Research Article

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Immobilization of laccase on itaconic acid grafted and Cu(II) ion chelated chitosan membrane for bioremediation of hazardous materials

Gulay Bayramoglu,[a]* Ihsan Gursel,[b] Meltem Yilmaz[c] and Mehmet Yakup Arica[a]

Abstract

BACKGROUND: Chitosan membranes were formed through a phase inversion technique and then cross-linked with epichlorohydrin (CHX). Heterogeneous graft copolymerization of itaconic acid (IA) onto membrane was carried out with different monomer concentrations (CHX-g-p(IA)). The membrane properties such as equilibrium swelling ratio, porosity, and contact angle were measured, together with analysis by scanning electron microscopy (SEM), energy dispersive analysis of X-rays (EDAX), atomic force microscopy (AFM), and Fourier transform infrared (FTIR) spectroscopy.

RESULTS: The Cu(II) ion incorporated membranes (i.e. CHX-g-p(IA)-Cu(II)) were used for reversible immobilization of laccase using CHX-g-p(IA) membrane as a control system. Maximum laccase adsorption capacities of the CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes (with 9.7% grafting yield) were found to be 6.3 and 17.6 mg mL^{-1} membrane at pH 4.0 and 6.0, respectively. The K_m value for immobilized laccase on CHX-g-p(IA)-Cu(II) (4.16×10^{-2} mmol L^{-1}) was 2.11-fold higher than that of free enzyme (1.97×10^{-2} mmol L^{-1}). Finally, the immobilized laccase was used in a batch system for degradation of three different dyes (Reactive Black 5, RB5; Cibacron Blue F3GA, CB; and Methyl Orange, MO). The immobilized laccase on CHX-g-p(IA)-Cu(II) membrane was more effective for removal of MO dye than removal of CB and RB5 dyes.

CONCLUSION: Flexibility of the enzyme immobilized grafted polymer chains is expected to provide easy reaction conditions without diffusion limitation for substrate dye molecules and their products. The support described, prepared from green chemicals, can be used for the immobilization of industrially important enzymes.

Keywords: chitosan; itaconic acid; graft copolymerization; enzyme immobilization; laccase; dye degradation

INTRODUCTION

Enzymes are well known green catalysts that are highly specific and efficient. However, the application of enzymes suffers from various problems, e.g. instability, non-reusability, and high cost.[1–4] Enzyme immobilization has become an effective way to partially overcome these limitations.[5–8]. The reversible immobilization of enzymes onto functionalized polymeric supports with high-density ion-exchange groups has been proposed as a very suitable method.[9–11] In this way, a very strong adsorption of enzyme on the polymer surface is obtained with an intense multipoint non-covalent attachment.[12,13] For reversible enzyme immobilization, ion-exchangers, hydrophobic and/or metal ion chelated supports have been used.[14–16] For the latter case, reversible enzyme immobilization on the metal ion chelated support is based on multipoint interactions between chelated metal ion and histidine, cysteine, glutamic acid, aspartic acid or tyrosine residues of the enzyme.[17–20] Most transition metal ions such as Fe(III), Fe(II), Ni(II), and Cu(II) can form stable complexes with electron-rich compounds.

In general, graft copolymerization is an attractive way to decorate polymeric supports with functional groups. For example, grafting of a functional group carrying vinyl monomer such as itaconic acid onto chitosan backbone could introduce novel oxygen rich ligands for chelating Lewis metal ions.[15,21–23] The green chemicals are almost ideal to prepare support materials for use in enzyme immobilization technology. Chitin is the second-most abundant green polymer after cellulose. The presence of amino groups in chitosan besides hydroxyl groups is highly advantageous for providing distinctive biological functions and

* *Correspondence to: Gulay Bayramoglu, Gazi University, Faculty of Sciences, Biochemical Processing and Biomaterial Research. E-mail: g_bayramoglu@hotmail.com; yakuparica@tnn.net*

a *Gazi University, Faculty of Sciences, Biochemical Processing and Biomaterial Research*

b *Bilkent University, Molecular Biology and Genetics Department, Bilkent 06800, Ankara, Turkey Laboratory, 06500 Teknikokullar, Ankara, Turkey*

c *Gazi University, Institute of Science and Technology, Department of Environmental Sciences, 06570 Ankara, Turkey*

for modification reactions.[24] Itaconic acid is also a green chemical and its formation was observed for the first time in acid medium of *Aspergillus itaconicus*.[25] Itaconic acid is an unsaturated C5 dicarboxylic acid, which can be used in industry as monomer or co-monomer in the manufacture of plastics, resins, synthetic fibers, and grafting of various polymers.[26–28] Based on its industrial potential, it was selected by the Department of Energy in the USA as one of the 12 building block chemicals, which are the most interesting compounds to be produced by industrial biotechnology.[29] Chitosan and itaconic acid were preferred over other materials because of their various advantages such as biodegradability, hydrophilic properties, presence of functional groups (i.e. amine and carboxylic groups), and natural origin. These are very important because polymers of petroleum origin are non-degradable, making them a major cause of pollution.

Laccases (EC 1.10.3.2) are blue multi-copper-containing enzymes and they are also green chemicals that catalyze the oxidation of a variety of organic substances coupled to the reduction of molecular oxygen.[30,31] Because of their broad specificity for the reducing substrates, laccases from white-rot fungi have received increasing attention as a potential enzyme in various biotechnological applications, such as in chemical or medicinal synthesis, in the analytical field as a component of biosensor systems, and in the bioremediation of contaminated water.[32,33] In addition, laccase in the presence of redox mediators is able to extend its oxidative action over the various aromatic organic compounds.[34,35] These mediators are low molecular mass compounds oxidized by the laccase acting as electron carriers between the enzyme and other compounds. White-rot fungi laccase has been used for the degradation of phenols and multi-cyclic aromatic compounds such as chlorinated phenols, textile dyes, and pesticides.

In the present study, cross-linked chitosan membranes were decorated with fibrous itaconic acid polymer, and then chelated with Cu(II) ion as a metal ligand. The CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes were used for reversible immobilization of laccase. The immobilized laccase was used for degradation of three different dyes (Reactive Black 5, Cibacron Blue F3GA, and Methyl Orange) in the presence and absence of mediator (i.e. acetosyringone) in batch mode.

