RESEARCH ARTICLE

Structure and property of immobilized lipase on chitosan attached to macroporous resin

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Transesterification catalyzed by lipase is an available method to produce biodiesel. In order to acquire efficient catalysts used in biodiesel synthesis, a novel immobilized lipase was prepared in this study by using macroporous resin modified by chitosan as the immobilization carrier. Chitosan was attached to macroporous resin prior to lipase immobilization to improve chitosan particles' poor mechanical strength. The structure of the immobilized carrier and lipase was characterized by scanning electron micrograph (SEM) and Fourier transform infrared spectroscopy (FT-IR). SEM demonstrated that the surface of the immobilized carrier was smoother than that of resin, and lipase was distinctly immobilized onto the carrier. FT-IR demonstrated that lipase was immobilized onto the carrier by covalent binding. The activity of immobilized lipase remained 80% of its initial activity after it was kept at 80℃ for 1 h, while the activity of free lipase dropped to 35% of its initial activity under the same conditions. After 30 days of storage, the residual activity was 79% for the immobilized lipase higher than 54% for free lipase. Moreover, immobilized lipase was not easily denatured and inactivated in the presence of non-polar organic solvents, but it's the opposite in polar organic solvents. Its hydrolysis rate of olive oil remained 70% after 10 cycles in batch operation. With waste oil as substrate, the esterification rate of the products was achieved about 81%. The synthesis of biodiesel catalyzed by the immobilized lipase in organic media provided the basis for the industrial applications.

Keywords: lipase; immobilization; chitosan; scanning electron micrograph (SEM); Fourier transform infrared spectroscopy (FT-IR).

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Introduction

With the worldwide shortage of fossil energy and environmental pressure, the development and application of the renewable energy has become the world focus of scientific and technical community. As a typical sustainable energy source, biodiesel has become more attractive recently due to its environmental friendliness. Transesterification of natural oils by chemical catalysis is one of the most effective preparation methods to synthesize biodiesel [1]. However, the chemical-catalyzed transesterification showed disadvantages such as overuse of short-chain alcohols, complex process, difficulty of product separation, and drainage pollution. Enzymatic synthesis of biodiesel has become a prime interest in recent reports owing to mild reaction conditions, low energy consumption, simple post-processing, and non-pollutant

emissions [2]. Unfortunately, free lipase is usually unstable and cannot be reused. Comparing to free lipase, the immobilized lipase can be recycled and, therefore, can reduce the output cost and become more stable in temperature, pH, and storage. The immobilization of free lipase becomes an inevitable trend of future development [3].

Commercial immobilized lipases, like Novozyme 435 and Lipozyme RMIM from Novozymes (China) Biotechnology Co., LTD, Tianjin, China, have displayed several mentioned requirements, such as conferring high activity and stability and allowing biocatalyst reuse [4]. Transesterification catalyzed by lipase is an available method to produce biodiesel. However, the price of commercialized immobilized lipase is prohibitively high for bio-energy industry, which is the main hurdle to commercial production of biodiesel. In view of the current high cost of lipases, the ideal feature of immobilized lipase is that the support should significantly decrease the cost of biocatalyst. So, it has become a very important research topic to find viable materials with low cost in the immobilized lipase industry [5, 6].

Chitosan (polyglucosamine (1-4)-2-amino-B-D glucose) is made by deacetylation of Chitin, which is widely found in nature. As a kind of promising natural biomaterial, the actual and potential applications of chitosan in biology have aroused more and more interest. Nowadays, chitosan is becoming a very interesting raw material due to its chemical versatility, natural abundance, and ecological compatibility. It is also well known for its non-toxicity, water insoluble properties, and being chemically modified easily [7]. Many researchers used chitosan as immobilized carrier to get a high activity immobilized lipase [8]. Tan, *et al*. immobilized lipase on polyvinyl alcohol (PVA)/chitosan composite [8] and Yi, *et al*. immobilized lipases on chitosan-tethered poly hollow fiber membrane [9]. Hung, *et al*. firstly immobilized lipase on chitosan beads by activating its hydroxyl groups with carbodiimide followed by cross-linking more lipase to the amino groups with glutaraldehyde [10].

