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Immobilization of *Pseudomonas fluorescens* Lipase on Hollow Poly(o-phenylenediamine) Microspheres and Its Application in the Preparation of Citronellyl Acetate

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Abstract: In order to address the challenges associated with free lipase in organic solvents, including aggregation, poor stability, and low catalytic efficiency, this study developed two types of poly(o-phenylenediamine) microspheres (solid and hollow) as supports for immobilizing lipase. The immobilization process utilized an adsorption method, with the poly(o-phenylenediamine) hollow microspheres being identified as the optimal support in a 2:5 enzyme-to-support ratio. On this basis, the lipase was immobilized by the covalent binding method. The immobilization conditions consisted of treating the support with 2% glutaraldehyde and immobilization at 40 °C for 2 h in pH 7.0 buffer. The specific activity of the immobilized enzyme was 5.3 times higher than that of the free enzyme. Covalent-binding immobilized lipase was also used for the preparation of citronellyl acetate by transesterification reaction, and, in optimized reaction conditions where the amount of immobilized enzyme was 0.1 g/mL, the reaction temperature was 50 °C and the shaking speed was 200 r/min during the reaction. Under these conditions, the citronellyl acetate yields can exceed 99% after 2 h. Furthermore, the stability of the immobilized lipase was investigated, and the residual activity of the immobilized enzyme was 95% after seven repetitions, while that of the free enzyme was only 70%. After 56 days of storage at room temperature, the immobilized enzyme retained 60% of its original viability, while the free enzyme retained only 31%.

Keywords: poly(o-phenylenediamine); lipase; immobilization; covalent binding method; citronellyl acetate; transesterification reaction

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1. Introduction

Lipase is the most widely used enzyme in industrial enzyme catalysis, which has shown great application potential in organic synthesis, pharmaceutical, food, biosurfactant, and other fields, and has become a research hotspot for industrial enzymes [1,2]. Among lipases, *Pseudomonas fluorescens* lipase (PFL) is a commercial enzyme that is easily produced via fermentation, which has the typical structure of lipase and plays an important role in hydrolysis [3], esterification [4], transesterification [5], the resolution of racemic mixtures [6], and other applications such as the enzymatic biodegradation of many polymers [7]. Its active site is formed by serine, aspartate, and histidine amino acids, over which there is a lid that isolates the active site from the reaction medium, rendering two main conformations: open and closed forms. If a hydrophobic interface is presented, the lid is opened, resulting in the open form and, therefore, high activity levels, since the active center of the lipase is fully exposed to catalyze the substrates. PFL plays an important role in biotechnological and industrial processes. Since the free enzyme is difficult to recycle and reuse, and given its stability is poor, its large-scale application is limited [8]. Therefore, the application of lipase depends on immobilization techniques to some extent. Immobilization can confine the enzyme to a specific spatial region in order to maintain its catalytic activity and can be reused many times [9]. The selection of support materials is an important factor in

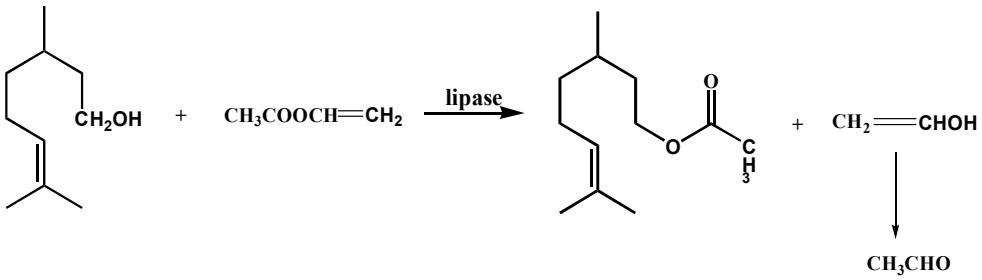
determining whether the enzyme can be successfully immobilized. It also affects the activity of immobilized enzymes. At present, inorganic materials and organic polymers are commonly used as support materials for immobilized enzymes [10–12]. Among them, organic polymers are rich in functional groups that can react with enzymes and bind them to supports.

Poly(o-phenylenediamine) (PoPD) is a polyaniline conductive polymer material [13]. Due to its advantages, such as the easy regulation of the doping mechanism and its low cost, it has become one of the most rapidly progressing micro-nano-polymeric materials in research and is expected to make a breakthrough in practical application fields; in addition, poly(o-phenylenediamine) also has very special advantages, such as easily available raw materials, simple synthesis, good environmental stability, and chemical stability [14–16]. As a conductive polymer material, poly(o-phenylenediamine) has been widely used in biosensing research. Horseradish peroxidase was immobilized on the surface of poly(o-phenylenediamine) nanoprobe for the detection of thrombin [17]. Poly(o-phenylenediamine)/carbon-coated nickel nanoparticles, modified with BSA, were prepared as glassy carbon electrodes, which can be used to detect and monitor bovine serum albumin and papaverine [18]. A glassy carbon electrode with immobilized horseradish peroxidase, anti-carcinoembryonic antigen antibodies, and bovine serum albumin was created with label-free immunosensors for the rapid detection of the carcinoembryonic antigen (CEA) using chronoamperometry, which was electropolymerized with poly(o-phenylenediamine) and modified by PANC metal composite [19]. Bu's group introduced a self-assembled bienzymatic electrochemical biosensor, in which acetylcholinesterase (AChE) and choline oxidase were immobilized on a poly(o-phenylenediamine)-modified Au electrode [20]. This as-presented biosensor was competent in the in situ detection of nerve agents (NAs) and in the recording of the inhibition and reactivation processes of AChE. In addition to the above studies, biomolecules such as glucose oxidase [21], glutamate oxidase [22], ascorbate oxidase [22], and DNA [23] have also been immobilized on poly(o-phenylenediamine) and its derivative supports for biosensor studies, all with good experimental results. These studies show that poly(o-phenylenediamine) has good biocompatibility with biological macromolecules and can be used for enzyme immobilization. However, there is no research report on the immobilization of industrial enzymes, especially lipases, with the help of poly(o-phenylenediamine), let alone the application of poly(o-phenylenediamine)-immobilized industrial enzymes.

