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A pH-response Polyacrylate Amphiphilic Copolymer as The Carrier to Immobilize Laccase

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Abstract: In this study, a pH-response polyacrylate amphiphilic copolymer P_{MDB} was used as the carrier to immobilize laccase from *trametes versicolor.* Under optimized conditions, the activity recovery of the immobilized laccase is 71.90%. The infrared spectroscopy results indicate laccase is successfully immobilized on the polymer $P_{MDB.}$ Also the properties of the immobilized laccase were studied. The recovery of the immobilized laccase is 96.8% (wt%) by adjusting the pH of the immobilized laccase solution to 3.3. And the immobilized laccase remains 78.13% of the initial activity after 5 batches reactions. Compare to the free laccase, the stabilities like thermal and storage stabilities of the immobilized laccase are enhanced. Moreover, the relative activity of immobilized laccase is nearly 110% when the concentration of urea in the solution is 0.02 M. The work in this manuscript can provide valuable references for enzyme immobilization.

Keywords: pH-response copolymer; Laccase; Immobilization.

1. Introduction

Laccase (EC 1.10.3.2) belongs to the group of blue copper enzymes [1, 2], and has been widely used in many fields like environmental protection [3], medicine [4] and food [5]. However the free laccase is unstable and difficult to reuse, thus limit the utility of laccase in industry. To solve these problems, the laccase is immobilized on different carriers. The immobilization of laccase has widely attracted attentions in recent years. Laccase is usually immobilized on various solid carriers, like porous glass [6], silica nanoparticles [7] and magnetic nanoparticles [8]. However, due to diffusion-controlled mass transfer and steric hindrance in biphasic systems with water-insoluble substrates [9], the bioconversion efficiency of laccase immobilized on solid carriers is low. Although immobilized laccase on soluble carriers can solve this problem, enzyme immobilized on soluble carriers is hard to recover form the reaction solution.

The reversibly soluble-insoluble carriers like pH response polymers can easily dissolve and precipitate in the solution by adjusting the pH of the solution. Laccase immobilized on the pH response carriers can not only easily precipitate in the reaction solution and recover by centrifugation, but also increase the mass transfer and reduce the reaction time. Therefore immobilized laccase on pH response carriers is a good attempt.

P_{MDB} is a pH-response polyacrylate amphiphilic copolymer and synthesized with three monomers, which are methacrylic acid, 2-(dimethylamino) ethyl methacrylate and butyl methacrylate [10]. P_{MDB} is a reversible water-soluble copolymer. When adjusts the pH of the aqueous solutions to 3.1, more than 97% P_{MDB} will precipitate out of the solution. Moreover the pH response polymer P_{MDB} can dissolve in the aqueous phase in a large pH range ($pH \leq 2.5$ or $pH \geq 4.1$), and has been successfully used as the carrier in cellulose immobilization [10]. Because of the pH-response property, also there is no report about the immobilization of laccase on pH response polymer, we choose the pH-response polymer P_{MDB} as the carriers to immobilize laccase.

In this study, we used the pH response polymer P_{MDB} as the carrier to immobilize laccase. The properties of laccase immobilized on P_{MDB}, such as the optimum reaction pH, thermo-stability and reusability are studied. The work in this study will provide valuable references for researchers studying in enzyme immobilization.

2. Methods

2.1. Enzyme and chemicals

Laccase from *Trametes versicolor* (Powder, EC 1.10.3.2), 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide (EDC) and 2,6-dimethoxy phenol (DMP) were purchased from Sigma Chemical Co. (Shanghai, China). Methacrylic acid (MAA), butyl methacrylate (BMA), ammonium persulfate (APS), sodium hydrogen sulfite ($NaHSO_3$) were purchased from Lingfen Chemical Co. Ltd. (Shanghai, China). 2-(dimethylamino) Ethyl methacrylate (DMAEMA) was synthesized according to the reference [11]. Other reagents were obtained from Guoyao Chemical reagent Co. Ltd. China. Deionized water was used throughout the experiments. All reagents were of analytical grade.

2.2. Preparation of pH-response polymer PMDB

The pH-response polymer P_{MDB} was prepared according to the former research of our group [10].