According to the statistics, lipase immobilized on the chitosan carrier shows a promising result on improving efficiency of catalysis. However, the existing preparation methods of the chitosan carrier generally require complicated manual operation and the carrier is not easy to recycle due to the poor mechanical strength [7, 11]. Therefore, a cost-efficient preparation of chitosan carrier is developed by many scientists in order to improve the defects of chitosan [12]. In this study, a new type of immobilized carrier was prepared by combining chitosan and macroporous resin. Comparing to the relevant studies domestically and internationally, the immobilized carrier prepared in this study has great advantages in terms of carrier strength and immobilized area. To the best of our knowledge, the immobilization of lipase onto chitosan attached to macroporous resin has been rarely reported. The novel immobilized lipase has great potential as catalysts for use in biodiesel synthesis.

Materials and methods

Preparation of chitosan carrier

Macroporous resin D301R (Chemical Plant of Nankai University, Tianjin, China) had been soaked for 12 hours with 75% ethanol before washed to neutral with distilled water, then dried to constant weight in a BPZ-6063LCB vacuum oven (Shanghai Yiheng Scientific Instrument Co., LTD, Shanghai, China) [12]. 1 g chitosan with a degree of deacetylation of 90% (Macklin, Shanghai, China) was added into 100 mL of 2% acetic acid solution and was completely dissolved with magnetic stirring [13]. 5 g resin and 25 mL of chitosan solution were mixed in a rotating bottle connected to vacuum pump system to obtain chitosan carrier at 50 rpm, 1,300 Pa vacuum, for 10 min. Subsequently, the chitosan carrier was extensively washed with distilled water until solution showed neutral. The chitosan carrier was then cross-linked with 15 mL of 0.1%

glutaraldehyde solution at 4℃ for 2 h and was dried to constant weight.

Immobilization of lipase

Before immobilization, the chitosan carrier was wetted by a small amount of deionized water. Lipase (fermented by *Aspergillus niger* with the specific activity of 5,000 U/g) (Shenzhen Leveking Biological Engineering Co., Ltd, Shenzhen, Guangdong, China) was then sufficiently mixed with the carrier at a mass ratio of 1:10 (lipase: carrier) in isooctane at 4℃ for 5 h. The immobilized lipase was subsequently dried at room temperature and stored at 4℃ for further use.

Scanning electron microscopy (SEM) analysis and Fourier transform infrared spectroscopy (FT-IR) measurement

The surface morphology of the macroporous resin, modified macroporous resin (chitosan carrier), and immobilized lipase were scanned by using HITACHI S-4800 FE-SEM (HITACHI, Tokyo, Japan) with 1 μm resolution, operated at 15 kv. Samples were dried under vacuum and coated with gold operated at 15 mA for 50 s before observation. The formation of chitosan carrier and immobilized lipase were scanned by using Nicolet FT-IR 6700 (Thermo Electron Corporation, Madison WI, USA). Samples were prepared as KBr pellets and were scanned at wavelength range of 4,000 to 400/cm.

Lipase activity assay

The activities of free and immobilized lipases were measured by the classical olive oil emulsion method [14]. Olive oil was purchased in a local market. A unit of lipase activity (U) was defined as one milligram or one milliliter of lipase that released 1 μmol fatty acid from hydrolyzing olive oil as substrate per minute under certain conditions (pH 7.5, 40℃). Each of the assays was performed in triplicate and control experiment was carried out with inactivated lipase.

Properties of free and immobilized lipase

Thermal stability was assayed for residual lipase activity by incubating the free and immobilized lipases in a water bath for 1h at temperature 30℃, 40℃, 50℃, 60℃, 70℃, and 80℃, respectively. Organic solvent stability was evaluated by storing 10 g immobilized lipase in 50 mL of organic solvent including isooctane, n-hexane, petroleum ether, cyclohexane, and methanol, respectively, at 4℃ for 1 h. The storage stability was evaluated by storing the immobilized enzyme at room temperature for 30 days with the residual lipase activity was examined every five days [15, 16]. The reusability of the immobilized lipase was determined by the hydrolysis of olive oil using the recovered immobilized lipase separated by centrifugation at 4,000 rpm for 20 min and comparing to the first run. The relative lipase activity was adopted as the ratio of lipase activity after and before treatment.