Citronellyl acetate is a colorless liquid with a lemon odor and is an important compound in flavor and fragrance [24]. Therefore, citronellyl acetate is widely used in the food, beverage, cosmetics, perfume, and pharmaceutical industries and has great application value [25]. The natural raw materials of citronellyl acetate are scarce, and the natural extraction method is not suitable for large-scale industrial production [26]. The traditional chemical synthesis method involves toxic chemicals and is prone to other side reactions, which limits its application in the food and beverage industry [27]. Lipase-catalyzed transesterification for citronellyl acetate is a typical green and environmentally friendly process, with the advantages of mild reaction conditions, high catalytic efficiency, and strong catalytic specificity. For its production, inexpensive raceme straw was processed into powder and filaments, on which *Pseudomonas fluorescens* lipase was immobilized by physical adsorption to synthesize citronellyl acetate via transesterification of citronellol and vinyl acetate [28]. The results showed that 99.8% conversion could be achieved after 12 h at 37 °C and 160 rpm. Yadav et al. studied the transesterification of citronellol and vinyl acetate to citronellol acetate catalyzed by Novozym 435 lipase in toluene under conventional heating and microwave-assisted conditions [29]. The conversion rates can reach 90% and 93%, respectivley. Macedo et al. studied the transesterification of citronellol with ethyl acetate and butyl acetate to citronellol acetate catalyzed by *Rhizopus* sp. lipase in a solvent-free system, and the conversions were 58% and 48%, respectively [30]. In the above studies, the use of free enzymes in the reaction has problems, including difficult recovery

and poor stability. On the other hand, the reaction time is too long when the immobilized enzyme is used for the reaction.

In this paper, *Pseudomonas fluorescens* lipase was used as the research object, and poly(o-phenylenediamine) hollow microspheres were used as the support to immobilize the lipase by the covalent binding method in order to improve the activity and stability of the lipase. Moreover, immobilized lipase was applied to the catalytic synthesis of citronellyl acetate by the transesterification of citronellol and vinyl acetate in a water-free organic system of citronellol dissolved in vinyl acetate (Scheme 1), which provided a new reference route for the industrial synthesis of citronellyl acetate.



Scheme 1. Lipase-catalyzed transesterification synthesis of citronellyl acetate.

2. Materials and Methods

2.1. Chemicals

The lipase powder used in the experiments was derived from *Pseudomonas fluorescens* and was purchased from Wako Pure Chemical Industries, Ltd in Osaka, Japan. Bovine serum albumin, citronellol, p-nitrophenol (p-NP), and p-nitrophenol palmitate (p-NPP) were obtained from Sigma-Aldrich. Standard citronellyl acetate was supplied by Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). The other chemicals and solvents are commercially available analytical-grade reagents. All chemicals and enzymes used were not further treated.

2.2. Preparation of Support

The droplet template method [31] was used to prepare the poly(o-phenylenediamine) supports. Both 0.1 g and 0.3 g of o-phenylenediamine monomer were dissolved in 20 g of deionized water, respectively, and 20% ammonium persulfate (APS) of equal molar mass was added and left to react at 0–5 °C for 12 h. After the above reaction, the solutions were filtered, washed, and dried, respectively, to obtain the dried supports for backup.

2.3. Immobilization of Lipase by Adsorption

The adsorption immobilization of lipase was performed in a closed conical flask. Unless otherwise specified, lipase immobilization by adsorption was performed as follows: 0.02 g of lipase was dissolved in 15 mL of pH 7.0, 50 mM phosphate buffer; 0.5 g of poly(o-phenylenediamine) support was added to start the adsorption reaction; and the shaker parameters were set at 40 °C and 150 rpm. After 4 h of adsorption, the mixture was filtered, and the solid was washed several times with the same buffer until the protein content in the solution was zero, and then placed in a vacuum drying oven for 24 h at room temperature.

2.4. Immobilization of Lipase by Covalent Binding

The lipase immobilization using the covalent binding method was performed in a closed conical flask. If not otherwise specified, the covalent binding immobilization of lipase was carried out as follows: 0.5 g of poly(o-phenylenediamine) support was added into 20 mL of phosphate buffer, with pH 7.0 and 50 mM; 2% glutaraldehyde was added; the solution was reacted at 40 °C for 2 h; and then filtered and washed after the reaction. Then, we dissolved 0.02 g of free lipase in pH 7.0, 50 mM phosphate buffer; took 15 mL in the glutaraldehyde-modified support and reacted it at 40 °C, 150 rpm for 4 h; filtered until

there was no protein in the solution; and, finally, put it in a vacuum drying oven to dry at room temperature for 24 h.

2.5. Determination of Enzyme Activity

The protein content was determined using the Coomassie brilliant blue method [32], and bovine serum albumin (BSA) was used as the standard protein. Using p-NPP as the substrate, the hydrolysis activity of the enzyme was determined by colorimetry [33]. A total of 0.0378 g of p-NPP was dissolved in 5 mL of acetone and 1 mL of Triton-100 was added and made up to 25 mL with phosphate buffer (pH = 7.5, 0.2 M). A total of 1 mL of the p-NPP solution and 14 mL of the phosphate buffer solution were mixed in a triangular flask and pre-heated for 5 min at 40 °C. A total of 20 mg of immobilized PFL, or 3 mg of free PFL, was added to the reaction solution, and the reaction was carried out at 40 °C and 200 r/min for 5 min. The reaction was terminated by addition of 1 M NaOH (5 mL), followed by centrifugation at 10,000 rpm for 10 min. The absorbance was recorded at 410 nm in a UV-spectrophotometer and compared with the standard curve of p-NP (the end product of enzymatic reaction). An enzyme activity unit is defined as the amount of p-NP produced per milligram of enzyme per minute. Specific activity is defined as the enzyme activity per milligram of protein.

2.6. Synthesis of Citronellyl Acetate in the Reaction of Transesterification Using Immobilized PFL

Unless otherwise specified, the reaction conditions were as follows: the reaction was carried out in a closed stopper bottle, the citronellol concentration was 300 mmol/L, and 3 mL of vinyl acetate was added as the reactant and solvent. The reaction solution described above was preheated in a constant temperature shaking table for 10 min and then 0.3 g of immobilized lipase was added, and the reaction began with the addition of the lipase. The parameters of the shaking table were set as follows: the reaction temperature was 50 °C and the shaking table speed was 200 rpm. The samples were sampled with a microinjector at regular intervals, and the amount of citronellyl acetate was analyzed by gas chromatography.

