Covalent Immobilization of Lipase on Novel Nanofibrous Membrane for Catalysis of the Organic Synthesis

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ABSTRACT

Enzymes are green biocatalysts which have been widely used in many fields. Immobilization enzymes on nanofibrous membrane possessed easy recycling and high stability which would broaden their applications. Covalent immobilization of lipase could endow them higher stability than other protocols. In this study, a novel nanofibrous membrane containing epoxy groups and hydrophilic polyethylene oxide branch was used as a support for lipase immobilization. The immobilized lipase was used as the biocatalyst to catalyse Rap. stroermer reaction. The results showed that it obtained the high product yield of 88% when the volume ratio of methanol and water was 4:1, the dosage of immobilized lipase was 40~50 mg, the reaction temperature and time were 30~35 °C and 10 h.

KEY WORDS : *Enzyme immobilization, Lipase, Electrospinning, Biocatalyst, Organic synthesis*

1. INTRODUCTION

Most of the organic reactions must be conducted by the catalysis action of catalysts. The commonly used catalysts for these organic reactions are noble metals, transition metals and organic catalysts. The synthesis reaction of 2-bromoacetophenone and salicylaldehyde as one of them should be catalyzed by the metal catalysts [1]. However, most of catalysis reactions by metals exist many disadvantages, such as the harsh reaction conditions, hard to reuse, easy to case the environmental pollution, high price and low selectivity [2-4]. Enzymes as green and

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sustainable catalysts possess biocompatibility and biodegradability which could catalyze the organic reactions under mild conditions. Enzymatic processes with high reaction rates and selectivities are more environmentally friendly, more cost-effective and more sustainable than the conventional synthesis reactions. Consequently, enzymes as the biocatalysts have been widely studied and used in the past two decades due to the growing demand of green and sustainable production [5-9].

Lipases are one of the most important enzymes possessing high specificity and efficient which are widely used in hydrolysis, esterification, transesterification and other organic synthesis reactions [10-12]. However, lipases are water soluble and poorly stable in organic solvents which result in the costs of enzymes, nonreusable and enzyme activity loss. These drawbacks could be overcome by immobilization on solid supports or membranes [13-15]. Several protocols have been used to immobilize lipase, the physical methods and chemical binding on the hydrophobic supports have been widely studied [16-20]. Whereas chemical binding methods are more stable than physical methods due to the stable covalent bond between the supports and enzymes [21]. Covalent immobilization of enzymes on supports by the reaction of the side chain amino acids of enzyme protein and the reactive functional groups on the supports including nitrile, amino, carboxyl and epoxy groups which have been studied extensively [22-26]. The supports containing reactive epoxy groups could offer multipoint covalent attachment with enzymes, thus to reduce the enzyme mobility and improve the stability. However, the hydrophobic and rigid surface of these supports would result in the enzyme activity loss. Improving the hydrophilicity of the supports is an effect way to improve the enzyme activity[27-28]. A novel poly (glycidyl methacrylate-co-methylacrylate)-g-polyethylene oxide (P(GMA-co-MA)-g-PEO) nanofibrous membranes (NFMs) containing reactive epoxy groups and hydrophilic polyethylene oxide branch chain was prepared by electrospinning which was used to immobilize lipase in our previous research [29]. The immobilized lipase on this novel NFMS possessed high enzyme loading, activity and stability.

In this study, the covalently immobilized lipase on the novel NFMs was used as the biocatalyst to catalytze the Rap-Stoermer reaction which the reaction mechanism is to form the -C=C- bond through a series reactions of nuclear substitution, nucleophilic addition and elimination between the 2-bromoacetophenone and salicylaldehyde. The volume ratio of methanol and water, the dosage of immobilized lipase, the reaction temperature and time effect on the product yield were investigated.