2.3. Enzyme assay

The activities of free and immobilized laccase were measured using DMP as substrates [12]. DMP, free and immobilized laccase were dissolved in the HAc-NaAc buffer (pH 4.5, 10 mM) respectively. The concentration of DMP in the solution (pH 4.5, 10 mM HAc-NaAc buffer) was 40 mM. 10 μL of DMP solution and equal volume laccase solution were added into 2980 μL of the buffer solution (pH 4.5, 10 mM HAc-NaAc buffer). After quick mixing, the increase in

the absorbance of the product at 470 nm was recorded. The reaction lasted 3 min (25 °C). The molar extinction coefficient for the oxidation of DMP at 470 nm is 49600 L/(mol·cm). One unit of the laccase activity is defined as the amount of laccase that is required to catalyze 1 μmol of DMP per minute. The activity of laccase was calculated according equation (1).

$$A = \frac{10^6 \times V_t \times \Delta A}{V \times \varepsilon \times \Delta t} \quad (1)$$

A is the activity of laccase (U/mL); V_t is the volume of the reaction solution (mL), V is the volume of the laccase solution (mL), ΔA is the increase in the absorbance of the product during the reaction time (dimensionless), Δt is the reaction time (min), ε is the molar extinction coefficient for the oxidation of DMP at 470 nm and it is 49600 L/(mol·cm).**Immobilization of laccase on P_{MDB}**

The preparation of P_{MDB} solution was similar with the reference [10], the final pH of the P_{MDB} solution was 4.5~6.5 and the P_{MDB} concentration was 0.5~2.5% (w/v). Crosslinking agent EDC was added to the P_{MDB} solution (the amount of EDC was 200~450 mg/g P_{MDB}) and stirred 15 min. Then laccase was added to the solution (the amount of laccase was 150~300 mg/g P_{MDB}) and stirred 1~6 h (100 rpm) for immobilization. After immobilization, 3 M HAc solution was added to adjust the pH of the solution to 3.3, and centrifuged 20 min (4000 rpm) at 4 °C. The solids were washed three times with 0.02 M acetic acid (containing 1 M NaCl and 1 M $CaCl_2$) solution to remove unbound enzyme and residual EDC [13]. The immobilized laccase (P_{MDB}-laccase) was dissolved in pH 4.5 HAc-NaAc buffer (0.1 M) and stored at 4 °C.

The recovery rate of immobilized laccase was calculated using the equation (2):

$$R(\%) = \frac{U_I}{U_F} \times 100 \quad (2)$$

R is the recovery rate of P_{MDB}-laccase (%); U_I is the activity of immobilized laccase (U), U_F is the activity of free laccase (U).

2.5. Infrared spectroscopy of PMDB and the immobilized laccase

FTIR spectroscopy analysis (KBr tablet) was carried out to evaluate the differences between the structure of pure polymer P_{MDB} and the immobilized laccase (P_{MDB}-laccase). Spectra measurements were performed on a Nicolet 320 FTIR (Nicolet Instrument Corporation, Charleston, WV, America), using a Zn-Se crystal and OMNIC software (Thermo Fisher Scientific, Waltham, MA), over a range of 400~4000 cm^{-1}. A uniform resolution (2 cm^{-1}) was maintained in all cases.

3. Results and Discussion

3.1. Optimal conditions for laccase immobilization

The effect of pH on the recovery rate of laccase immobilization was studied within a pH range of 4.5~6.5 (Fig. 1a). When the pH of the immobilization solution is lower than 4.5, the crosslinking agent EDC in the solution will start to precipitate. And when the pH value is higher than 4.5, the recovery rate of immobilized laccase decreases. The optimum pH for laccase immobilization is 4.5.