2.7. Analysis Method of Citronellyl Acetate

The analyses of citronellyl acetate were performed using a GC apparatus that was equipped with a flame ionization detector and an SGE AC10 stainless steel column. The area of the internal standard method was adopted, and hexadecane was used as the standard. The following specific test conditions were used: the N_2 flow rate was 44 mL/min; the H_2 flow rate was 40 mL/min; the air flow rate was 400 mL/min; the tail blowing rate was 25 mL/min; the split ratio was 10:1; the column temperature was kept at 170 °C; the injector temperature was 250 °C; and the detector temperature was 250 °C.

The initial rate of the reaction is defined as the amount of citronellyl acetate generated per unit of time and per unit volume. Enzyme transesterification activity is defined as the amount of citronellyl acetate that is generated by the enzyme per unit of time and per unit amount of enzyme. The yield of citronellyl acetate was calculated as the percentage of the actual amount of citronellyl acetate produced compared to the theoretical amount.

2.8. Determination of Enzyme Reaction Stability

The transesterification of citronellol was used as a model reaction to investigate the reaction stability of the immobilized enzyme. First, 100 mmol/L of 95% citronellol was weighed in a 50 mL volumetric flask, then, the volume was fixed with vinyl acetate, and 3 mL was taken in a stopper flask. The free lipase powder (insoluble in vinyl acetate), or the immobilized lipase, was added to the reaction at 50 °C at 200 rpm, the reaction time was 3 h, and the initial rate was measured by taking the solution 5 min after the reaction. After each reaction, the reaction solution was filtered out and the immobilized enzyme was collected and reused.

2.9. Determination of Enzyme Storage Stability

The free lipase and the immobilized enzyme were kept at room temperature, and a certain amount of enzyme was taken every week to study the stability using the citronellol transesterification reaction as a model reaction. The reaction conditions were the same as those in Section 2.8.

3. Results and Discussion

3.1. Characterization of Support Morphology

The morphology and size of various poly(o-phenylenediamine) supports can be observed by electron microscopy. Figure 1A–E are SEM and transmission electron microscopy images of the poly(o-phenylenediamine) supports that were prepared with 0.3 g of o-phenylenediamine. The spherical morphology of poly(o-phenylenediamine) is visible in the figures, with an open cavity on the surface of each spherical shell and its hollow structure. The diameter of the hollow microspheres ranges from 100 to 800 nm, with an average diameter of about 400 nm and an open pore size of about 150 nm. It can be seen from the electron microscope images (Figure 1C,D,F) that, when the dosage of o-phenylenediamine is 0.1 g, the obtained poly(o-phenylenediamine) support is a solid sphere with an average diameter of about 400 nm. Poly(o-phenylenediamine) supports with different morphologies can be obtained by polymerization with different doses of o-phenylenediamine, which is consistent with the literature [31].

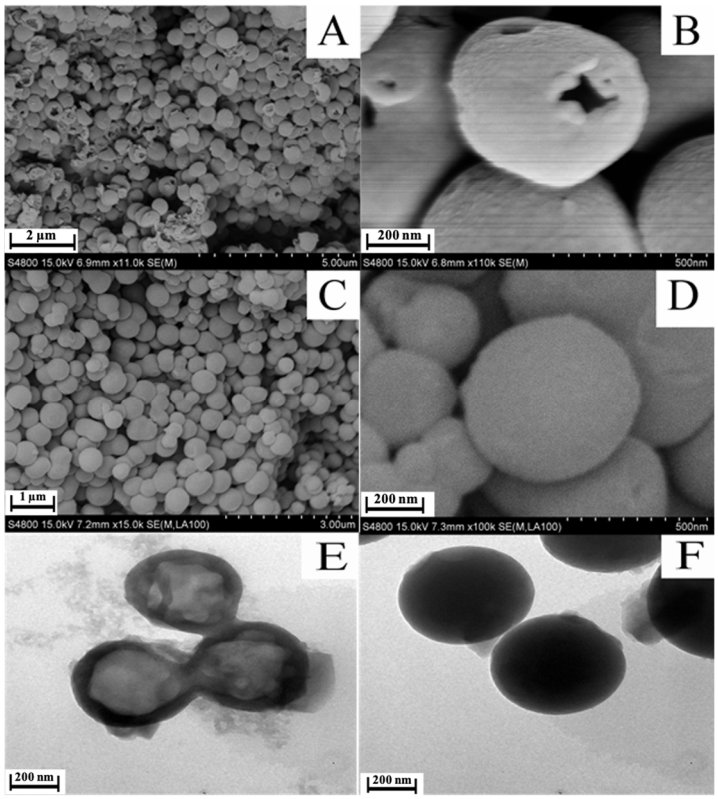


Figure 1. Scanning electron microscope and transmission electron microscope images of poly(o-phenylene diamine) supports: (**A**,**B**) are scanning electron micrographs of a hollow support; (**C**,**D**) are scanning electron micrographs of a solid support; (**E**) is transmission electron micrographs of a hollow support; and (**F**) is transmission electron micrographs of a solid support.

3.2. Effect of Different Supports on Lipase Immobilization by Adsorption

The effect of lipase immobilization by the adsorption of two supports was investigated and the results are shown in Table 1. As can be seen from Table 1, the amount of protein immobilized on the hollow support is greater than that on the solid support. After

immobilization, the specific activity of the immobilized lipase is much higher than that of the free enzyme. Compared to free lipase, the specific activity of the immobilized solid support lipase is 2.2 times, while that of the immobilized hollow support lipase is 4.1 times. This is because the hollow support has a hollow structure and an opening on the surface, and the hollow support has a larger surface area so that the protein molecules can enter the support and be fixed. This shows that the structural characteristics of the support itself have an important influence on the immobilization results. Therefore, the hollow poly(o-phenylenediamine) support was selected in the follow-up experiment.

Table 1. Effect of different supports on immobilized lipases.