MATERIALS AND METHODS

Materials

Chitosan powder, ammonium persulfate (APS) and itaconic acid monomer were obtained from Fluka AG (Buchs, Switzerland). Laccase (EC 1.10.3.2: p-diphenol : dioxygen oxidoreductase; 20 U mg^{-1}) from *Tramates versicolor,* acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone), Reactive Black 5 (RB5; Remazol Black B; empirical formula: $C_{26}H_{21}N_5Na_4O_{19}S_6$; MW: 991.82; CAS Number: 241-164-5, and λ_{max}:597 nm), Cibacron Blue F3GA (CB; Reactive Blue 2; empirical formula : $C_{29}H_{17}N_7Na_3O_{11}S_3Cl$; MW:840.11; CAS Number:12 236-82-7, and λ_{max}:610 nm), Methyl Orange (MO; empirical formula : $C_{14}H_{14}N_3NaO_3S$; MW:327.33; CAS Number:208-925-3, and λ_{max}:560 nm), 4-hydroxy-3,5-dimethoxy-hydroxybenzaldehyde (syringaldazine), and epichlorohydrin were obtained from Sigma–Aldrich Chem. Co. (Heidelberg, Germany). All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

Preparation of itaconic acid grafted chitosan membranes

The preparation of CHX membranes was carried out as reported previously.[36] The graft co-polymerizations of itaconic acid on the CHX membranes were carried out in a two-neck round bottomed glass reactor. In a typical grafting, cross-linked chitosan membrane disks (about 2.0 g) and APS solution (6.58×10^{-3} mol L^{-1}, 20 mL) were transferred into the reactor and stirred magnetically at 100 rpm for 10 min. Then, the monomer was added drop-wise to the reactor and its concentration was varied between 0.2 and 1.0 mol L^{-1}. The grafting reaction was carried out at 60 °C for 3.0 h under nitrogen atmosphere. Different grafting ratios were obtained by varying the initial monomer concentration. The grafted chitosan membranes were washed with purified water, and then dried under reduced pressure at 60 °C. The grafting percentage was determined from the mass of dried membranes before and after grafting using following equation:

$$\text{poly(itaconic acid) grafting (\%)} = (W_2 - W_1)W_1 \times 100 \quad (1)$$

where W_1 and W_2 are the mass of cross-linked chitosan membrane and p(IA) grafted membranes, respectively.

Incorporation of Cu(II) ions on CHX-g-p(IA) membrane

Incorporation of Cu(II) ion on the membrane was carried out as follows: 5.0 g of the membranes were transferred in a flask containing of Cu(II) ion solution (50 mL, pH 5.0). The flask was stirred magnetically for 2.0 h. The amount of incorporated Cu(II) ions was calculated by using the concentrations of the Cu(II) ions in the initial solution and in supernatant solution. The concentration of Cu(II) ion was determined using atomic absorption spectrophotometry.

Immobilization of laccase onto CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes

Immobilization of laccase via adsorption on the CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes from aqueous solutions was studied in batch mode. The membranes (1.0 mL) were incubated with enzyme solution (1.0 mg mL^{-1}, 25 mL) at 25 °C for 2 h, the flask was agitated magnetically at 100 rpm. To determine the effect of pH on the immobilization efficiency, pH of the medium was changed between 3.0 and 8.0 in either acetate (50 mmol L^{-1}, pH 4.0–5.5) or phosphate buffer (50 mmol L^{-1}, pH 6.0–8.0). The arithmetic mean values and standard deviations were calculated and the margin of error for each data set was determined according to a confidence interval of 95% using Excel for Windows. The amount of laccase immobilized on the membranes was determined by measuring the initial and final concentrations of protein in the immobilization medium using the Bradford protein assay method.[37] The range of laccase concentration selected for the adsorption isotherm study was 0.1–2.0 mg mL^{-1}. The maximum capacity (q_{max}) data for the adsorption of laccase was obtained from the experimental data, and the equilibrium data was analyzed using the Langmuir isotherm model. The Langmuir isotherm model is represented as following equation:

$$q = (q_{max}\, C_{eq})/(K_d + C_{eq}) \quad (2)$$

where the apparent dissociation constant, K_d, (i.e., the adversely of dissociation constant of the ligand/surface interaction or equal to association constant, K_a, ($K_a = 1/K_d$)) is the energy of adsorption dissociation constant (M). K_d has dimension of concentration, and the protein binding is stronger when it is smaller. q is the amount of adsorbed laccase on the supports (mg mL^{-1}), C_{eq} the equilibrium laccase concentration in solution (mg mL^{-1}), and the constant q_{max} represents the maximum binding at the complete saturation of adsorbent binding sites.

Activity assays of free and immobilized laccase

Laccase activity was measured according to the Ride method[38] based on measurement of the change in optical density of syringaldazine at 530 nm. The enzyme activity unit was defined as the amount of enzyme that oxidizes syringaldazine to quinone and increases of absorbance by 0.001 optical density (OD) per min. The laccase activity assays were carried out over the pH range 3.5–8.0 and temperature range 15–55 °C to determine the pH and temperature profiles for the free and the immobilized enzymes. The results were converted to relative activities (percentage of the maximum activity obtained in that series). The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized laccase were determined as reported previously.[22]

Storage stability of free and immobilized laccase

The storage stability of the free and immobilized laccase preparations was determined as change of activity over time. The free and immobilized enzyme samples were stored at 4 °C in citrate buffer pH 5.5, and the samples were taken in same time intervals from the stored enzymes preparations. The activity determined at the first measurement was set to 100%.

Degradation of dyes by immobilized laccase

The reaction mixture containing 5.0 mL of RB5, CB and/or MO solution (100 mg L^{-1}) and 5.0 mL of acetate buffer solution (pH 5.5, 100 mmol L^{-1}) or same buffer solution containing 0.2 mmol L^{-1} acetosyringone (as a mediator) was incubated with 0.2 mL free enzyme solution or 0.2 mL of the laccase immobilized membrane. The free and immobilized laccase activities of the reaction medium were 51 U per 2.5 mg protein in 0.2 mL enzyme solution and/or 58 U on 0.2 mL membrane, respectively. The contents were shaken at 150 rpm at 35 °C for 12 h using an orbital shaker. After a predetermined time period, the decrease in the absorbance of the reaction medium was measured using a UV/visible spectrophotometer at 597 and 560 nm for RB5 and MO dyes, respectively. A calibration curve was plotted of absorbance versus concentration using each dye solution with known concentrations.