The effect of P_{MDB} concentration on the recovery rate of the laccase immobilization is shown in Fig. 1b. When the P_{MDB} concentration of the immobilization solution is 0.5~1.5% (w/v), the recovery rate increases as the P_{MDB} concentration increases. It is because when the P_{MDB} concentration is low, the free carboxyl groups on the carriers (P_{MDB}) is not enough to bind with the amino groups of laccase molecules in the solution, resulting in a waste of laccase. With increasing P_{MDB} concentration, much more carboxyl is provided, leading to more laccase immobilized on the carriers, thus recovery rate increases. However, when the P_{MDB} concentration increases above 1.5% (w/v), the amount of crosslinking agent EDC is increased, and a small amount of EDC will precipitate in the solution at pH 4.5, leading to low recovery rate of immobilized laccase. Therefore the optimum P_{MDB} concentration for laccase immobilization is 1.5% (w/v).

Figure 1c shows the effect of the amount of laccase on the recovery rate of laccase immobilization. The recovery rate increases and reaches a maximum when the amount of laccase is 230 mg/(g P_{MDB}). Thereafter the activity decreases as the amount of laccase further increases. This may because as the amount of the laccase increases, more and more laccase is attached to the carrier, causing some activity sites hidden or damaged, leading to the activity of immobilized laccase decreases [14]. The optimum amount for laccase immobilization is 230 mg/(g P_{MDB}).

The effect of the amount of crosslinking agent EDC on the laccase immobilization is shown in Fig. 1d. The recovery rate first slightly increases and reaches a maximum when the amount of EDC is 250 mg/(g P_{MDB}). When the amount of EDC increases further, there is a decrease in the recovery rate. This is because when the EDC concentration is high in the reaction solution, will greatly promote the binding between the free carboxyl groups of P_{MDB} and the amino groups of laccase molecules, resulting in tighter combination between laccase and the carrier (P_{MDB}), causing the denaturation of immobilized laccase.

Fig. 1e shows the effect of immobilization time on the laccase immobilization. The laccase immobilization achieves a maximum recovery rate when the reaction time is 5 h. When the reaction time continues to increase, the recovery rate decreases. It may because long-term shaking in the acidic reaction solution makes the laccase denature, causing recovery rate decreases [14].