2. EXPERIMENTAL

2.1 Materials

Polyethylene glycol methacrylate (PEGMEMA, M_n=950), glycidyl methacrylate (GMA), methyl acrylate (MA), monopotassium phosphate (KH_2PO_4), monosodium phosphate(Na_2HPO_4), silica gel (200 mesh), petroleum ether (PE) and ethyl acetate (EA) were purchased from Sinopharm Chemical Reagent Co.,Ltd (Shanghai, China). 2-Bromoacetophenone and salicylaldehyde were purchased from Shanghai Aladdin biochemical Polytron Technologies Inc (Shanghai, China). Dimethylformaide (DMF) and n-hexane were purchased from Wuxi City Yasheng Chemical Co.,Ltd (Jiangsu, China). Candida antarctica lipase B (CALB) (1U/mg) was of biological

grade and purchased from Hangzhou Novocata Biotechnology Co., Ltd (Zhejiang, China).

2.2 Preparation of enzyme biocatalyst

The immobilized lipase was prepared according to our previous research[29], P(GMA-co-MA)-g-PEO terpolymer was synthesized by PEGMEMA, GMA and MA with the mass ratio of 12.5%, 12.5% and 75%. Then the P(GMA-co-MA)-g-PEO nanofibrous membrane was prepared by electrospinning. The CALB was covalently immobilized on the nanofibrous membrane. The chemical structure of P(GMA-co-MA)-g-PEO terpolymer was as shown in Fig.1, and the properties of the immobilized lipase has also been studied in our previously research.

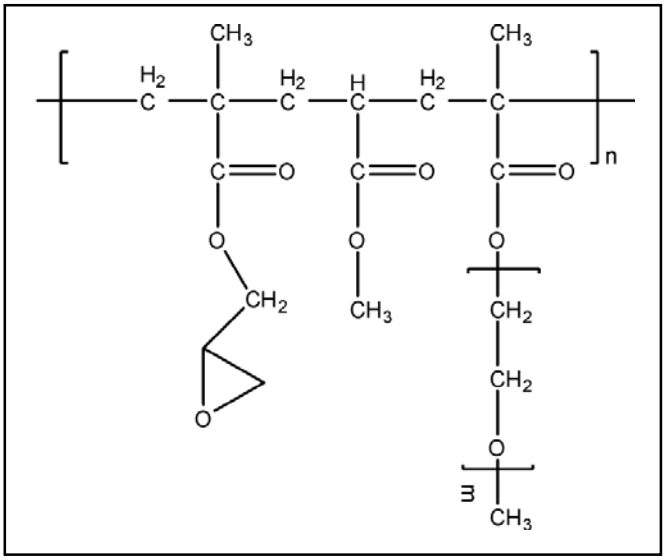

Fig. 1. Chemical structure of P(GMA-co-MA)-g-PEO

2.3 Catalytic synthesis reaction of 2-bromoacetophenone and salicylaldehyde

Salicylaldehyde 1.22 g and 2-bromoacetophenone 1.99 g were added into the three-necked round bottom flask containing 50 mL methanol and water mixed solvent. Then a certain amount of immobilized lipase on P(GMA-co-MA)-g-PEO nanofibrous membrane was added into the flask. The reaction mixture was heated to a certain temperature for synthesis reaction under the magnetic stirring. After the reaction, the immobilized lipase was removed by filtration. Then the reaction mixture was obtained by extraction of water which was separated by silica gel using PE/EA (v/v, 20:1) as the mobile phase. The collected effluent was evaporated to remove the solvent and the reaction product of *benzofuran-2-yl(phenyl)methanone* was obtained. The synthesis route was as shown in Fig. 2.

The product yield was calculated according to Equation 1.

$$W = \frac{m_1}{m_0} \times 100\% \quad (1)$$

Where W is the product yield (%), m_1 is the mass of the product (g), m_0 is the total mass of reactants (g).