Percentage dye removal was calculated by using the following expression:

$$\text{Removal dye (\%)} = [(C_0 - C)/C_0] \times 100 \quad (3)$$

where C_0 and C are the concentration of RB5, CB or MO dye in the initial solution and in the final solution for a certain period of time (mg L^{-1}).

Characterization studies

The amount of available surface functional carboxyl groups of the CHX-g-p(IA) membrane was determined by the back titration method. About 1.0 g of dry p(IA) grafted membranes was immersed in 20 mL of 0.1 mol L^{-1} NaOH for 24 h at room temperature. The H^+ ions of the membranes are replaced with Na^+ ions, and the residual solution is back titrated with 0.1 mol L^{-1} HCl.

FTIR spectra of the CHX and CHX-g-p(IA) membranes were obtained using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry sample (about 0.01 g) mixed with KBr (0.1 g) and pressed into a tablet form. The FTIR spectrum was then recorded. Contact angles to water of the dry CHX, CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes were measured by sessile drop method as described previously.[36] The water content of the CHX and CHX-g-p(IA) membranes was determined at 25 °C in phosphate buffer (50 mmol L^{-1}, pH 7.0) with a gravimetric method.

Surface morphology of the membrane samples were examined using SEM and the presence of the elements on membrane samples were also verified using EDAX analysis. Imaging and EDAX analysis were performed with a FEI Quanta 200F scanning electron microscope (FEI Company, Hillsboro-Oregon, USA) equipped with an EDAX-detector (Ametek Process and Analytical Instruments). EDAX was performed at an acceleration voltage of 20 kV.

Atomic force microscopy (AFM) was used for the morphological characterization of the membrane surfaces. AFM images of dry plain chitosan and p(IA) grafted chitosan membranes were taken on an AFM instrument (PSIA, XE 100 E model) with a 0.56 Hz scanning rate. The resonance frequency and the force constant were 75 kHz and 3 N m^{-1}, respectively.

The pore volume of the CHX-g-p(IA) membranes was measured by a mercury porosimeter. The specific surface area of membranes was measured by a surface area apparatus and calculated using the BET (Brunauner–Emmett–Teller) method.

RESULTS AND DISCUSSION

Preparation and characterization of CHX-g-p(IA)-Cu(II) membranes

Chitosan membrane was prepared by a phase-inversion method and cross-linked with epichlorohydrin and the chemistry of the membrane is presented in Fig. 1. Heterogeneous graft copolymerization of itaconic acid onto cross-linked chitosan membrane was carried out in aqueous solution with APS as an initiator. The complex formed by the reaction between $-NH_2$, $-OH$ and $-Cl$ groups of cross-linked chitosan decomposed to generate the free radical sites at about 60 °C, facilitating the reaction for the monomer.[39] The effect of itaconic acid concentration on the grafting efficiency of the CHX membrane was also studied. The grafting efficiency of the membrane was increased with increasing itaconic acid concentration from 0.2 to 0.6 mol L^{-1} (data not shown). After a certain limit, the increase in itaconic acid concentration accelerates the homopolymerization reaction. For this reason, in the remaining study, the initial concentration of itaconic acid (0.6 mol L^{-1}) was used in the polymerization reaction and the percentage grafting efficiency under this condition was about 9.7%.

The free amino group content of the CHX membrane was found to be 4.21×10^{-3} mmol mL^{-1} membrane. After grafting with itaconic acid, there was no detectable free amino group of the membrane. The carboxyl group concentration of the CHX-g-p(IA) was determined as 1.96×10^{-3} mmol mL^{-1}. The Cu(II) ion chelating capacity of the CHX-g-p(IA) membrane (grafting efficiency 9.7%) was 7.3×10^{-4} mmol mL^{-1}.

Compared with CHX membrane (54 ± 2%), the water content of CHX-g-p(IA) membrane increased to 103 ± 3%. This appears reasonable when it is remembered that the CHX is not a highly polar compound. After grafting p(IA), negatively charged carboxyl groups were introduced on the polymer structure, and should cause more water uptake.

The energy dispersive X-ray analysis (EDAX) was performed to determine the elemental composition of the CH, CHX, CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes, and the results of EDAX analysis are presented in Fig. 2(A)–(D). In Fig. 2(A), only carbon, nitrogen and oxygen (100%) are observed for chitosan membrane. However, in Fig. 2(B), an additional chlorine peak is apparent due

