Research Article

Preparation of Laccase Immobilized Cryogels and Usage for Decolorization

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Poly(methyl methacrylate-*co*-glycidyl methacrylate) (poly(MMA-*co*-GMA)) cryogels were synthesized by radical cryopolymerization technique. Then, laccase enzyme was covalently attached to the cryogel and characterized by using swelling studies and SEM and EDX analyses. Kinetic properties and optimum conditions of the immobilized and free laccase were studied and it was found that $V_{\max}$ of the immobilized laccase was lower than that of free laccase. K_m of the immobilized laccase was increased upon immobilization. Optimum pH was found to be 4.0 for each type of laccase, while optimum temperature was shifted to the warmer region after the immobilization. It was also found that thermal stability of the immobilized laccase was higher than that of free laccase. Immobilized laccase could be used for 10 times successive reuse with no significant decrease in its activity. Also, these laccase immobilized cryogels were successfully used for the decolorization of seven different dyes.

1. Introduction

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a copper bearing enzyme in multicopper family which is produced by various plants and secreted by diverse fungi species which have lignin degradation capability [1]. Recently some bacterial species which demonstrate laccase activity were also characterized. Laccase oxidize a wide variety of the phenolic compounds and aromatic amines by catalyzing the reaction of the one electron oxidation of phenols, anilines, and aromatic thiols to their radicals with parallel reduction of oxygen to water [2]. Substrate specificity of the laccase is broad and it can oxidize not only these compounds but also nonphenolic substances [3]. Laccase can be used in various industrial applications such as pulp delignification, wood fiber modification, dye or stain bleaching, chemical or medicinal synthesis, and contaminated water or soil remediation due to its wide specificity behavior against its nonspecific substrates [4]. Besides all these, one of the unique properties of laccase is the capability for degradation of dyes. The enzyme can decolorize various dyes such as azo, anthraquinone, and triphenylmethane, through nonspecific

free radical mechanism with creation of phenolic compounds [5, 6].

Using the laccase in industrial applications has some limitations such as low stability and productivity and its high production cost [7]. In order to improve the reusability of the enzymes, increase the enzyme stability, and reduce the cost, laccase has been immobilized successfully on various support materials such as magnetic chitosan microspheres [1], nonporous poly(GMA/EGDMA) beads [8], polyamide 6,6 fibers [9], poly(glycidyl methacrylate) brush grafted poly(hydroxyethyl methacrylate) films [10], methylene blue modified mesoporous silica MCM-41/PVA [11], magnetic mesoporous silica nanoparticle [7], alginate/gelatin blend with PEG [12], epoxy-activated Sepabeads EC-EP3 and Dilbeads NK [4], alginate-chitosan microcapsules [13], and PVA cryogel [14].

There is a great interest in the area of the improvement and development of new materials which are used for the bioseparation processes due to the ever growing demands for the biologically active pure compounds (low molecular weight compounds, biopolymers such as DNA and proteins,

viruses, cell organelles, and cells) [15]. For this reason, development of macroporous polymeric materials has attracted great attention especially in biomedical, biotechnological, and medical purposes [16]. One of the new types of polymeric materials which have significant potential in biotechnology is cryogels. They are synthesized at frozen temperature and thus are named as cryogel (in Greek, frozen or ice). Cryogels are extremely porous polymeric materials and can be synthesized in various morphology and porosity by using any of the gel producing precursors [15]. Nowadays, cryogels find too many usages in various biotechnological applications as a chromatographic material, a support material for immobilization of molecules and cells, a matrix for electrophoresis and immunodiffusion, and a gel support for solid culture media [17]. Basic application areas of the cryogels are biocatalysis with immobilized enzymes and cells; bioseparation for purification of target molecules; chromatography of cell organelles, viruses, microbial, and mammalian cells; and three-dimensional matrix for mammalian cell culture [16].