Synthesis of biodiesel

To explore the feasibility of the immobilized lipases in synthesis of biodiesel, esterification of waste oil (Guizhou Jinjiang Bioenergy Science and Technology Co. LTD, Bijie, Guizhou, China) with methanol was chosen as a model reaction [17]. The waste oil is usually made from discarded kitchen waste containing a lot of impurities, moisture, colloids, and pigment, *etc*. There was a whole set of procedures for preprocessing waste oil [18]. First, most of the impurities were removed by either gravity or centrifugal force at a speed of 8,000 rpm. The refined oil was obtained by bleaching with oxidation, degumming with phosphoric acid, and removing moisture in turn. The ester synthesis was carried out in screw-capped flasks (50 mL) containing 6 g of refined oil, 400 mg of immobilized lipase, and 420 μL of methanol appended for three times at 0 h, 8 h, 16 h, respectively, in the presence of 3 mL of hexane. The reaction mixture was incubated at 40℃ in HZQ-X300 shaking incubator (Shanghai Ranhui Industrial Co. LTD, Shanghai, China) at 200 rpm for 24 h.

Analyses of biodiesel were confirmed by using Gas Chromatograph Mass Spectrometer (GC/MS) system equipped with a series 5975 B Insert MSD mass-selective detector (Agilent technologies,

Santa Clara, CA, USA). 2 µL of product was analyzed after splitless injection employing a HP-5MS Phenyl Methyl Siloxane capillary column (30 m × 250 µm × 0.25 µm nominal) (Agilent Technologies, Santa Clara, CA, USA). Helium was used as a carrier gas at a constant rate of 1 mL/min. The temperatures of the injector and detector were 250℃ and 240℃, respectively. The following temperature program was applied as 120℃ for 5 min, increasing to 180℃ at the speed of 3℃/min, increasing to 220℃ at the speed of 10℃/min, and then 220℃ for 31 min.

Statistical Analysis

All the experiments were carried out in triplicate, and the results were shown as mean ± standard deviation. Statistical analysis was conducted by using one-way ANOVA followed by LSD multiple-range test by using IBM SPSS Statistics software (version 19.0 for Windows) (IBM, Armonk, NY, USA).

Results and discussion

Scanning electron microscopy (SEM) analysis

The morphologies of macroporous resin D301R, chitosan carrier (modified D301R), and immobilized lipase were examined by using SEM [19]. Micrographs showed that microsphere, modified or not, exhibited good sphericity. D301R is a macroporous styrene-based resin with coarse and poriferous surface (Figure 1A). The surface of modified D301R was smooth and less porous showing that the surface of the resin was coated with a layer of chitosan (Figure 1B). Comparing to the surface of modified D301R, the immobilized lipase had some obvious protrusion attachments proving the effective adhesion of the lipase molecules (Figure 1C).

Fourier transform infrared spectroscopy (FT-IR) measurement

The FT-IR analysis of the nascent and modified D301R as well as the free and immobilized lipase were showed in Figure 2. The chitosan's characteristic peaks in FTIR spectrum were in agreement with the ones reported before [20], as N-H and O-H (3,440/cm), CH_3 and CH_2 (2,920/cm), C=O (1,583/cm and 1,650/cm), NH_2 (1,590/cm), C-O-C (1,081/cm) were observed for chitosan. As chitosan carrier was the modified product of D301R, its basic skeleton structure of modified chitosan should be consistent with D301R. However, the increased peak intensity at about 2,853/cm, 2,923/cm, and 1,590/cm was observed for chitosan carrier in comparison with the spectrum of D301R. The absorbing peaks of the groups displayed small changes, demonstrating that D301R and chitosan had weak intermolecular interaction.