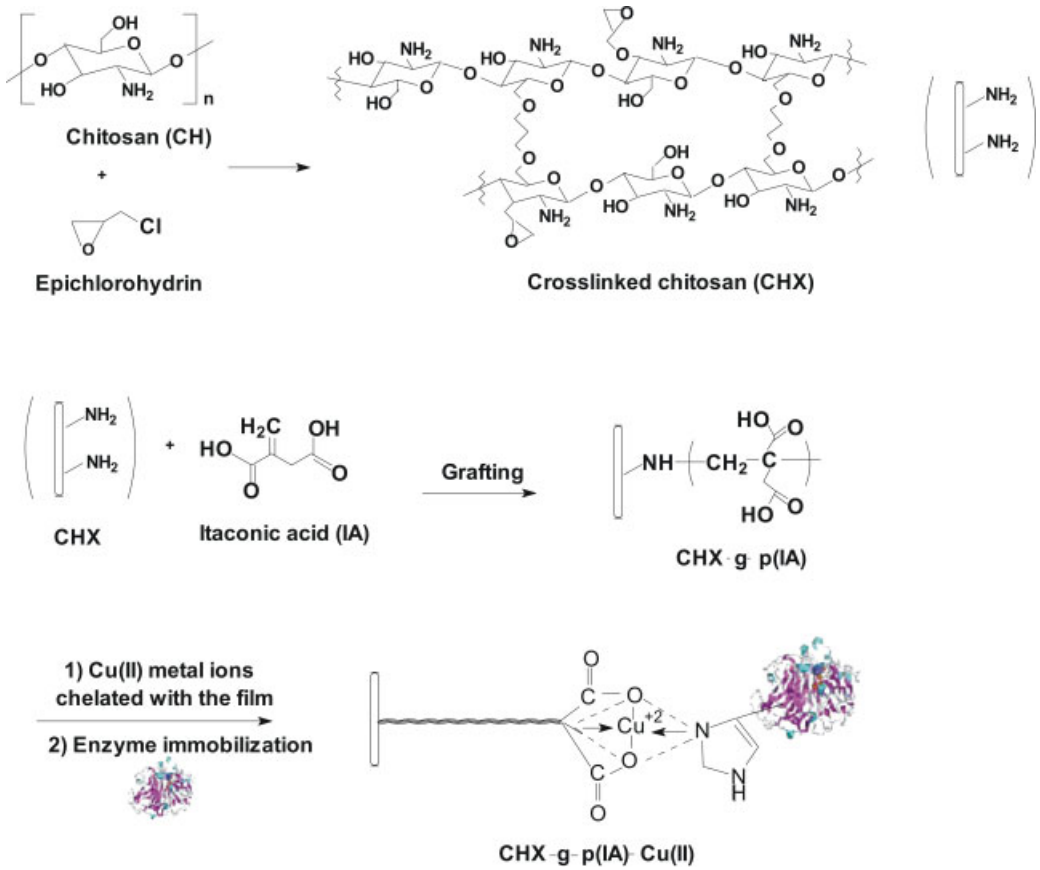

Figure 1. Chemistry of the support material.

to the cross-linking of chitosan membrane with epichlorohydrin. In Fig. 2(C), carbon, nitrogen, oxygen, and chloride ratio are changed for CHX-g-p(IA) membrane compared with CHX membrane. In addition, the presence of copper peak for CHX-g-p(IA)-Cu(II) membrane is observed in Fig. 2(D). The presence of copper in the EDAX spectrum confirms the incorporation of copper ions to the polymer matrix and this is in corroboration with AAS results.

The surface of cross-linked chitosan membranes with varying degrees of graft copolymerization were observed by SEM, and microphotographs are presented in Fig. 3(A)–(C). Figure 3(A) shows the surface morphology of CHX membrane before grafting. One can see that the CHX membrane (Fig. 3(A)) has a porous surface and relatively heterogeneous appearance. The pores on the surface of the CHX membrane start to disappear when the itaconic acid grafting reaches about 9.7% (Fig. 3(B)). With increasing p(IA) grafting on the CHX, the grafted fibrous polymer chains p(IA) seem to fill pores of the membrane. The pores became less obvious when p(IA) grafting percentage reached about 15.8%, giving a smooth appearance on the surface of the grafted membrane (Fig. 3(C)), showing proof of the grafting.

AFM images of CHX and CHX-g-p(IA) membrane samples are shown in Fig. 4(A) and (B), respectively. The images were obtained over an area of 40 μm × 40 μm for the CHX and CHX-g-p(IA) membranes. The surfaces of both membrane samples have typical hills and valleys morphology. However, the surface topography of the p(IA) grafted membrane is significantly different in surface roughness compared with CHX membrane. Figure 4(B) shows that the polymer brushes have been grafted onto the CHX surface and a nano-metric composite structure has been formed, and the coating thickness is around 400 nm. The grafted polymer chains p(IA) seem to fill the valleys, and cause a less rough appearance to the grafted membrane compared with the base CHX membrane.

The Brunauer–Emmett–Teller (BET) surface area of the CHX membrane has been calculated to be 13.2 $m^2\ mL^{-1}$, which is higher than that of CHX-g-p(IA) membrane (7.84 $m^2\ mL^{-1}$). It should be noted that base CHX membrane showed a higher surface roughness and pore volume compared with the p(IA) grafted CHX membrane. The surface property is a more important parameter for enzyme immobilization than that of the surface area (i.e. surface roughness and porosity) due to the substrate and product diffusional limitation. Thus, the grafted membrane could be more available for enzyme immobilization.

The FTIR spectra of CHX-g-p(IA) membrane has a carboxyl group band at 1768 cm^{-1}, different from than that of the CHX. The appearance of the new peak evidenced the successful grafting of p(IA) onto the CHX membrane. The broad bands at ~3400 cm^{-1} corresponds to stretching vibration of –OH and $-NH_2$ groups, and the peak at 1657 cm^{-1} is due to N–H deformation in the amino group of chitosan. A relatively high intense peak at around 1084 cm^{-1} is the characteristic peak of polysaccharides.

The surface wettability of the membrane sample was determined with contact angle measurements. The values of contact angle to water were 60.8° ± 1.2, 50.3° ± 0.8, and 73.6° ± 1.7 for the CHX, CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes. The p(IA) grafted membrane surface resulted in a significant difference in the value of contact angle to water compared with CHX membrane. On the other hand, Cu(II) ions chelation resulted in an