Support	Bound Protein (μg/g Support)	Activity (μmol/(min·mg Lipase))	Specific Activity (μmol/(min·mg Protein))
Solid support	313.59 ± 6.07	$1.06 \pm 0.08 \times 10^{-3}$	3.39 ± 0.013
Hollow support	387.24 ± 3.94	$2.48 \pm 0.02 \times 10^{-3}$	6.41 ± 0.005
Free lipase	—	$5.40 \pm 0.02 \times 10^{-3}$	1.56 ± 0.008

3.3. Effect of Different Enzyme/Support Ratios on Adsorption Immobilization

The effects of different enzyme/support ratios (r) on immobilization were investigated, and the results are shown in Figure 2. With the increase in the ratio of the enzyme to the support, the activity and specific activity of the immobilized lipase increased initially and then decreased. When the ratio of lipase to support was 2:5, they reached the maximum. However, the bound protein increased significantly at enzyme-to-support ratios of less than 2:5. As the enzyme ratio was further increased, the increase in immobilized protein became smaller. As the amount of enzyme increases, the adsorption sites on the support are occupied by enzymes, and the support cannot bind more proteins. At the same time, the immobilization of lipase by unit mass support is limited. When the amount of lipase immobilized on the support reaches a certain level, increasing the amount of lipase cannot improve the bound protein. On the contrary, enzyme aggregates, or even multilayers of enzymes, are easily formed on the surface of the support, leading to a decrease in enzyme activity and specific activity [34]. Therefore, the optimal ratio of lipase to support is 2:5.

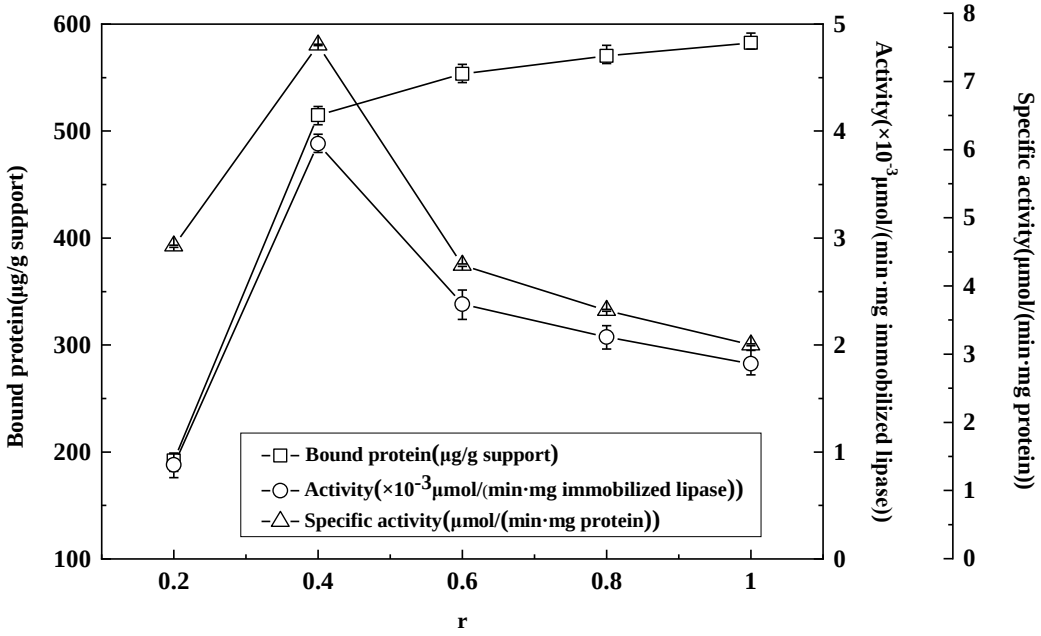


Figure 2. Effect of enzyme/support ratio (r) on amount of adsorbed protein and activities of immobilized lipase.

3.4. Effect of Glutaraldehyde Concentration on Lipase Immobilized by Covalent Binding

In order to further improve the activity and stability of the immobilized enzyme, glutaraldehyde was used to modify the support on the basis of the immobilized enzyme by adsorption, and the free enzyme was bound to the hollow poly(o-phenylenediamine) support by the covalent binding method. Figure 3A shows the effect curves of enzyme activity, specific activity, and protein binding amount on the hollow poly(o-phenylenediamine) particles that were modified by the glutaraldehyde solution at different concentrations. It can be seen from the figure that, with the increase in glutaraldehyde concentration, the amount of protein binding the immobilized lipase increased slightly. The activity and specific activity of the immobilized lipase first increased and then decreased, which was more than that of the immobilized enzyme without glutaraldehyde crosslinking. When the glutaraldehyde concentration reached 2%, the activity and specific activity of the immobilized lipase reached the maximum amount. This is due to glutaraldehyde treatment, and glutaraldehyde and poly(o-phenylenediamine) reacted, forming imine. Another aldehyde group in glutaraldehyde reacts with an amino group in proteins to form imine, which strengthens the bond between the support and the lipase. However, as the concentration of glutaraldehyde increases, further treatment may destroy the favorable conformation of the enzyme molecules and lead to a decrease in enzyme activity.