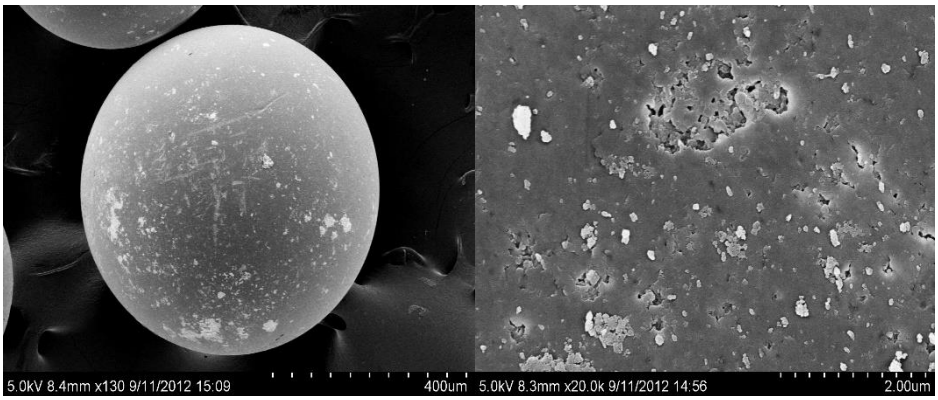
The characteristic absorption peaks of free lipase are O-H and N-H (3,353/cm), C-H (2,918/cm), C=O (1,650/cm), C-O (1,002/cm). After lipase was immobilized on chitosan carrier, the absorption peaks of O-H and N-H broadened and shifted to 3,353/cm. The molecular interaction between the free lipase and carrier is the main reason leading to a decrease in the N-H bond and weak stretching vibrations. The characteristic absorption peak of Schiff base (1,630~1,690/cm) appeared at the immobilized lipase because of the covalent interaction between the free lipase and glutaraldehyde.

Properties of immobilized lipase

(1) Thermal stability:

The rate of enzyme-catalyzed reactions is influenced by temperature. Although free lipase has high catalytic activity in the biocatalysis reaction, its activity will decrease significantly when it has been continuously heated. Enzyme immobilization has been proven an effective method to improve thermal stability of enzyme [21]. As seen in Figure 3, lower temperature had no impact on catalytic activity of the immobilized lipase and free lipase. The thermostability of the lipase changed with an increase of treating temperature. The immobilized lipase showed good thermal stability, but that of the free lipase decreased significantly. When the immobilized lipase was stored at 80℃ for 1 h, catalytic activity remained 80% of initial activity. In contrast, free lipase dropped to nearly 35% of its initial activity under the same temperature. This observation

A.



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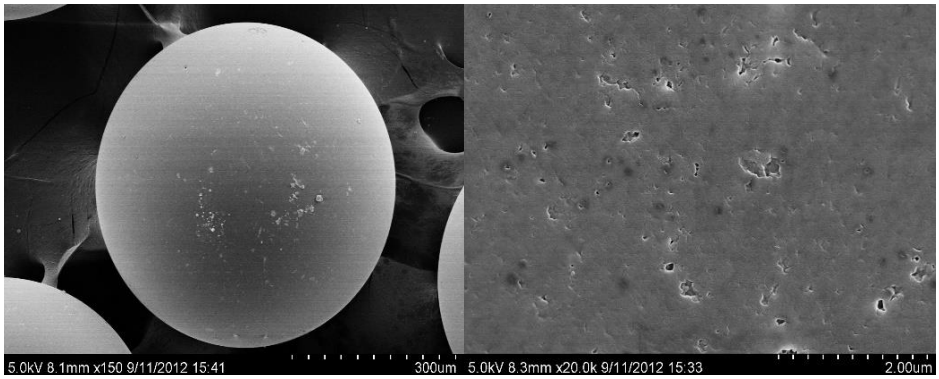