Dyes are widely used in textile, paper, cosmetic, pharmaceutical, and dyeing and printing industries, and the effluents from these industries were released to the environment. This effluent contains environmentally hazardous polluting agents such as pesticides, heavy metals, pigments, and dyes. The degradation of these dye bearing effluents is not too easy and conventional treatment techniques (such as activated sludge, trickling filters, adsorption, coagulation-flocculation, ion-exchange, oxidation, electrochemical methods, etc.) does not effectively degrade them due to the complex structure of the effluent, besides these techniques may also generate hazardous byproducts [3, 18]. These treatment techniques are highly expensive and because of this their application in industrial waste treatment is also limited. In recent years, there is a great attention to the use of microbial enzymes for dye decolorization applications due to their effective treatment behavior [19]. For these purposes, laccase has been widely used for decolorization of variety of synthetic dyes such as Remazol Brilliant Blue [4, 20–22], Acid Green 27, Acid Violet 7 and Indigo Carmine [23], Neolane Yellow, Maxilon Blue, Neolane Pink, Basacryl Yellow, Neolane Blue and Bezaktiv Yellow [3], Remazol Black-5 [19, 21], Acid Blue 62, Acid Blue 40, Reactive Blue 81, Direct Black 22, Acid Red 27 [24], Reactive Red 251, Reactive Orange 122 [21], Reactive Black 5, Acid Blue 25, Methyl Orange, Methyl Green, Acid Green 27 [4], Direct Red 28, Acid Orange 74, Reactive Blue 15, Acid Blue 74, Reactive Blue 19, Azure B [25], Bromophenol Blue, Naphthol Blue, and Methyl Red [26].

In this paper, decolorization efficiency of the immobilized laccase was studied. For these purposes, poly(methyl methacrylate-co-glycidyl methacrylate) (poly(MMA-co-GMA)) cryogels were synthesized with cryopolymerization technique. Prepared cryogels were characterized by using SEM and EDX analyses and swelling studies. Then, laccase was covalently immobilized onto these newly synthesized cryogels. Kinetic parameters of free and immobilized laccase were also investigated and optimum pH and temperature profiles were studied. Also, thermal stability of the free and immobilized form of laccase was investigated. Finally,

decolorization capability of the immobilized laccase was studied with various dyes.

2. Experimental

2.1. Materials. Laccase (from *Trametes versicolor*), methyl methacrylate, glycidyl methacrylate, N,N'-methylene-bisacrylamide (MBAAm), ammonium persulfate (APS), and N, N,N',N'-tetramethylene diamine (TEMED) were supplied from Sigma (St. Louis, USA). All other chemicals were of reagent grade and purchased from Aldrich (Steinheim, Germany).

2.2. Synthesis of Poly(MMA-co-GMA) Cryogel. Poly(MMA-co-GMA) cryogel was synthesized with free radical cryopolymerization technique [27]. Polymerization procedure was explained as follows. Firstly, 0.283 g of MBAAm was dissolved in 10.0 mL of distilled water and mixed with 5.0 mL of MMA and GMA solution which was prepared by dissolving 1.07 mL of MMA and 100 μ L of GMA in 10.0 mL of distilled water. Then, 20.0 mg of APS and 25 μ L of TEMED were added to this mixture and polymerization reaction was initiated. Resulting solution was immediately poured to the syringe and was frozen at -12°C for 24 h. After this polymerization period, prepared cryogel was washed with distilled water in order to remove unreacted monomers and initiators. Chemical structure of the poly(MMA-co-GMA) cryogel was shown in Figure 1.

2.3. Immobilization of Laccase onto Poly(MMA-co-GMA) Cryogel. Laccase immobilization experiments were carried out according to the literature described [28]. Briefly, poly(MMA-co-GMA) cryogel was equilibrated with pH 8.0 phosphate buffer (50 mM) for 2 h. Then, 20.0 mL of laccase solution (2.0 mg/mL in pH 8.0 phosphate buffer) was passed through the cryogel column (0.335 g-dry weight) by using a peristaltic pump at 22°C for 18 h. Covalent attachment between the cryogel and laccase molecule was carried out by the help of epoxy groups of the GMA monomer in the cryogel structure. Immobilization of the laccase onto poly(MMA-co-GMA) cryogel was schematically demonstrated in Figure 2. Immobilized amount of laccase was determined by measuring the initial and final laccase concentrations with the method of Bradford [29].