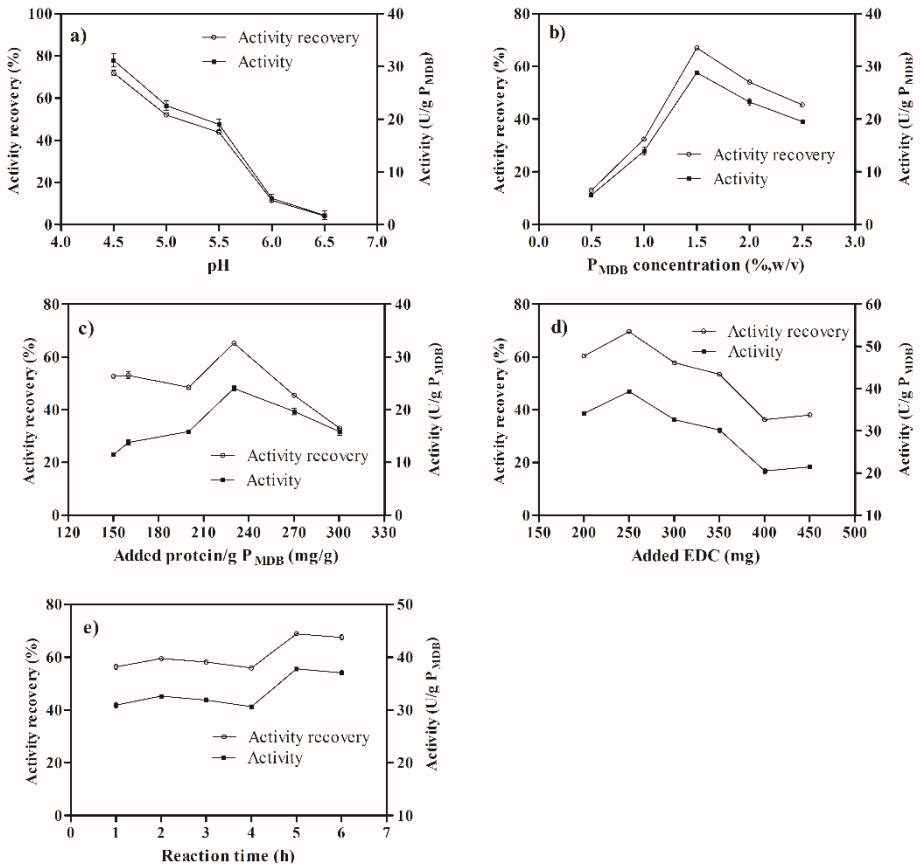

Figure 1. Effect of pH, P_{MDB} concentration, the amount of protein, the amount of crosslinking agent EDC, and reaction time on laccase immobilization. a) The effect of pH on laccase immobilization. Conditions of laccase immobilization: 0.1 M HAc-NaAc buffer with different pH (4.5~6.5), the P_{MDB} concentration is 1.5% (w/v), the reaction time is 5 h (100 rpm), the amount of laccase and EDC are 230 mg/(g P_{MDB}) and 250 mg/(g P_{MDB}) respectively. b) The effect of P_{MDB} concentration (w/v) on laccase immobilization. Conditions of laccase immobilization: pH 4.5 0.1 M HAc-NaAc buffer with different concentration of P_{MDB} (0.5~1.5%, w/v), the reaction time is 5 h (100 rpm), the amount of laccase and EDC are 230 mg/(g P_{MDB}) and 250 mg/(g P_{MDB}) respectively. c) The effect of the amount of laccase on laccase immobilization. Conditions of laccase immobilization: pH 4.5 0.1 M HAc-NaAc buffer, the P_{MDB} concentration is 1.5% (w/v), the reaction time is 5 h (100 rpm), the amount of EDC is 250mg/(g P_{MDB}). The amount of laccase in the reaction solution is different (150~300 mg/(g P_{MDB})). d) The effect of the amount of EDC on laccase immobilization. Conditions of laccase immobilization: pH 4.5 0.1 M HAc-NaAc buffer, the P_{MDB} concentration is 1.5% (w/v), the reaction time is 5 h (100 rpm), the amount of laccase is 230mg/(g P_{MDB}). The amount of EDC in the reaction solution is different (200~450 mg/(g P_{MDB}). e) The effect of the reaction time on laccase immobilization. Conditions of laccase immobilization: pH 4.5 0.1 M HAc-NaAc buffer, the P_{MDB} concentration is 1.5% (w/v), the amount of laccase and EDC are 230mg/(g P_{MDB}) and 250mg/(g P_{MDB}) respectively. The reaction time (100 rpm) is different (1~6 h).

3.2. Effects of pH on solubility of immobilized laccase

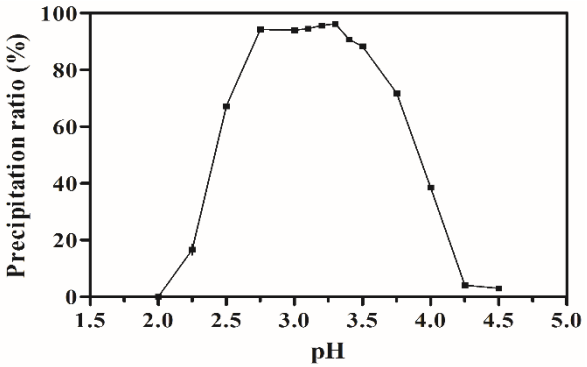

Figure 2. Solubility of P_{MDB}-lacacase as a function of pH.

Fig. 2 shows the effect of the pH on solubility of the immobilized laccase (P_{MDB}-laccase). When the pH of the solution is 2.3~4.2, some of the P_{MDB}-laccase in the solution will precipitate. More than 95% P_{MDB}-laccase precipitates at pH 3.3. And when the pH of the solution is above 4.5 or below 2.0, the precipitation of P_{MDB}-laccase will redissolve.