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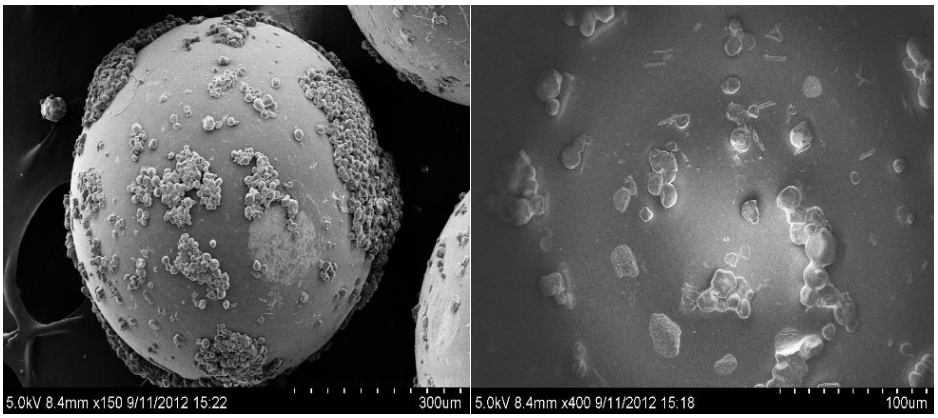

Figure 1. Micrographs of resin D301R (A), modified D301R (B), and immobilized lipase (C). Magnifications: 130× on the left panel; 20,000× on the right panel.

confirmed that the immobilized lipase was more stable to temperature than free lipase because the presence of the carrier prevented the extensional deformation and self-denaturing of the lipase molecule. There are many examples in the previous studies, showing that utilization of surface-binding type immobilization strategy could enhance thermal stability of the enzyme [22]. The high thermal stability of immobilized lipase provides an additional benefit of using immobilized lipase in practical applications.

(2) Storage stability:

The storage stability of lipase was directly related to the industrial application, and immobilization technology is an effective measure to improve

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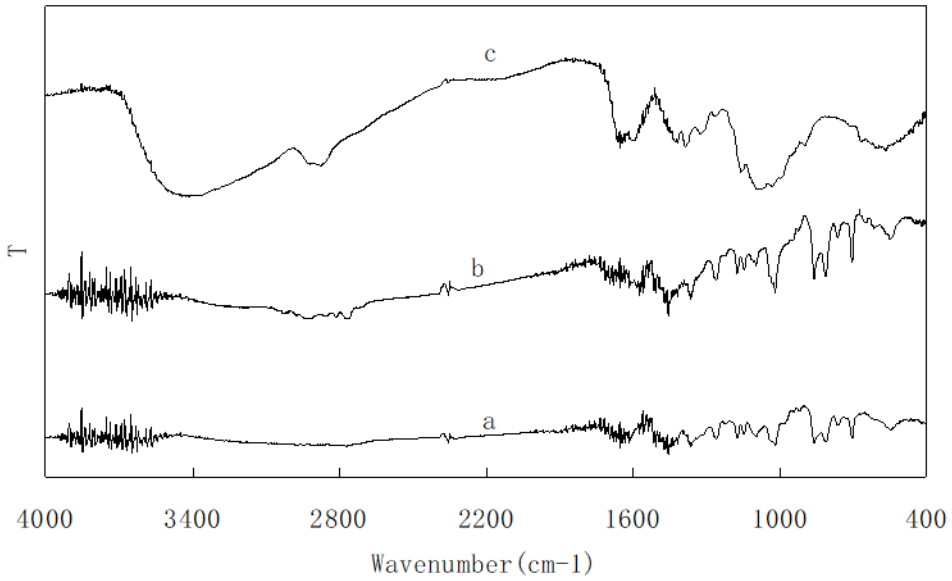