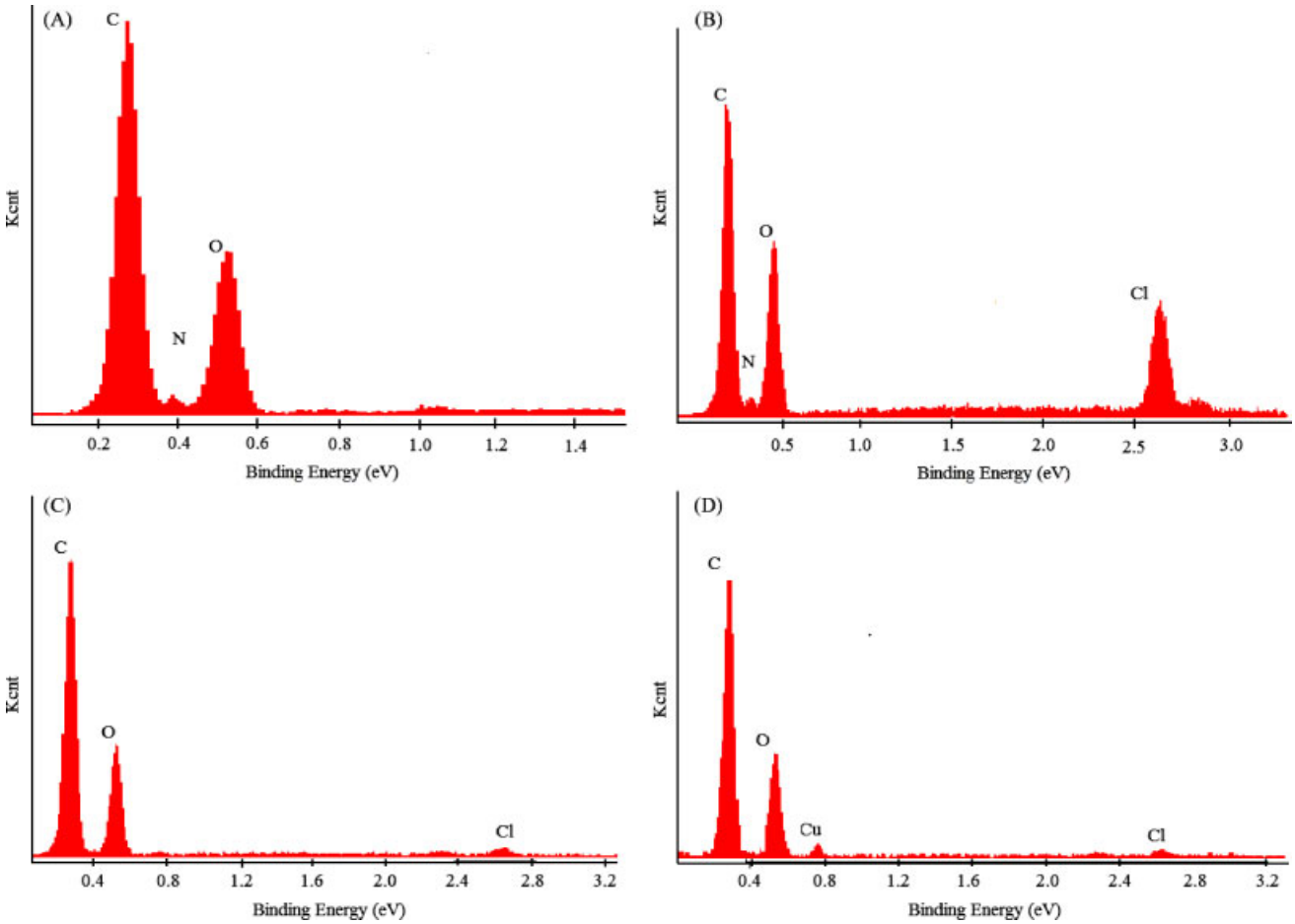

Figure 2. SEM–EDAX spectrum for (A) chitosan; (B) cross-linked chitosan; (C) poly(itaconic acid) grafted chitosan with 9.7% grafting efficiency; (D) poly(itaconic acid) grafted chitosan (9.7% grafting efficiency) with copper Cu(II) ion.

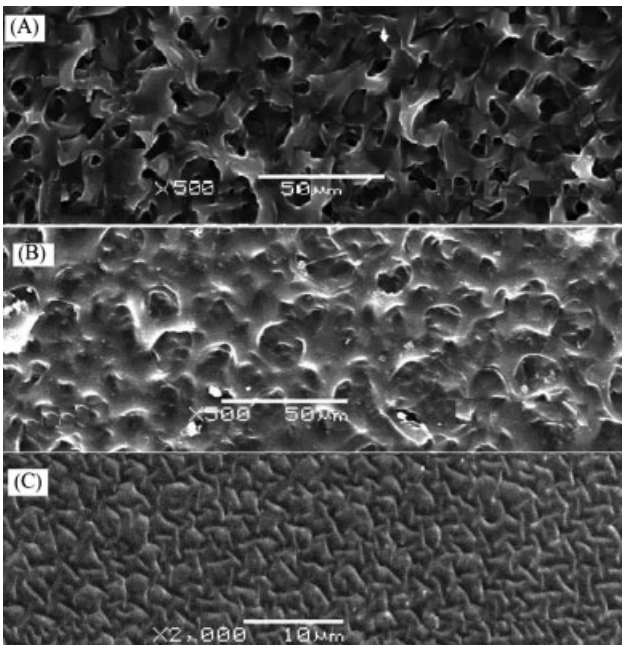

Figure 3. SEM image of (A) cross-linked chitosan; (B) poly(itaconic acid) grafted chitosan with 9.7% grafting efficiency; (C) poly(itaconic acid) grafted chitosan with 15.8% grafting efficiency.

increase in the hydrophobic character of the membrane compared with CHX-g-p(IA).

Immobilization of laccase on membranes

Laccase was immobilized on CHX-g-p(IA)-Cu(II) membranes with different graft yields (%), and the results are presented in Fig. 5. The amount of immobilized laccase increased significantly with increasing graft yield up to 13.1% (about 20.8 mg protein mL^{-1} membrane), and then reached a plateau with increase in graft yield to 15.8% (21.1 mg protein mL^{-1} membrane). Increasing graft yield increases the number of functional adsorptive groups. As seen from the figure, the recovered enzyme activity is higher at smaller graft yield. This may be explained by the fact that fibrous polymer chains of the membrane should be covered with more adsorbed laccase as the graft yield increases, resulting in a sticky web-like coating over the fibrous polymer surface. Thus, these layers may prevent diffusion of the substrate molecules from the inner layer of the polymer brushes where the enzyme was immobilized.[40]

Effect of pH and initial laccase concentration on immobilization efficiency

The effect of pH on the laccase immobilization capacity of the membrane (grafting efficiency 9.7%) was investigated in the pH range 3.0–8.0 (data not shown). The maximum enzyme loading was obtained as 17.6 mg mL^{-1} at around pH 6.0 with the