3.3. FT-IR spectrum analysis of PMDB and immobilized laccase

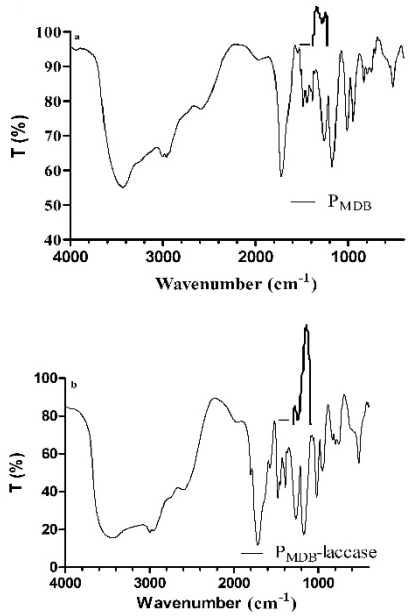

Figure 3. The FT-IR spectra of P_{MDB} and P_{MDB}-lacacase. a) the FT-IR spectrum of P_{MDB}. b) the FT-IR spectrum of P_{MDB}-lacacase.

The FT-IR spectra of P_{MDB} and immobilized laccase (P_{MDB}-lacacase) are shown in Fig. 3. Compare Fig. 3a and Fig. 3b, there is an apparent absorption peak at 1500 to 1600 cm^{-1} in the FT-IR spectrum of P_{MDB}-lacacase (Fig. 3b). Because absorption peaks at 1500 to 1600 cm^{-1} indicate the structure of amide, which belongs to the enzyme. The FT-IR spectra of P_{MDB} and P_{MDB}-lacacase confirm laccase is immobilized to the polymer P_{MDB}, thus the laccase immobilization is successful.

3.4. Properties of immobilized laccase

The effect of pH in reaction solution on the activities of free laccase and immobilized laccase (P_{MDB}-lacacase) were investigated (Fig. 4a). The relative activities of free laccase and P_{MDB}-lacacase under optimal conditions are defined as 100% (Fig. 4a, 4b). The optimum reaction pH for free laccase is pH 3.5, while the optimum pH for P_{MDB}-lacacase is pH 3.8 (Fig. 4a). Similar results were reported when immobilized laccase on surface-modified magnetic silica particles [14]. The shift in the optimum pH value for free laccase and P_{MDB}-laccase (pH 3.5→3.8) may due to the unequal partitioning of H^+ and OH^- concentrations occurring in the microenvironment of the immobilized laccase and bulk solution [15]. Moreover, compare to free laccase, the activity of P_{MDB}-lacacase is higher in a pH range of 3.8~6.0, which indicate P_{MDB}-lacacase has higher stability than free laccase in an acidic pH range.