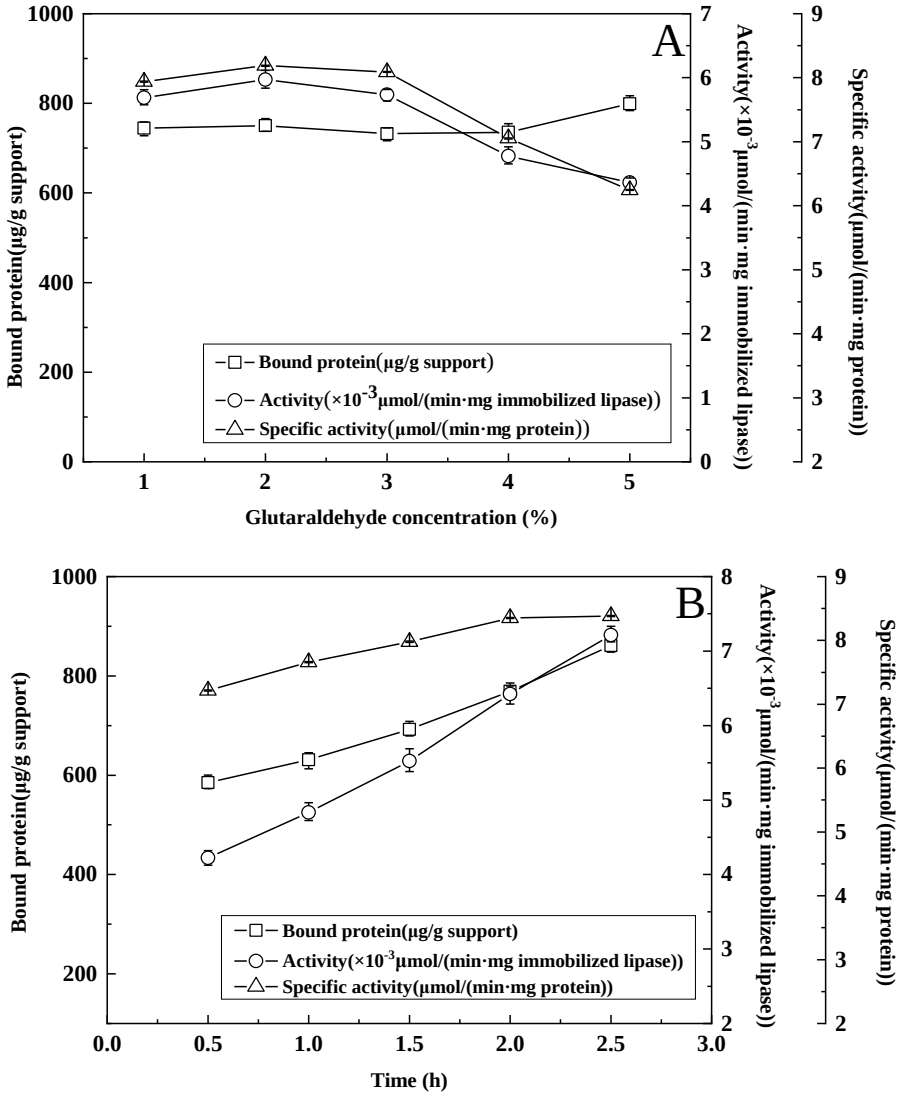


Figure 3. *Cont.*

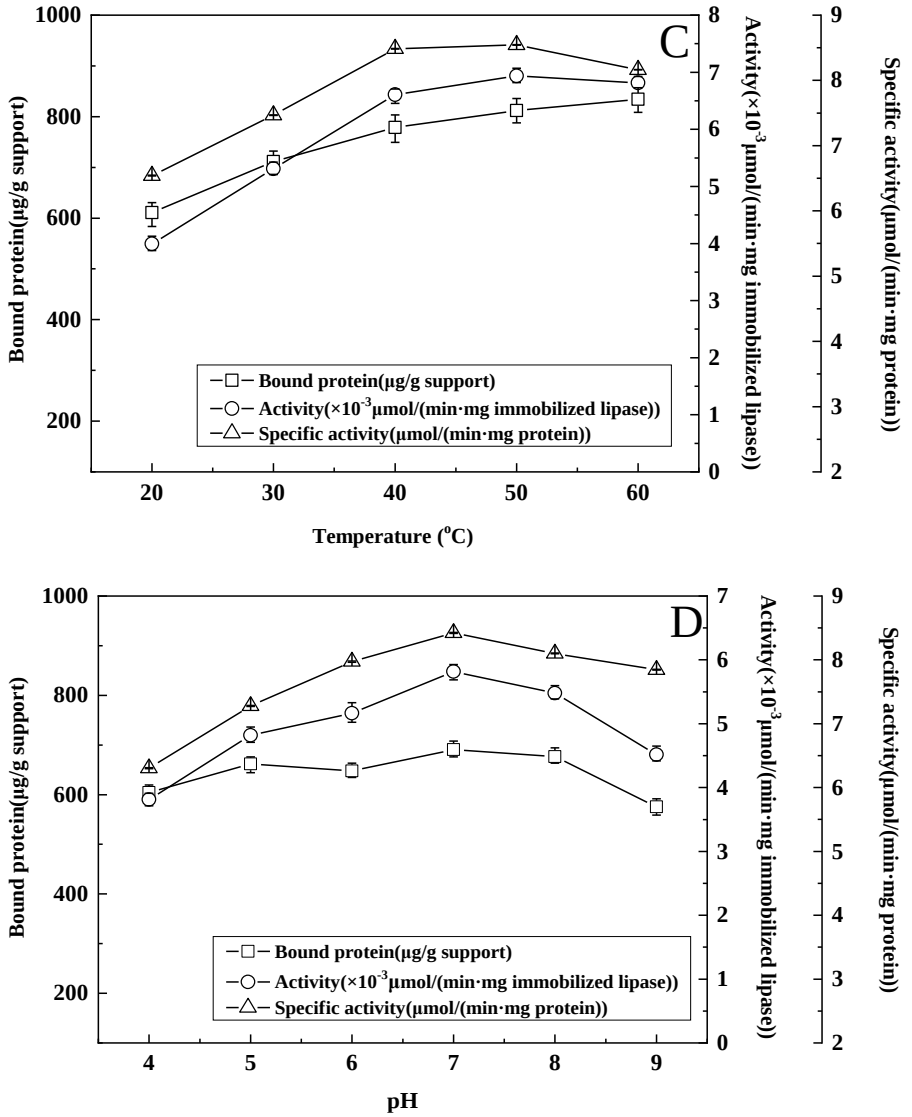


Figure 3. Effect of immobilized conditions on the amount of adsorbed protein and activities of immobilized lipase ((**A**): r 0.4, 30 °C, pH 7, 2.5 h; (**B**): r 0.4, 30 °C, pH 7, glutaraldehyde 2%; (**C**): r 0.4, 2 h, pH 7, glutaraldehyde 2%; and (**D**): r 0.4, 40 °C, 2.5 h, glutaraldehyde 2%).

3.5. Determination of Covalent Binding Time

It can be seen from the effect of the reaction time (Figure 3B) that, with increasing covalent binding time, the amount of protein binding of the immobilized lipase increased linearly. At the beginning of immobilization, the activity of the immobilized lipase increased with the prolongation of the covalent binding time. After immobilization for 2 h, the activity of the immobilized enzyme remained unchanged with increasing reaction time. Therefore, 2 h was selected as the best immobilization time.