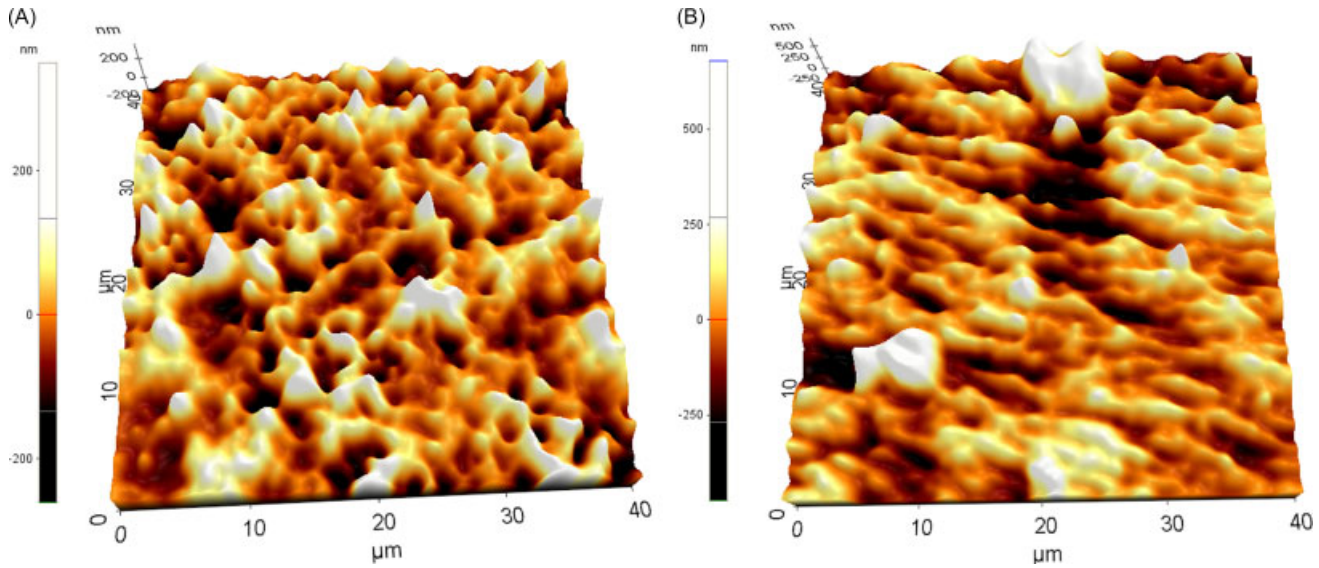

Figure 4. AFM images of (A) cross-linked chitosan membrane surface before grafting; (B) after grafting of cross-linked chitosan membrane with poly(itaconic acid). The grafting was about 9.7%. The size of the images is 40 μm × 40 μm.

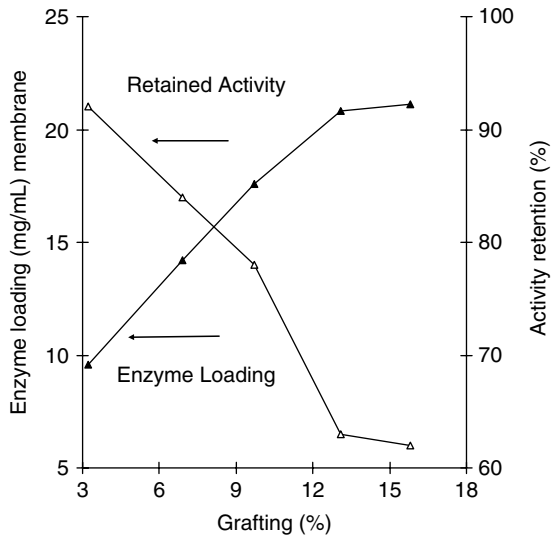

Figure 5. Effect of graft yield on enzyme loading efficiency and retained enzyme activity.

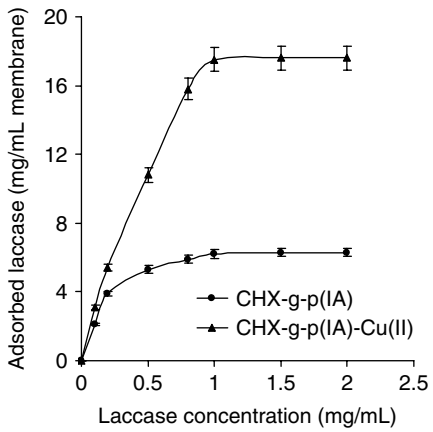

Figure 6. Experimental equilibrium adsorption isotherm for the CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes.

CHX-g-p(IA)-Cu(II) membrane. Significantly lower enzyme loading 6.3 mg mL^{-1} was obtained for CHX-g-p(IA) membrane at more acidic pH 4.0 regions compared with Cu(II) ion incorporated counterpart membrane. Laccase is classified as a multi-copper-containing blue-enzyme and it has four copper ions in clusters of histidine residues (His-x-His).[41] This phenomenon could also provide additional high binding affinity for laccase to itaconic acid grafted and/or Cu(II) chelated fibrous polymer. Transition metal ions such as Cu(II) are considered strong Lewis acids and interact with strong Lewis bases such as nitrogen and oxygen. It is known that electrostatic interactions and hydrogen bonds are the main forces responsible for specific adsorption. With increasing pH values, the adsorbed amount of laccase was increased because the histidine residue exposed at the surface of laccase molecules form coordination sites for Cu(II) ion, and it acts as a ligand, at pH around 6.0.

Experimental equilibrium adsorption curves were obtained for laccase adsorption on the CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes with different initial laccase concentration at pH 4.0 and 6.0, respectively (Fig. 6). The adsorption capacity was increased with the initial protein concentration in the medium (from 0.1 to 2.0 mg mL^{-1}), and reached a maximum adsorption capacity (about 17.6 mg mL^{-1} membrane) at 1.0 mg mL^{-1} laccase concentration; above this initial concentration value capacity remained stable. The amount of laccase adsorbed onto the CHX-g-p(IA) membrane was 6.3 mg mL^{-1}, which was about 2.79-fold lower than for CHX-g-p(IA)-Cu(II) membrane.

The Langmuir adsorption is expressed by Equation (2). The corresponding transformations of the equilibrium data for laccase gave rise to linear plots (data not shown), indicating that the Langmuir equation could be applied to these systems. The model gave a very good fit to the adsorption data of laccase on both membranes with high correlation coefficients (up to $R_2 = 0.998$). The maximum capacity (q_{max}) was calculated as 6.62 and 18.1 mg mL^{-1} for CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes, respectively. The K_d values were found to be

1.24×10^{-6} and 5.23×10^{-7} mol L^{-1} with CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes, respectively. The apparent dissociation constant (K_d) estimated from the intercept is a measure of the stability of the complex formed between a protein and an adsorbent under specified experimental conditions. For example, a large K_d value indicates that the protein has a low binding affinity for the adsorbent. In this case, a very low K_d value obtained for stable binding of laccase on the CHX-g-p(IA)-Cu(II) membrane.