2.4. Characterization of Poly(MMA-co-GMA) Cryogel. Energy Dispersive X-Ray (EDX) analysis of cryogel was carried out by using an EDX instrument (LEO, EVO 40, Carl Zeiss NTS, USA). Pore size and cryogel morphology were investigated with Scanning Electron Microscopy (SEM). For this, cryogel was dried and coated with one layer of gold and then, SEM photograph of cryogel was taken by using an SEM device (Philips XL-30S FEG, The Netherlands). Swelling degree (S) of poly(MMA-co-GMA) and laccase immobilized poly(MMA-co-GMA) cryogels were also determined. For this, cryogels were dried at 60°C for 3 days and weighed (m_d). Then, cryogels were placed in 50.0 mL of distilled water at

FIGURE 1: Chemical structure of poly(MMA-co-GMA) cryogel.

FIGURE 2: Schematic presentation of the immobilization of laccase onto cryogel structure.

25°C for 2 h. Swelled cryogels were taken out from water and weighed (m_s) and the swelling degree was calculated as [27]

$$S = \frac{m_s - m_d}{m_d} \quad (1)$$

2.5. Laccase Activity Studies. Laccase activity was determined by using the 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as a substrate [30]. For free enzyme, 0.1 mL of ABTS solution (10.0 mM) was mixed with 880 μL of pH 4.0 acetate buffer solution (100 mM) and incubated at 25°C for 20 min. Then, enzymatic reaction was initiated with addition of 20 μL of enzyme solution. Activity of the immobilized laccase was determined in continuous system. For this, 10 mL of ABTS (10.0 mM) in acetate buffer (pH 4.0, 100 mM) was used as a substrate solution and passed through the laccase immobilized cryogel. The activity of the free and immobilized form of laccase was measured by using the increase in absorbance at 420 nm. One unit of laccase activity is defined as the required enzyme amount for the oxidation of 1.0 μmol of ABTS per min at 25°C.

2.6. Determination of Kinetic and Optimal Properties of Free and Immobilized Laccase. In order to determine the K_m and $V_{\max}$ values of the free and immobilized form of laccase, initial ABTS concentrations were changed between 1.0 and 10.0 mM in pH 4.0 acetate buffer (100 mM) at 25°C. Activity studies of the free and immobilized form of laccase were performed in the pH range of 3.0–6.0 by using 0.1 M of acetate buffer (for pH 3.0–4.5) and 0.1 M of phosphate buffer

FIGURE 3: SEM photograph of the poly(MMA-co-GMA) cryogel.

(for pH 5.0–6.0), in order to determine the optimum pH profiles of two laccase forms. In order to determine the optimum temperature of the laccase, medium temperature was changed between 4.0 and 60°C. Thermal stability of free and immobilized laccase was also determined at 55 and 65°C. For this, enzyme preparations were incubated at 55 and 65°C and activities of the enzymes were measured with the above mentioned method with defined time intervals for 5 h. In order to investigate the operational stability of the immobilized laccase, activity measurements were repeated for 10 times. Storage stability of free and immobilized laccase was also investigated for 30 days. For this purpose, enzyme preparations were stored at +4°C and activities of the enzymes were determined at the beginning and at the end of 30 days of storage. After each activity experiments, immobilized cryogels were washed with water and equilibrated with pH 4.0 acetate buffer (100 mM) for next activity study.

2.7. Decolorization Studies. Seven different dyes Procion Red ($\lambda_{\max}$: 536 nm), Reactive Green 5 ($\lambda_{\max}$: 674 nm), Reactive Brown 10 ($\lambda_{\max}$: 526 nm), Reactive Green 19 ($\lambda_{\max}$: 631 nm), Cibacron Blue F3GA ($\lambda_{\max}$: 605 nm), Alkali Blue 6B ($\lambda_{\max}$: 587 nm), and Brilliant Blue 6 ($\lambda_{\max}$: 607 nm)) were used for the investigation of the decolorization efficiency of the laccase immobilized poly(MMA-co-GMA) cryogel. For this, 10.0 mL of dye solution (0.1 mg/mL) was passed through the laccase immobilized cryogel column by using a peristaltic pump at

FIGURE 4: EDX spectrum of the laccase immobilized poly(MMA-co-GMA) cryogel.

