

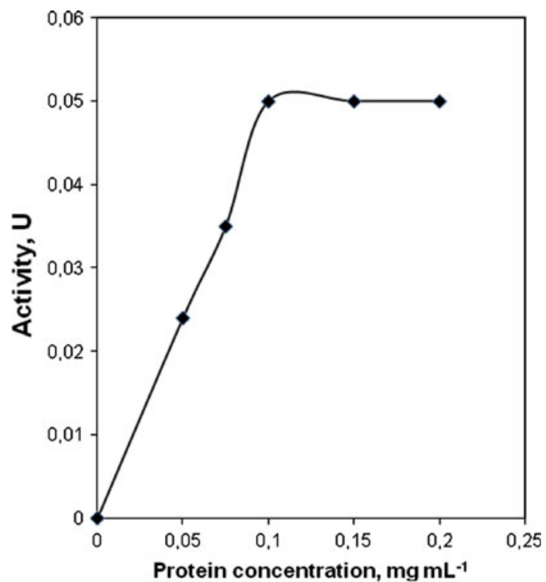


Fig. 6 The effect of protein concentration on enzyme activity ($T = 25$ °C, pyrogallol concentration = 10 mmol L^{-1})

Effect of pH on enzyme activity

The pH of a solution plays an important role on enzymatic activity in an aqueous medium. Effect of pH on the activity of HRP for free enzyme and immobilized enzyme was studied by varying the pH of the reaction medium between 3 and 9 and results are shown in Fig. 7. Free HRP showed maximum activity at pH 8 whereas

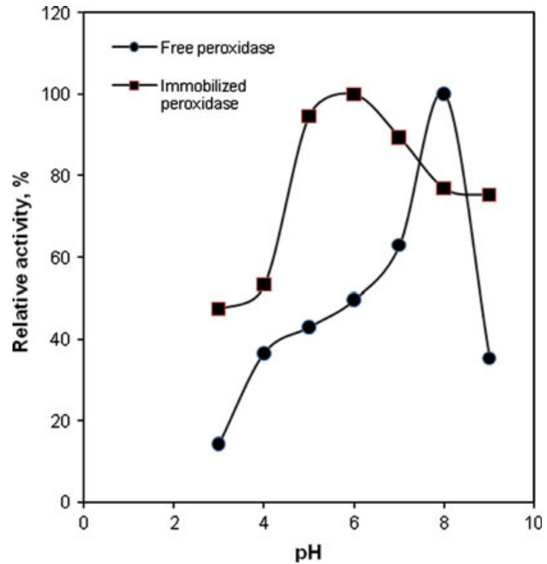


Fig. 7 The effect of pH on enzyme activity ($T = 25$ °C, pyrogallol concentration = 10 mmol L^{-1})

covalently bound HRP showed maximum activity 6. A small change in pH, ionic strength of aqueous medium may cause loss of activity of the HRP. The pH of the reaction medium can have several effects of the structure and activity of HRP. This can lead to altered protein recognition or the enzyme might become inactive because substrate cannot bind to the active sites or it cannot undergo catalysis. The immobilization of enzyme usually results in stabilization of over a broader range of pH [46, 47].

Effect of temperature on enzyme activity

The effect of temperature on the activity of free and immobilized HRP at pH 6 in temperature of 20–60 °C is shown in Fig. 8. Maximum activity was observed 40 °C for the both free HRP and the immobilized HRP. The results showed that temperature is effective on enzyme activity. The temperature profile for the immobilized HRP on activated PET fibers was very similar to that of the free HRP. It affects the speed of molecules of enzyme and substrate, the activation energy of the catalytic reaction and the thermal stability of the HRP (free and immobilized) and substrate. At low temperature the rate of enzyme reactions is very slow. The molecules have low kinetic energy and collisions between them are less frequent. Thus, both the free HRP and the immobilized HRP are deactivated at low temperatures. An increase in temperature causes an increase in kinetic energy. The rate of enzyme activity of free and immobilized HRP is the highest at 40 °C. After 40 °C the rate of reaction starts to decrease. The enzyme molecules are denatured, causing the shape the active site to change. The free HRP and immobilized HRP molecules are denatured causing the shape of the active site to change. At 60 °C the free HRP and immobilized HRP are almost completely denatured [28, 48, 49].