The ΔG° values for laccase adsorbed on the membranes were calculated using the following equations; $\Delta G^\circ = -RT \ln K_a$. The negative ΔG° values for each membrane indicated that adsorption of laccase on the CHX-g-p(IA) and CHX-g-p(IA)-Cu(II) membranes was a favourable process, and these were −33.7 and −35.8 kJ mol^{-1} at 25 °C, respectively. The negative adsorption enthalpy values at given temperatures indicates the spontaneous nature of the adsorption and confirm the feasibility of the adsorption process.

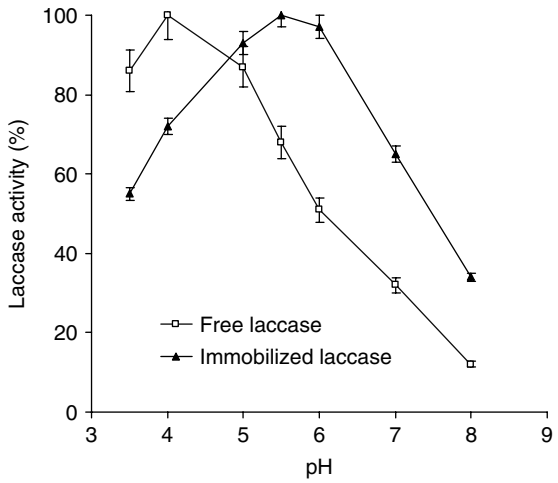

Figure 7. Effect of pH on free and immobilized laccase enzyme activity.

Kinetic parameters

The laccase immobilized on CHX-g-p(IA)-Cu(II) membrane exhibited a higher value of Michaelis constant K_m than the free counterpart, reflecting higher apparent affinity of the substrate for the free laccase. The K_m values of the free and immobilized laccase were found to be 1.97×10^{-2} and 4.16×10^{-2} mmol L^{-1}, respectively. The V_{max} values were estimated from the data as 20.4 and 16.5 U mg^{-1} protein for the free and adsorbed enzymes on the CHX-g-p(IA)-Cu(II) membrane. The decrease in V_{max} value as a result of immobilization is considered to be associated with the K_m value since the lower the value of K_m, the greater the affinity between the enzyme and substrate.[42,43] In the literature, different kinetic parameters (i.e. K_m and V_{max}) for free and immobilized laccase have been reported. It is noteworthy that in such immobilization processes there are several parameters that determine the kinetic parameters of the laccases, such as source of enzymes, substrates, experimental temperatures, method of calculation used by other researchers, so that comparison of our results with those reported earlier proved somewhat difficult.[44] Wang *et al*. immobilized laccase (from *Pycnoporus sanguineus*) on the magnetic Cu(II) chelated particles with iminodiacetic acid as chelating ligand. The value of the Michaelis constant (K_m) of the immobilized laccase (1.60 mmol L^{-1}) was higher than that of the free one (0.76 mmol L^{-1}), whereas the V_{max} values for free and immobilized enzymes were 1.13 and 0.77 U g^{-1}, respectively.[45] Lu *et al*. reported that the activity of laccase entrapped in alginate/chitosan microspheres was about 6.84 U per 1.5 g polymers.[46] Kunamneni and Alcade studied the immobilization of laccase on epoxy-activated acrylic polymers, and V_{max} value was about 203 U g^{-1} of support.[47] When compared with the values of kinetic parameters reported in the literature, the novel Cu(II) chelated membranes used for immobilization of laccase in this work are promising for immobilization of other enzymes with non-covalent interactions.

In addition, in this work, V_{max}/K_m values of the immobilized laccase were lower than that of free laccase, which implies that the accessibility to the active sites on the immobilized laccase was hindered to some extent as a result of mass transport and diffusion limitation. In this study, the catalytic efficiencies (V_{max}/K_m) of the free and immobilized laccase onto membrane were found to be 1035 and 397, respectively. The catalytic efficiency of laccase was decreased about 2.61-fold upon immobilization.

Effect of pH and temperature

The effect of pH on the activity of free and immobilized laccase was studied in the pH range 3.5–8.0 (Fig. 7). For free laccase, the activity is strongly pH-dependent, and optimum pH was observed at pH 4.0. After immobilization, as pH is increased, activity increases rapidly first then decreases; the optimum value was pH 5.5, and the curve shape and the optimal pH were significantly changed. The shift in optimum pH toward a to less acidic pH value upon immobilization may be due to the difference in the hydronium ion concentration of the micro-structures of the polymeric support and in the bulk of the solution. In addition, the optimum pH range of the immobilized laccase was broader than for the free enzyme, which could be attributed to the formation of multipoint non-covalent interactions, which may limit the transition of enzyme conformation with the change of pH.[48,49] Therefore, the immobilized laccase maintained a relatively high activity over a broader pH range.

The effect of temperatures on the activity of free and immobilized laccase was studied in the temperature range 15–55 °C. For free and immobilized laccases, the activities increase gradually first with increasing temperature up to an optimum temperature at around 35 °C and 40 °C, respectively, and then decreased rapidly. The shift of the optimum temperature indicates an increase in the thermal stability of the immobilized laccase, and this is due to the change of physical and chemical properties of the immobilized enzyme.[48] In particular, the non-covalent multipoint interactions between the enzyme and the CHX-g-p(IA)-Cu(II) support may reduce the degrees of freedom of the molecular structure of the enzyme, thus, protecting it to some extent from denaturation by high temperature.[50]

Storage stability

Free and immobilized laccase were stored in an acetate buffer (50 mmol L^{-1}, pH 5.5) at 4 °C and the activity measurements were carried out for a period of 8 weeks (data not shown). The free enzyme lost all of its activity within 5 weeks; the immobilized laccase lost 37% of its activity in 8 weeks. The result readily indicates that the immobilized laccase exhibits improved stability over the free enzyme. Of the immobilization methods, fixation of enzyme molecule on a surface often gives rise to the highest stabilization effect on enzyme activity, because the active conformation of the