3.6. Effect of Temperature on Immobilization

Temperature affects the speed of molecular thermal motion, and in the same immobilization time, it will directly affect the efficiency of enzyme immobilization on the support. Moreover, a high temperature can also cause protein denaturation and even inactivation. Therefore, it is very important to choose the correct temperature when immobilizing enzymes. The experimental results (Figure 3C) show that the amount of protein immobilized on the support is relatively small when it was immobilized at a lower temperature. With the increase in immobilization temperature, the thermal motion gradually intensified, and the amount of protein that was immobilized on the support increased. However, because

the protein will be inactivated and denatured at higher temperatures, although the amount of immobilization of the protein support increases, the enzyme activity and specific activity will decrease instead. Therefore, it was finally determined that 40 °C is the best adsorption temperature for the immobilization process.

3.7. Effect of Buffer pH on Immobilization

The effect of buffer pH on enzyme activity is due to a large number of acidic and basic amino acid residues on enzyme protein molecules, and the change in pH directly affects the dissociation state of the side chain groups of these residues, which can directly affect the binding of substrates and further catalytic reactions [35]. In an enzyme immobilization reaction, both the immobilized enzyme and the support exist in a buffer solution, and the pH value of the buffer solution can change the ionization state of the enzyme molecule and the support. In addition, as a kind of protein, when the pH value of the solution exceeds a certain range, the microstructure of the enzyme will change, which will cause the inactivation of the enzyme. Therefore, the pH value of the buffer is one of the key factors affecting enzyme immobilization. In this experiment, the effect of pH on the activity of immobilized lipase was investigated. The experimental results are shown in Figure 3D. The results show that the activity of the immobilized lipase first increased and then decreased with increasing pH and reached maximum at pH 7.0. As the pH continues to increase, because the stability of free lipase in a high pH environment is poor and enzyme immobilization is a relatively slow process, long-term exposure to the high pH environment will change the spatial structure of the lipase, causing most of the enzyme molecules to lose most of their activity before being immobilized on the support, and ultimately leading to a decline in immobilized enzyme activity. Therefore, the pH of the buffer during immobilization was set at 7.0.

3.8. Comparison of Some Immobilized Lipases

The activity and specific activity of the immobilized PFL that was prepared under optimized conditions were compared with those reported in the literature over the last three years and the results are presented in Table 2. Free and immobilized PFLs have a wide range of activities and specific activities due to the difference in substrate. It is difficult to compare them directly. Activity recovery is defined as the percentage of the ratio of the specific activity of the immobilized enzyme to the specific activity of the free enzyme. The comparison with activity recovery is very convincing. As shown in Table 2, even for the same type of support, the activity recovery varies from one support to another. This is due to a variety of factors, such as the properties, structure, and immobilization method of the support. The activity recovery ranged from a maximum of 2125% to a minimum of only 50%. The activity recovery level in the present study was high compared to that reported in the literature and has good potential for application.

Table 2. Comparison of activities between immobilized PFL on PoPD and immobilized PFL on other supports reported in the last five years.

Free Lipase		Support	Immobilized Lipase		Activity Recovery (%)	Substrate	Reference
Activity	Specific Activity		Activity	Specific Activity			
0.005	1.56	PoPD	0.003	8.26	530	p-NPP	This study.
0.02	0.47	SBA-15	0.05	0.92	196		[36]
0.02	0.47	Na/SBA-15	0.06	1.24	264	sunflower oil/ethanol	[36]
0.02	0.47	Ca/SBA-15	0.06	1.24	264		[36]
-	-	AGMNP-Co^{2+}	-	-	2125	dodecanoic acid/1-dodecanol	[37]
-	445.6	P(GMA-HEMA)/SiO_2	3773	1033.70	232	1-dodecanol/dodecanoic acid	[38]
-	445.4	MNCMMS	4897	1136.19	255	p-NPL	[39]
-	5.56	AuNPs-BI	-	5.03	90	tributyrin	[40]
-	5.56	AgNPs-BI	-	5.92	106	tributyrin	[40]
-	5.56	AuNPs-Pt-DEBP	-	2.87	52	tributyrin	[40]

Table 2. *Cont.*

Free Lipase		Support	Immobilized Lipase		Activity Recovery (%)	Substrate	Reference
Activity	Specific Activity		Activity	Specific Activity			
-	5.56	AgNPs-Pt-DEBP	-	2.78	50	tributyrin	[40]
-	5.56	poly-Pt-DEBP	-	4.79	86	tributyrin	[40]

This study: unit for activity is μmol/(min·mg lipase); unit for specific activity is (μmol/(min·mg protein)). Reference [36]: unit for activity is mg/min; unit for specific activity is mg/(min·g protein). Reference [37]: AGMNP-Co^{2+}: Co^{2+}-chelated magnetic nanoparticles. Reference [38]: unit for activity is μmol/(min·g lipase); unit for specific activity is μmol/(min·mg protein); P(GMA-HEMA)/SiO_2: poly(glycidyl methacrylate-2-hydroxyethyl methacrylate)/SiO_2 nanofilm. Reference [39]: unit for activity is μmol/(min·g lipase); unit for specific activity is μmol/(min·mg protein); MNCMMS: mesoporous nanofilm-constructed millimeter-sized macroporous SiO_2; p-NPL: 4-Nitrophenyl Laurate. Reference [40]: unit for specific activity is μmol/(min·mg protein). MNPs (AuNPs-Pt-DEBP, AuNPs-BI, AgNPs-Pt-DEBP, AgNPs-BI): Au- and Ag-based nanoparticles (AuNPs or AgNPs) stabilized with trans, trans-[dithiodibis (tributylphosphine) diplatinum(II)-4,4′-diethynylbiphenyl] (Pt-DEBP) and dithiol 4,4′-dithiol-biphenyl (BI); poly-Pt-DEBP: organometallic oligomer of Pt-DEBP.

3.9. Effect of the Amount of Immobilized Enzyme on the Preparation of Citronellyl Acetate

From the influence of the immobilized enzyme dosage in Figure 4A, it can be seen that at the same reaction time, with the increase in the immobilized enzyme dosage, the yield gradually increased, especially in the initial stage of the reaction (before 90 min). At the same time, it can be seen from the figure that the yield of citronellyl acetate obviously increased at the initial stage of the reaction. After 2 h of reaction, the yield tended to be stable. In a certain range of enzyme concentrations, the higher the enzyme concentration, the higher the probability of enzyme contact with the substrate was, and the faster the catalytic rate. The amount of enzyme is related not only to the reaction rate, but also has an important impact on the production cost of products. Taking into account the factors of yield and economic benefit, 0.3 g of immobilized lipase/0.05 g substrate was selected as the catalyst addition.