2.4 Influence of the synthesis reaction product yield

The influence of product yield by different content of immobilized lipase from 0 mg to 50 mg was investigated

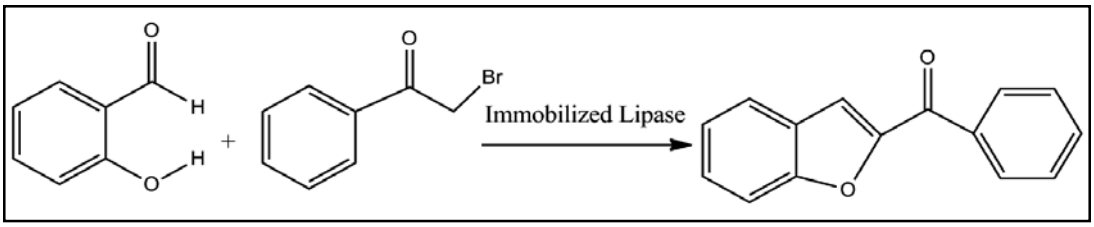

Fig. 2. Synthesis route of *benzofuran-2-yl(phenyl)methanone* by immobilized enzyme

when the reaction temperature was 35 °C, the volume ratio of methanol and water was 4:1 and the reaction time was 10 h.

The influence of product yield by different volume ratio of methanol and water (5:0, 4:1, 3:2, 2:3, 1:4, 0:5) was investigated when the reaction temperature was 35 °C, the content of immobilized lipase was 50 mg and the reaction time was 10 h.

The influence of product yield by different reaction temperatures from 20 to 50 °C was investigated when the volume ratio of methanol and water was 4:1, the content of immobilized lipase was 50 mg and the reaction time was 10 h.

The influence of product yield by different reaction time (2 h, 4 h, 6 h, 8 h, 10 h) was investigated when the volume ratio of methanol and water was 4:1, the content of immobilized lipase was 50 mg and the reaction temperature was 35 °C.

2.5 Characterization

The chemical structure of the reaction product was characterized by nuclear magnetic resonance (NMR). ^{1}H-NMR and ^{13}C-NMR were conducted on Bruker Avance spectrometer (400 MHz) at room temperature using $CDCl_3$ as the solvent.

3. RESULTS AND DISCUSSION

3.1 Characterization of synthesis product

The specifically reaction process of Rap-Stoermer reaction which was catalyzed by lipase as show in Fig. 3.