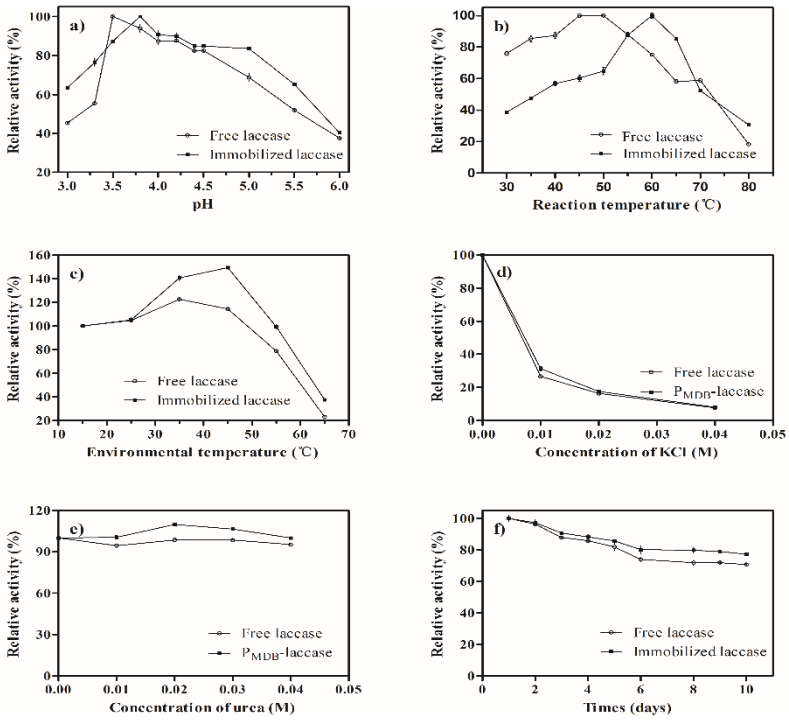

Figure 4. The properties of free and immobilized laccase (P_{MDB}-laccase). a) the effect of pH in reaction solution on the activities of free laccase and P_{MDB}-laccase. The activities of free laccase and P_{MDB}-laccase were measured at different reaction pH (pH 3.0~6.0 0.1 M HAc-NaAc buffer, 25 °C). b) the effect of reaction temperature on the activities of free laccase and P_{MDB}-laccase. The activities of free laccase and P_{MDB}-laccase were measured at different reaction temperature (30~80 °C, pH 4.5 0.1 M HAc-NaAc buffer). c) the effect of storage temperature on the activities of free laccase and P_{MDB}-laccase. The activities of free laccase and P_{MDB}-laccase were measured (pH 4.5 0.1 M HAc-NaAc buffer, 25 °C) after heat treatment at different temperature (15~65 °C) for 1 h. d) the effect of ionic strength (KCl) on the activities of free laccase and P_{MDB}-laccase. The activities of free laccase and P_{MDB}-laccase were measured (pH 4.5 0.1 M HAc-NaAc buffer, 25 °C) with different concentration of KCl (0~0.04 M) in the reaction solution. e) the effect of urea on the activities of free laccase and P_{MDB}-laccase. The activities of free laccase and P_{MDB}-laccase were measured (pH 4.5 0.1 M HAc-NaAc buffer, 25 °C) with different concentration of urea (0~0.04 M) in the reaction solution. f) the effect of storage time (days) on the activities of free laccase and P_{MDB}-laccase. The activities of free laccase and P_{MDB}-laccase were measured (pH 4.5 0.1 M HAc-NaAc buffer, 25 °C) after different storage time (1~10 days, 4 °C).

The effect of reaction temperature on activities of free laccase and P_{MDB}-laccase were investigated, and the results are shown in Fig. 4b. The reaction temperature value has an obvious influence on laccase activity. The optimum temperature for P_{MDB}-laccase is 60 °C, which is higher than that for free laccase. Similar results have been reported. The increase in the optimum reaction temperature may because the multipoint interactions lead to an increase in activation energy of immobilized enzyme [16].

The relative activities of free laccase and P_{MDB}-lacacase at initial are defined as 100% (Fig. 4c, 4d, 4e, 4f). Fig. 4c shows that the relative activities of free laccase and P_{MDB}-laccase are the same below 25 °C, however the relative activities of P_{MDB}-laccase is better than that of the free laccase above 65 °C. The free laccase remains 20.0% of its initial activity with heat treatment at 65 °C for 1 h, while the P_{MDB}-laccase remains 40% of its initial activity. According to the reference, the enhanced thermal stability is attributed to the combination between laccase and P_{MDB} [17].

Fig. 4d shows that the effect of ionic strength (KCl) on the activity of free laccase and P_{MDB}-laccase. The relative activities of free laccase and P_{MDB}-laccase decrease gradually with increasing ionic strength (KCl). And the relative activity of P_{MDB}-laccase is always higher than the free laccase at the same ionic strength.

As shown in Fig. 4e, the relative activity of free laccase decreases gradually with increasing urea concentration, whereas the activity of P_{MDB}-laccase increases firstly and then decreases gradually. Nevertheless, the relative activity of P_{MDB}-laccase is always above 100% when the urea concentration is below 0.4 mol/L. As we all know, urea is an extensively used denaturing agent in protein folding field. Urea can influence the three dimensional structure of proteins by non-covalent forces like hydrogen bonds and hydrophobic effects, thus denaturing enzyme at high concentrations [18, 19]. The results of the effect of urea on the activities of free laccase and P_{MDB}-laccase indicate that the hydrogen bonds and hydrophobic effects of laccase are influenced during immobilization, thus the stability of P_{MDB}-laccase is enhanced when adding denaturing agent urea at low concentration.

According to Fig. 4f, the times required for a 30% reduction in activity are 6 days and more than 10 days for free laccase and P_{MDB}-laccase, respectively. It is obvious that the stability of laccase is enhanced by immobilization, and is consistent with the thermal stability data.