FIGURE 5: Effect of pH on the activity of free and immobilized form of laccase.

the flow rate of 0.5 mL/min. Decolorization activity was monitored photometrically by using a UV-Vis spectrophotometer (Shimadzu, 1601, Japan) for 10 min.

All measurements were repeated three times and the average values were used for all calculations.

3. Results and Discussion

3.1. Synthesis and Characterization of Poly(MMA-co-GMA) Cryogel. Synthesized poly(MMA-co-GMA) cryogel had sponge-like morphology and was elastic and opaque. When compressed by hand, cryogel lost all water accumulated inside the pores. This cryogel exhibited fast swelling properties, and when dried cryogel was submerged in water,

FIGURE 6: Effect of temperature on the activity of free and immobilized form of laccase.

TABLE 1: Kinetic constants of the free and immobilized laccase.

Enzyme type	K_m (mM)	V_{max} ($\mu\text{mol}/\text{min}$)
Free	5.26	0.016
Immobilized	11.11	0.013

it swelled rapidly and restored its original shape and size within 1-2 min. Internal structure and morphology of the poly(MMA-co-GMA) cryogel was shown in Figure 3. As shown in the figure, cryogel had a macroporous structure and pore diameter was found in the range of 10–100 μm . EDX analysis of the laccase immobilized poly(MMA-co-GMA) cryogel was demonstrated in Figure 4. As seen here, synthesized laccase immobilized cryogel composed of C, O, N and S atoms. As clearly seen here that, while the poly(MMA-co-GMA) cryogel contained only C and O atoms, additional N and S atoms appeared here due to the incorporation of protein structured laccase onto the cryogenic structure. Immobilized amount of laccase was also investigated and it was found to be 51.7 mg/g cryogel. Specific activities of free and immobilized form of laccase were determined as 8.9×10^{-3} U/mg and 7.7×10^{-3} U/mg, respectively. As stated here, activity of laccase decreased slightly upon immobilization, and this decrease is carried out probably due to the certain conformational changes which were taken place upon immobilization. The equilibrium swelling degree of the poly(MMA-co-GMA) and laccase immobilized poly(MMA-co-GMA) cryogels were calculated as 8.21 g $\text{H}_2\text{O}/\text{g}$ cryogel and 9.93 g $\text{H}_2\text{O}/\text{g}$ cryogel, respectively. It can be concluded from this result that swelling degree of the cryogel increased with incorporation of laccase onto the cryogel structure.

3.2. Kinetic and Optimal Properties of Free and Immobilized Laccase. The kinetic constants of free and immobilized form of laccase were summarized in Table 1. As seen in the table, V_{max} value of laccase decreased upon immobilization from 0.016 to 0.013 $\mu\text{mol}/\text{min}$. The K_m value of immobilized

FIGURE 7: Thermal stability profile of the free and immobilized laccase at 55°C (a) and 65°C (b).

laccase (11.11 mM) was about 2 times higher than that of free laccase (5.26 mM). This increase in K_m value indicated that affinity of the laccase to its substrate decreased with immobilization. These decreases in the activity were probably due to the steric hindrances caused by the support or decrease in the enzyme flexibility or diffusional limitations of substrate [1]. Effect of pH on the activity of the free and immobilized laccase was demonstrated in Figure 5. As seen in figure, maximum activity was observed at pH 4.0 for both free and immobilized laccase. Above and below this pH value, enzymatic activity of the laccase decreased dramatically. Optimum temperature profile of free and immobilized laccase was shown in Figure 6. As seen here, optimum temperatures of free and immobilized laccase were found to be 25 and 45°C, respectively. This shift towards the higher temperature brings about very important property to the immobilized form of laccase. Dyeing and painting process generally carried out at high temperatures and effluents from these industries are also protecting their temperatures. Cooling is often time consuming and it is essential to treat them even if they are already hot. Immobilized laccase with optimum temperature at 45°C may be successfully applicable for the decolorization of such wastes. The same thermal property of the immobilized laccase was also monitored with the thermal stability studies. Thermal stability profiles of the free and immobilized laccase were investigated at 55 and 65°C and findings were demonstrated in Figures 7(a) and 7(b), respectively. As seen in the figure, while free laccase protected 64% of its initial activity at the end of 5 h incubation at 55°C, immobilized laccase showed 77% of initial activity. The same finding also monitored at 65°C, immobilized laccase demonstrated 67% of its initial activity at the end of 5 h incubation, while free laccase showed only 34%. From these results, it can be concluded that thermal stability and resistance of the laccase were increased with immobilization process. These findings can also enhance the usability of the immobilized form laccase in waste water