3.10. Effect of Reaction Temperature on the Preparation of Citronellyl Acetate

Temperature is an important parameter affecting enzyme-catalyzed reactions, and choosing the appropriate reaction temperature not only helps to prolong the life of the enzyme, but also increases the rate of reaction [4]. From the effect of the reaction temperature (Figure 4B), it can be seen that the yield of citronellyl acetate increases with increasing temperature in the experimental temperature range studied here. When the reaction temperature was 60 °C, the reaction course curve basically overlapped with that of 50 °C, indicating that the optimal reaction temperature for the immobilized enzyme to catalyze the reaction was 50 °C. Compared to the optimal reaction temperature of 40 °C for the free enzyme, it was increased by 10 °C. This also indicated that the thermal stability of the immobilized enzyme was greatly improved compared to that of the free enzyme. This is because the rigidity of the enzyme increases after it is immobilized on the support. In order to make it more active and combine it with the substrate molecules, it is necessary to increase the temperature in order to increase its flexibility and improve the contact of the enzyme with the substrate. Therefore, the catalytic reaction temperature of immobilized lipase is higher than that of free lipase.

3.11. Effect of Shaking Intensity on the Preparation of Citronellyl Acetate

From the effect of the rotation rate, as shown in Figure 4C, it can be seen that at the same reaction time, the yield increases with increasing rotation rate. At the same time, it can be seen that when the rotation rate is greater than 200 rpm, the yield growth rate tends to be basically stable, which indicates that when the rotation rate reaches 200 rpm, the external diffusion restriction in the reaction system is largely eliminated. The immobilized enzyme reaction system is a heterogeneous reaction system. The combination of the enzyme and the substrate molecules is restricted by external diffusion, which affects the catalytic efficiency of the enzyme [41]. The influence of external diffusion can be reduced, or even eliminated,

by studying the influence of the shaking table rotation rate. Considering that the increase in product yield was not obvious at a higher rate, 200 rpm was chosen as the reaction rate to reduce the energy consumption as much as possible without affecting the mass transfer effect. After the above optimization, the yield of citronellyl acetate can reach over 99% after 2 h of reaction.

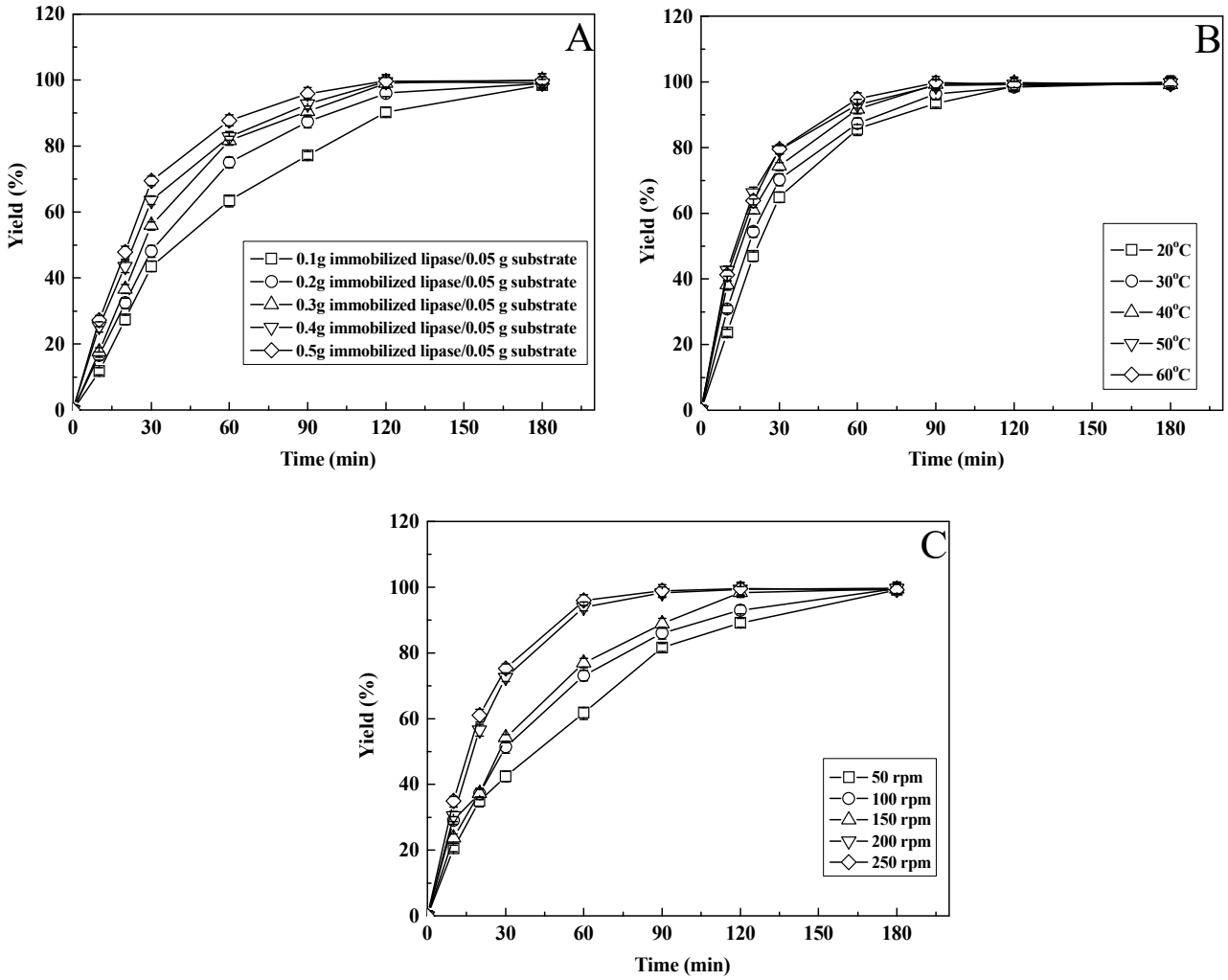


Figure 4. Effect of reaction conditions on the synthesis of citronellyl acetate catalyzed by immobilized lipase: (**A**) is the amount of immobilized lipase; (**B**) is reaction temperature; and (**C**) is shaker rotation rate.