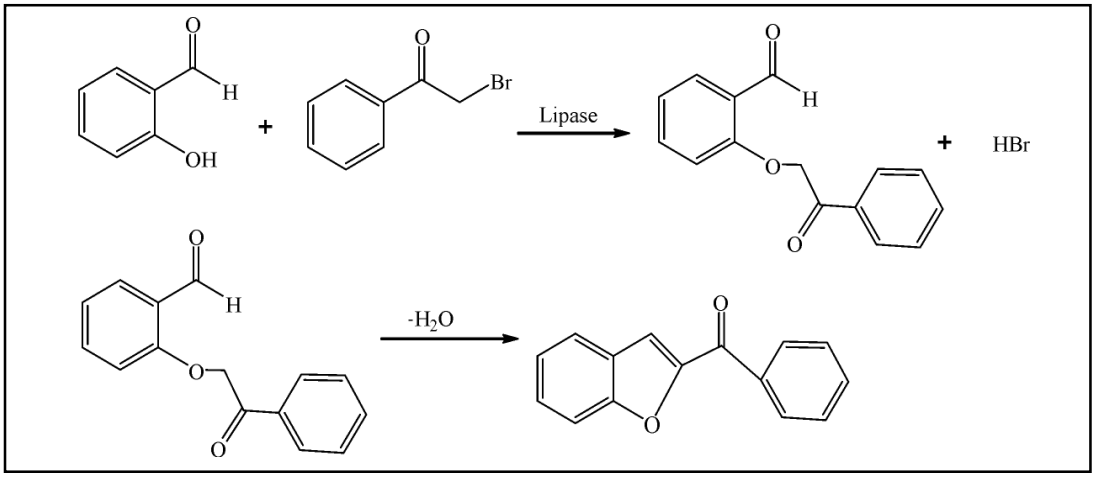

Fig. 3. The specifically reaction process of Rap-Stoermer reaction

The product of the synthesis reaction was separated and purified according to the experimental section. The structure of the product was characterized by nuclear magnetic resonance (^{13}C-NMR and ^{1}H-NMR). The result of the ^{13}C-NMR was as shown in Fig. 4A: 192.91 ppm (1C, C8), 159.63 ppm (1C, C13), 134.02 ppm (1C, C7), 132.25 ppm (1C, C9), 130.81 ppm (1C, C12), 129.31, 127.47 ppm (2C, C10), 125.68, 124.23 ppm (2C, C11), 123.10 ppm (1C, C5), 122.73 ppm (1C, C2), 121.29 ppm (1C, C3), 117.28 ppm (1C, C4), 112.89 ppm (1C, C6), 110.70 ppm (1C, C1).

The ^{1}H-NMR of the product was as shown in Fig.4B. The peaks at 8.06~8.13 ppm are assigned to 2 H atoms of C_6H_5-, the peak at 7.72 ppm is attributed to 1 H atom of -C_6H_4- , the peak 7.67 ppm is attributed to another 1 H atom of C_6H_5-, the peaks at 7.55-7.57 are assigned to 2 H atom of C_6H_5-, the peak at 7.51 ppm is assigned to 1 H atom of -C_6H_4-, the peak at 7.26 ppm belongs to 1 H atom of -CH=C-, the peak at 6.89-6.99 ppm belongs to 2 H atoms of -C_6H_4-.

The result of ^{13}C-NMR and ^{1}H-NMR demonstrated that the synthesis product was the aim product.

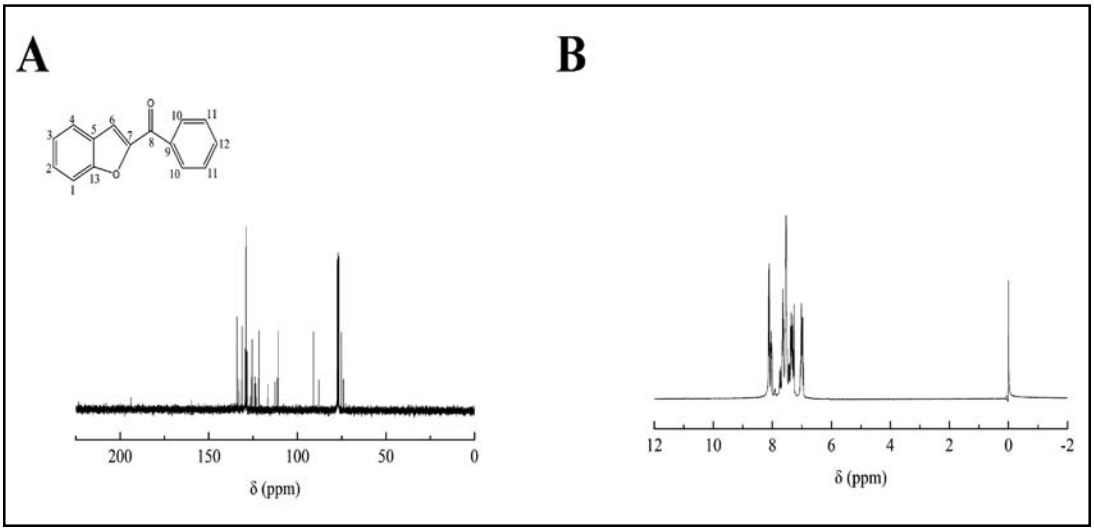

Fig. 4. (A)^{13}C-NMR and (B) ^{1}H-NMR spectra of organic synthesis products spectra of organic synthesis products