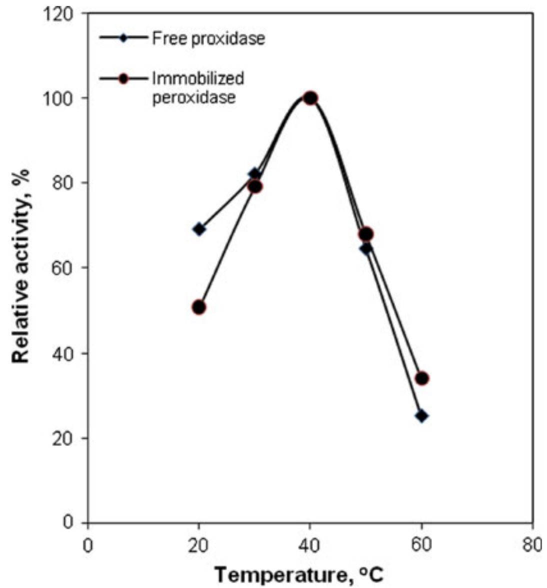


Fig. 8 The effect of temperature on enzyme activity (pyrogallol concentration = 10 mmol L^{-1})

Thermal stability

Thermal stability experiments were carried out with the free and the immobilized HRP in the buffer of optimum pH (6) at 40 °C for different time intervals (0–180 min). From the results illustrated in Fig. 9, it is observed that the covalently bound HRP showed better thermal stability than free HRP. It can be seen that the immobilized HRP retained relative activity of about 64.4% whereas the relative activity retained by the free HRP was only 19.2%.

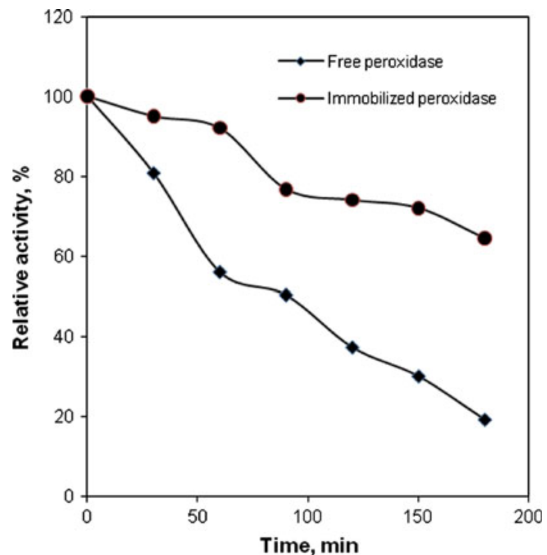


Fig. 9 The thermal stability of the free HRP and the immobilized HRP ($T = 40$ °C, pyrogallol concentration = 10 mmol L^{-1})

Reusability of immobilized enzyme

The most important advantage of immobilization is the reusability of immobilized HRP on the activated PET fibers. Therefore, investigations were carried out to assess repeated usability of immobilized HRP on the activated PET fibers. The results are shown in Fig. 10. Immobilized HRP on the activated PET fibers retained a relative activity of 69.6% after 5 cycles and remained constant for another 5 repeated use activity assay measurement. It should be noted that immobilized HRP on the activated PET fibers can effectively reduce the cost in degradation of industrially important dyes applications.