3.12. Operational Stability of Immobilized Enzyme

One of the key features of enzyme immobilization is the easy separation of the immobilized enzyme from the reaction medium and the reusability of the enzyme. Citronellol transesterification was used as a model reaction to investigate the operational stability of the immobilized enzyme and its comparison with free lipase. The results are shown in Figure 5A. It can be seen that, after immobilization, the operational stability is obviously enhanced. After seven repeated uses, the activity of the immobilized lipase by the covalent binding method is approximately 95%, while that of the free lipase is only 70%. The transesterification reaction of vinyl esters and alcohols can result in the formation of acetaldehyde, which is known to deactivate microbial lipases, presumably by structural changes caused by initial Schiff base formation at solvent-accessible lysine residues. Previous studies have shown that several lipases were sensitive toward acetaldehyde deactivation, whereas others were insensitive. PFL belongs to the latter. The activity of PFL remained nearly unaffected (−2%) upon overnight incubation with acetaldehyde (0.1 M) in toluene [42]. Therefore,

the effect of acetaldehyde on immobilized PFL can be ignored during reuse. Rice straw was processed into powder and filaments, on which PFL was immobilized (RSD-PFL and RSF-PFL) for a transesterification reaction to produce citronellyl acetate in a solvent-free system [28]. The highest yield reached 96%. The relative activity of RSD-PFL and RSF-PFL decreased to 85.7% and 87.2%, respectively, after being reused six times. The average attenuation rates of relative activity were 0.36%/h and 0.32%/h, respectively. In contrast, the average attenuation rate of the relative activity of immobilized PFL in this study was 0.24%/h, which was smaller than that reported in the literature.

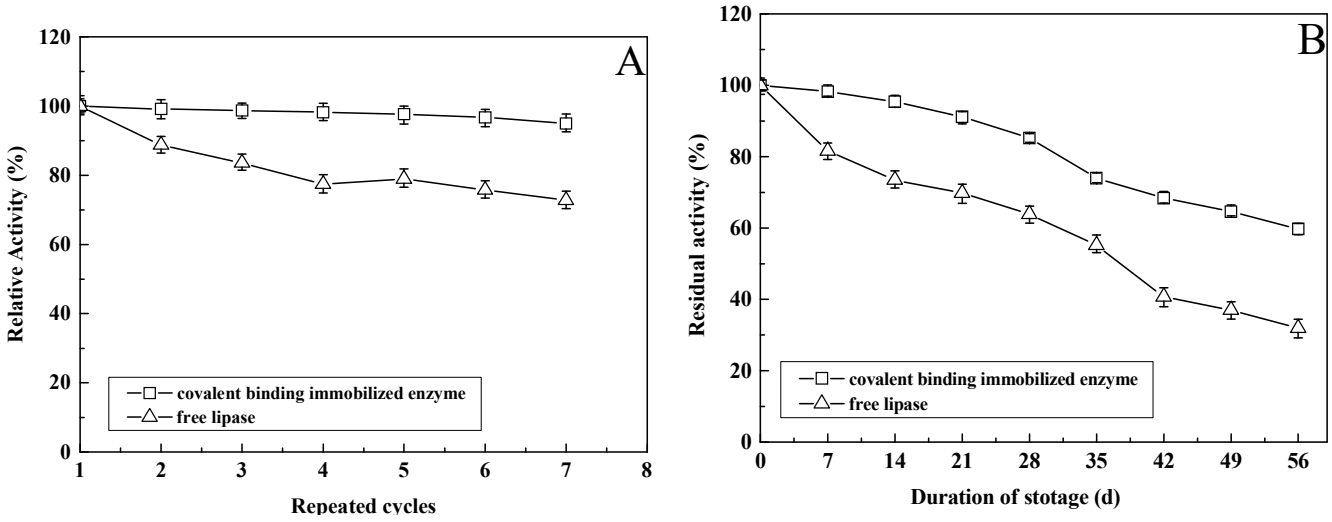


Figure 5. Comparison of the stability of free and immobilized lipases: (**A**) is operational stability and (**B**) is storage stability.

3.13. Storage Stability of Immobilized Enzyme

The storage stability of the immobilized enzyme is shown in Figure 5B. It can be seen that the storage stability of the immobilized lipase is better than that of the free lipase. After 56 days of storage, the lipase activity is still 60% of the initial activity, whereas the free lipase is only about 31% of the initial activity. Generally, enzyme preservation is performed at 4 °C. In this study, stability experiments were performed at room temperature to accelerate enzyme inactivation. It has been reported in the literature that PFL immobilized on carbon nanofibers (CNF-PFL) lost its activity by 60% of the initial value when at 4 °C for 90 days [43]. Similarly, the solution in the present study could only be stored for 56 days at the same rate of reduction. However, considering the difference in storage temperature, our stability should have been higher than that of CNF-PFL. Pitzalis et al. immobilized PFL on zeolitic imidazolate framework-8 (ZIF-8) supports and stored them at 4 °C for 15 days, whereafter the activity was retained at 99% [44]. The biocatalyst, immobilized on PoPD, was stored for 14 days, and its relative activity was still over 95%, which is basically the same as PFL@ZIF-8.

4. Conclusions

Lipase was immobilized on hollow poly(o-phenylenediamine) microspheres by the covalent binding method. By optimizing the immobilization conditions, the specific activity of the immobilized enzyme was 5.3 times higher than that of the free enzyme. At the same time, the stability of the immobilized enzyme was investigated, and the results showed that the immobilized lipase had greatly improved thermal stability, operational stability, and storage stability. The immobilized lipase was used in the transesterification reaction to produce citronellyl acetate. Under the optimized experimental conditions, the yield of citronellyl acetate was more than 99%. Compared to the free enzyme, the optimum reaction temperature of the immobilized enzyme was increased by 10 °C. After seven

recycles of reaction, the relative activity of the immobilized enzyme was 25% higher than that of the free enzyme. These results indicate that the hollow poly(o-phenylenediamine) microspheres are a good support for lipase immobilization.

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