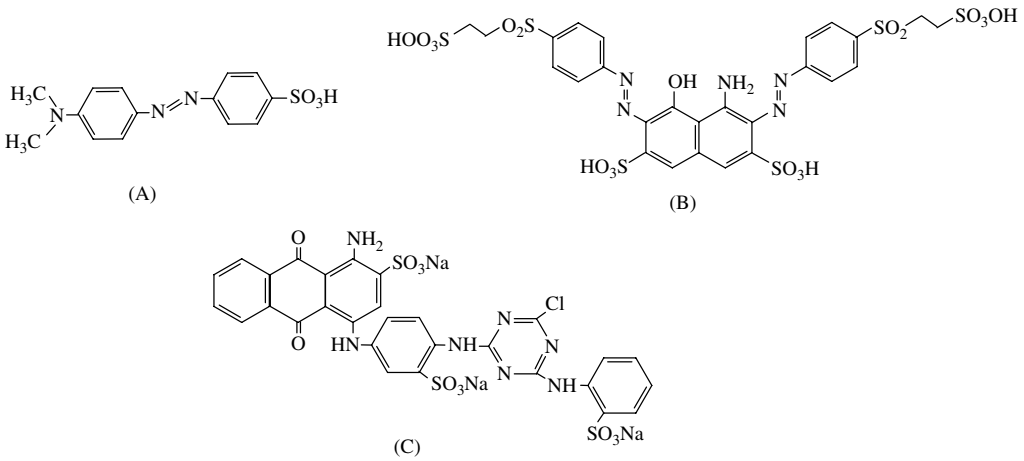

Figure 8. Chemical formula of the azo dyes: (A) Methyl Orange; (B) Reactive Black 5; (C) Cibacron Blue F3GA.

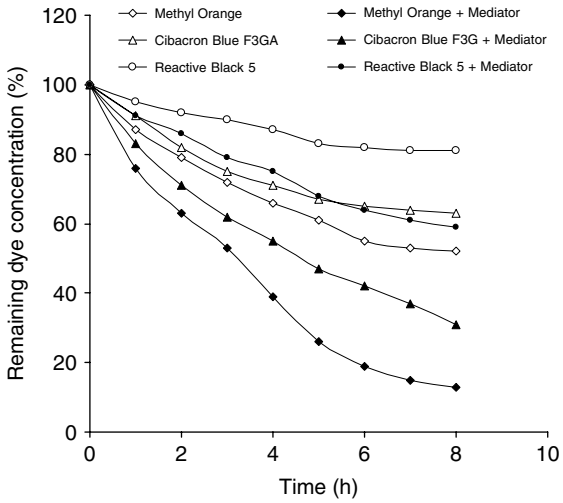

Figure 9. Degradation rate of immobilized laccase for Methyl Orange, Cibacron Blue F3GA and Reactive Black 5 in the presence and absence of acetosyringone mediator. (temperature: 35 °C, dye concentration: 100 mg L^{-1}).

immobilized enzyme is stabilized by multipoint interactions with the support.[51–53]

Reusability of CHX-g-p(IA)-Cu(II) membrane

For immobilized enzyme, one of the most important advantages is reuse stability, which can effectively reduce the cost in industrial applications. The reuse stability of immobilized enzyme in terms of repetitive use was investigated using 20 successive measurements on artificial substrate syringaldazine. To evaluate the reuse stability, the laccase-immobilized CHX-g-p(IA)-Cu(II) membrane was washed with acetate buffer (50 mmol L^{-1}, pH 5.5) after each run and reintroduced into a fresh solution. This process was repeated up to 20 cycles. It was observed that the residual activity of the immobilized laccase was 81% after 10 uses and remained constant for another 20 measurements.

Degradation of Reactive Black 5, Cibacron Blue F3GA and Methyl Orange dyes by immobilized laccase

Both free and immobilized laccase were used for the degradation of Reactive Black 5, Cibacron Blue F3GA and Methyl Orange dyes (Fig. 8) in the presence and absence of acetosyringone mediator. Under the same experimental conditions, a blank was used containing same quantity of membrane with denatured enzyme or mediator molecules. The degradation rate of dyes by the immobilized laccase was followed by sampling the solution at different times and analyzed as described above. The degradation rates of the tested dyes with time are presented in Fig. 9. As seen from the figure, immobilized laccase on CHX-g-p(IA)-Cu(II) membrane was highly efficient for the degradation of RB5, CB and MO dyes in the presence of mediator compared with a mediator-free system. In the mediator-free system, the amount of degradation of RB5, CB, and MO was 19%, 37% and 48% in 8 h, respectively. In the presence of acetosyringone mediator, 43%, 69% and 87% degradation of RB5, CB, and MO dye degradation was observed with immobilized laccase. The relative activity order of the immobilized laccase for the degradation of dyes for both cases, measured in terms of the time required to oxidize, was Methyl Orange>Cibacron Blue F3GA>Reactive Black 5. As is known from the literature, laccase is a useful green enzyme for the removal of several aromatic compounds (i.e. textile dyes, aromatic pesticides, chlorinated phenols and phenol derivatives) from wastewater. The degradation results of the tested RB5, CB and MO dyes were in accordance with the literature.[54–57]

CONCLUSION

In this study, chitosan membranes bearing p(IA) were synthesized by heterogeneous graft copolymerization of IA onto cross-linked chitosan membranes. The amount of grafted p(IA), which affects the performance of the resultant membranes, can be controlled by adjusting the reaction conditions. The grafted p(IA) acted as a metal-chelating ligand, and there is no need for any reaction step to activate the matrix for chelating-ligand immobilization. In addition, grafted p(IA) brush provides a hydrophilic microenvironment for the guest enzyme. The p(IA) grafted and/or Cu(II) ion incorporated membranes were used for reversible immobilization of laccase. The laccase adsorption capacity of the Cu(II) ion incorporated membrane is higher than that of the CHX-g-p(IA) membrane. The stability of laccase against pH and temperature after immobilization was improved by immobilization, demonstrating that the prepared CHX-g-p(IA)-Cu(II) membrane was suitable support for laccase immobilization. In addition, the regeneration of supports at the end of the life of the immobilized enzyme

can effectively reduce the cost of the immobilized enzyme system. Finally, the immobilized laccase on the CHX-g-p(IA)-Cu(II) membrane showed higher degradation efficiency (87%) in the presence of mediator for MO dye compared with CB **(69%)** and RB5 (43%) dyes.

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