FIGURE 8: Operational stability of the immobilized laccase.

management. Operational stability profile of the immobilized laccase was demonstrated in Figure 8. As demonstrated in figure, operational stability of the immobilized laccase was found to be very high. At the end of the 10th reuse, activity of the immobilized laccase decreased only about 6.7%. Storage stability of the free and immobilized form of laccase was also determined and it was found that while immobilized enzyme protected 85.5% of its initial activity, free preparation protected 52.8% of its initial activity, at the end of the 30 days.

3.3. Decolorization Studies. Decolorization efficiency of immobilized laccase was demonstrated in Figure 9. As seen here, immobilized laccase decolorized the studied seven dyes effectively. All dyes decolorized by using immobilized laccase at the rate of 50% at the end of 10 min. Decolorization percentage of the dyes were also given in Table 2. Murugesan et al. [19] used laccase from *Ganoderma lucidum* for decolorization of Remazol Brilliant Blue R and they found that Remazol Brilliant Blue R was decolorized by 77.4% within 2 h. Peralta-Zamora et al. [21] investigated that the

TABLE 2: Decolorization percentage of dyes at the end of 10 min.

Dyes	Procion Red	Reactive Green 5	Reactive Brown 10	Reactive Green 19	Cibacron Blue F3GA	Alkali Blue 6B	Brilliant Blue 6
Decolorization %	81.53	59.81	73.59	66.33	62.95	59.71	61.68

FIGURE 9: Decolorization efficiency of immobilized laccase onto poly(MMA-co-GMA) cryogel.

decolorization of Remazol Brilliant Blue R, Remazol Black B, Reactive Orange 122, and Reactive Red 251 dyes by using immobilized laccase within 30 min and decolorization capacities were found to be 35–45%, 10%, 10–30%, and 5–55%, respectively. Kunamneni et al. [4] used immobilized form of laccase in order to decolorize the synthetic dyes and they found 61–82% decolorization rates within 6 h. Claus et al. [25] used laccase from *Trametes versicolor* for decolorization of azo dyes and they reached 3.0%–82% decolorization efficiency within 16 h. Murugesan et al. [6] purified laccase enzyme from *Pleurotus sajor-caju* and used for the decolorization of three azo dyes. Investigators found 70–90% decolorization yield within 24 h. One of the most important features of laccase immobilized poly(MMA-co-GMA) cryogel was its speed. It reached high decolorization values only within 10 min and it can be concluded from these results that immobilized form of laccase was successfully used for the decolorization of dyes by using a continuous system and this system can be adapted to the industrial waste water management system as a decolorization agent.

4. Conclusion

Dye effluents cause serious environmental pollution and management of these effluents is difficult due to the complex structure of the dye wastes and used techniques for these purposes are very expensive. For these purposes, new decolorization techniques have been developed and used for management of the dye effluents. Laccase has been used extremely

for the decolorization process due to its unique enzymatic properties. Its immobilized form especially, has been used in various decolorization studies. One of the new polymeric materials which are used intensively in biotechnological area is cryogel. Preparation of these polymeric materials is easy and can be produced in desired shape, size, and functionalities. In this presented work, laccase was successfully immobilized onto poly(MMA-co-GMA) cryogel and decolorization properties of this preparation was investigated. It was shown that, this new immobilized laccase preparation was used for decolorization of seven different dyes and decolorized all studied dyes effectively. It can be concluded from these results that this new laccase immobilized cryogenic medium can be used for the decolorization of the dye and paint industry effluents and the other dye bearing waste waters.

Conflict of Interests

No conflict of interests was declared.

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