3.2 Effect of the volume ratio of methanol and water on the product yield

The stability of the enzyme conformation was greatly influenced by the non-covalent forces associating with water, thus to influence the catalysis activity. The water molecules exist in two forms on the supports, the one is existence between the pores of the supports, and the other is strong adsorption on the surface of the supports. Both of the two forms of water

molecules exist in the immobilized lipase which prepared in this study. The enzyme structure and function are greatly affect on the bound water of the enzyme molecule. The immobilized lipase possessed high activity due to the existence of the bound water. Therefore, the water ratio in the solvent may influence the immobilized lipase activity, and thus the catalysis reaction would be influenced. The effect of the volume ratio of methanol and water on the product yield was investigated which was as shown in Fig. 5. As can be seen from Fig. 5, the product yield reached the maximum when the volume ratio of methanol and water was 4:1. And then it would decrease when continuing increase the ratio. The reason about this may be due to that a certain amount water was beneficial for keeping the stability of enzyme conformation, while it would influence the enzyme active central structure which resulted in the decreasing of enzyme activity when the content of water was too high.

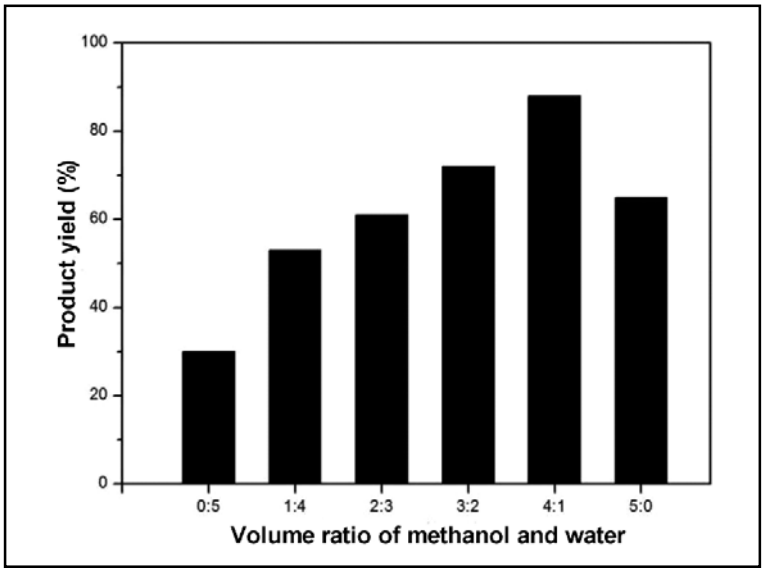

Fig. 5. Effect of volume ratio of methanol and water on product yield

3.3 Effect of immobilized lipase dosage on the product yield

The product yield of the synthesis reaction of 2-bromoacetophenone and salicylaldehyde which was influenced by the content of the immobilized lipase was investigated. As shows in Fig. 6, the reaction must be performed under the catalytic condition of the immobilized lipase. The product yield was increased with the increasing dosage of immobilized lipase. When the content of immobilized lipase was higher than 40 mg, continuing increasing the amount of immobilized lipase the product yield was increased slightly. Therefore, the optimal dosage content of immobilized lipase was 40~50 mg.