Decolorization of methyl orange dye by free HRP and immobilized HRP

Application of free enzyme in the decolorization of dye processes is not economical, while immobilized enzyme is more economical. The purpose of this test was to assess the use of an inexpensive immobilized HRP on activated PET fibers for decolorization of the azo dye. Both free HRP and immobilized HRP were used in a batch process for the degradation of azo dye. The results are shown in Fig. 11. It could be observed from this figure that immobilized HRP on activated PET fibers was more efficient in azo dye removal when compared to the free HRP. About 98% of azo dye removal was observed with immobilized HRP, while 79% of azo dye removal was found with the free HRP. 45 min of the contact time is sufficient for the maximum azo dye removal. As is known from the literature [9, 50], HRP is useful for the removal of dye from wastewater and enzymatic reaction of HRP is fairly rapid. Shaffiqu et al. [51] reported that methyl orange, an acidic mono azo dye at 200 mg L^{-1}, was degraded to 50% at pH 7.0 in 4 h of treatment by HRP.

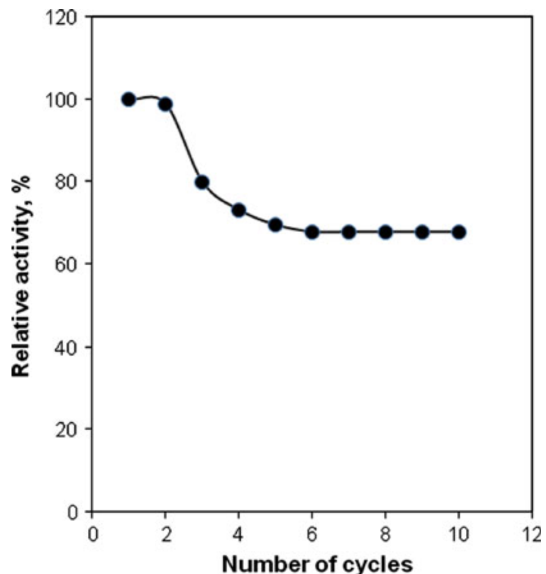


Fig. 10 The effect of repeated use of immobilized enzyme on activity ($T = 25$ °C, pyrogallol concentration = 10 mmol L^{-1})

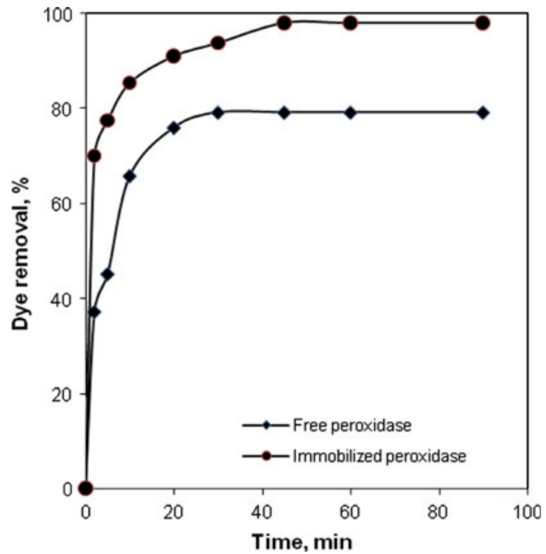


Fig. 11 Removal rate of free and immobilized HRP for methyl orange ($T = 40$ °C, dye concentration = 50 mg L^{-1})

Immobilized HRP on activated PET fibers showed the potential to be applied in the treatment of azo dye effluent.

Conclusions

Activated fibers were used as a new support material for the immobilization of HRP. PET fibers were grafted with GMA using benzoyl peroxide as initiator. HMDA was then covalently attached to this GMA grafted PET fibers. HMDA-GMA-*g*-PET fibers were activated with glutaraldehyde and HRP was successfully immobilized for the removal of azo dye. Removals of azo dye (methyl orange) in aqueous solutions by the immobilized HRP onto activated PET fibers were higher than the ones by the free HRP. The immobilized HRP could remove 98% methyl orange (50 ppm) in 45 min. The immobilized HRP onto activated PET fibers showed good decolorization of azo dye. Thus, the support material should be immobilized with other enzymes to remove dyes in textile wastewater.

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