3.5. Kinetics of enzyme reactions

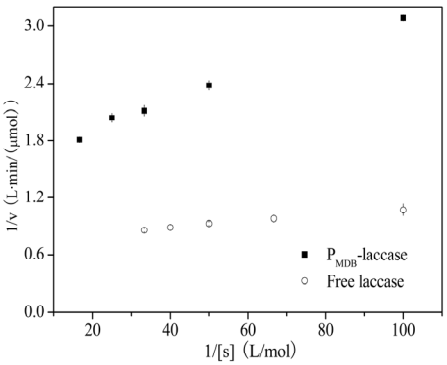

Figure 5. The Lineweaver–Burk plots of free laccase and P_{MDB}-laccase. Reactions were carried out in HAc-NaAc buffer (0.1 M, pH 4.5) with different concentration of the substrate DMP (10~60 mM) at 25 °C.

Fig. 5 is the Lineweaver–Burk plots of free laccase and P_{MDB}-laccase. The K_m of laccase changes from 4.07 to 9.29 mmol/L after immobilization. The Vm decreases from 82.64 μmol/(L·min) (for free laccase) to 39.22 μmol/(L·min) (for P_{MDB}-laccase). The increase in K_m means that the P_{MDB}-laccase has a lower affinity towards the substrate. This result is accorded with references [20]. The increase in the Km is due to the structural change of the enzyme during immobilization or the lower accessibility of the substrate to the active sites of the immobilized enzyme [21].

3.6. Reuseability of PMDB-laccase

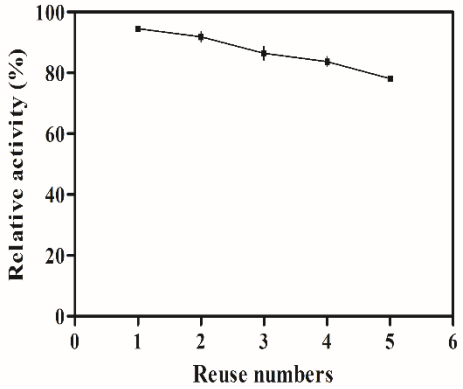

Figure 6. The Reusability of PMDB-laccase for hydrolysis of DMP. Reactions were carried out in HAc-NaAc buffer (0.1 M, pH 4.5) at 25 °C. Relative activities were calculated by defining the initial activity of PMDB-laccase as 100%.

P_{MDB}-laccase remains 78.13% of the initial activity after 5 batch reactions (Fig. 6). The decrease in activity of P_{MDB}-laccase may due to enzyme inactivation in reuse treatment [10]. Compare to free laccase, the immobilized laccase (P_{MDB}-laccase) remains high relative activity and can easily reuse.

4. Conclusion

In this study, a pH-response copolymer P_{MDB} was used as carriers to immobilize laccase, and the properties of immobilized laccase (P_{MDB}-lacacase) were studied. The recovery rate of the laccase immobilization is 71.90% and the PI of the immobilized laccase (P_{MDB}-lacacase) is 3.3. Over 95% P_{MDB}-lacacase is precipitated when the pH of the solution is 3.3. P_{MDB}-lacacase remains 78.13% of the initial activity after 5 batches reactions. Compare to the free laccase, the stabilities such as thermal and storage stabilities of P_{MDB}-lacacase are enhanced. Especially, the relative activity of immobilized laccase is nearly 110% when the concentration of urea in the solution is 0.02 M. Overall the laccase immobilization on the pH-response copolymer P_{MDB} is successful, thus indicate P_{MDB} is a suitable soluble-insoluble carrier for enzyme immobilization and has the potential to employ in enzyme immobilization widely. The optimal condition of laccase immobilization in this paper can provide valuable reference for enzyme immobilization.

5. Declaration of Competing Interest

There are no conflicts to declare.

6. Data Availability

Data will be made available on request.

7. Author Statement

Tingting Yu: Formal analysis, Methodology, Writing - original draft, Writing - review & editing. Xuejun Cao: Supervision, Writing - review & editing.

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