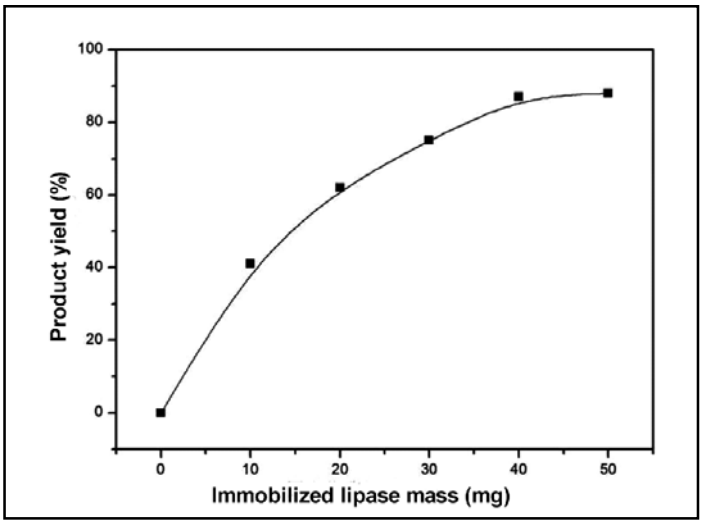

Fig. 6. Effect of the amount of immobilized lipase on product yield

3.4 Effect of the reaction temperature and time on the product yield

The characteristic of the catalysis organic synthesis reaction by enzyme was the mild reaction condition such as low temperature. Effect of reaction temperature on the product yield was investigated as shown in Fig. 7A. The product yield was greatly increased when the temperature was higher than 25 °C. It reached the maximum when the temperature was about 30~35 °C, and after then the product yield decreased with the increasing of temperature. The results can be explained due to the enzyme activity of the immobilized lipase would gradually loss with the increasing temperature. Thus the optimal temperature of this catalysis reaction was 30~35 °C.

The effect of reaction time on the product yield was as shown in Fig. 7B. The product yield was increased linearly with the reaction time which revealed the mild reaction condition of the enzyme catalysis reaction in a certain extent. The product yield changed slightly when the reaction time was higher than 10 h. Thus the optimal reaction time of this catalysis synthesis reaction was 10 h. The product yield of this reaction achieved 88% under the above optimal conditions. The 50mg free lipase was used to catalyzed this reaction under the optimum reaction condition, and the product yield was 88%. Rao et al [30] reported the product yield of this reaction through the microwave-mediated solvent free method was 93%. Therefore, the immobilized lipase could achieve almost the same product yield with free lipase and other method.

4. CONCLUSION

The covalently immobilized lipase on novel nanofibrous membrane containing epoxy groups and hydrophilic polyethylene oxide

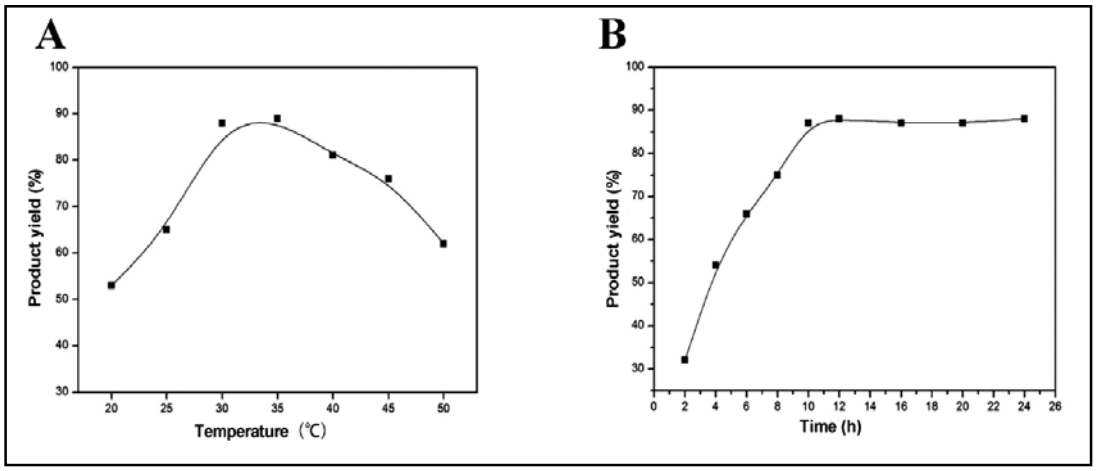

Fig. 7. Effect of (A)reaction temperature and (B) reaction time on product yield

branch chain was used as a biocatalyst for catalysis the organic synthesis, Rap-stoermer reaction. The result of ^{13}C-NMR and ^{1}H-NMR demonstrated that the synthesis product was the aim product. This reaction achieved the high product yield of 88% when the volume ratio of methanol and water was 4:1, the dosage of immobilized lipase was 40~50 mg, the reaction temperature and time were 30~35 °C and 10 h. The result revealed that the immobilized lipase on the P(GMA-co-MA)-g-PEO NFM would achieve a good perspective of application in the organic synthesis reactions.

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