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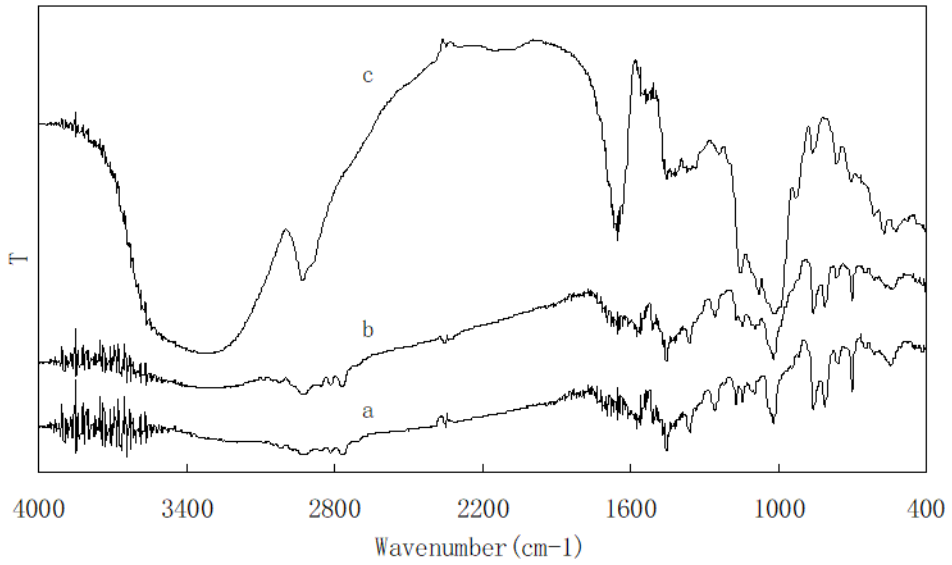

Figure 2. FT-IR analysis of D301R and lipase. **A.** nascent D301R (a), modified D301R (b), chitosan (c). **B.** modified D301R (a), immobilized lipase (b), free lipase (c).

the storage stability of enzyme. Lipase was stored at room temperature and the activity was analyzed every 5 days during total 30 days period. The variation of the relative activity of the lipase was shown in Figure 4. The relative activity of the immobilized lipase was about 79% as against 54% for free lipase during 30 days of storage. The experiments also showed that the immobilized lipase had better storage stability than free lipase, which could be attributed to an improved physical and mechanical stability of the lipase arising from the multipoint covalent binding between lipase molecule and the carrier [23].

(3) Organic solvent stability:

The stability of the lipase in organic solvent was important for the improvement of biodiesel production and application. As Hiroyasu, *et al*. mentioned, almost all natural enzymes are easily

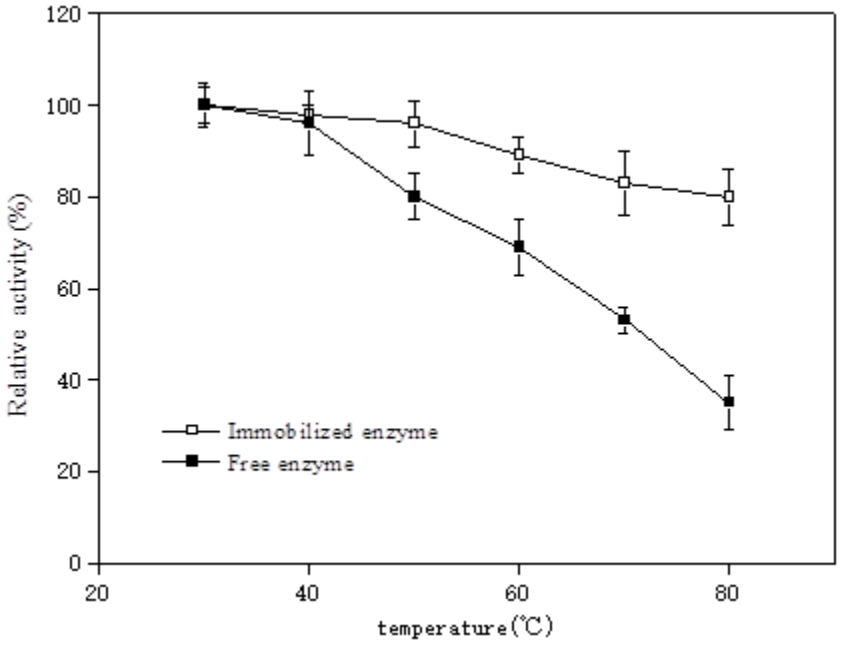

Figure 3. Effect of storage temperature on free and immobilized lipase activity. All reactions were carried out for 1 h at water bath, using 1 g of immobilized lipase. The relative activity of 100% for free lipase and immobilized lipase was 19.8 U/mL and 4.1 U/mg, respectively.

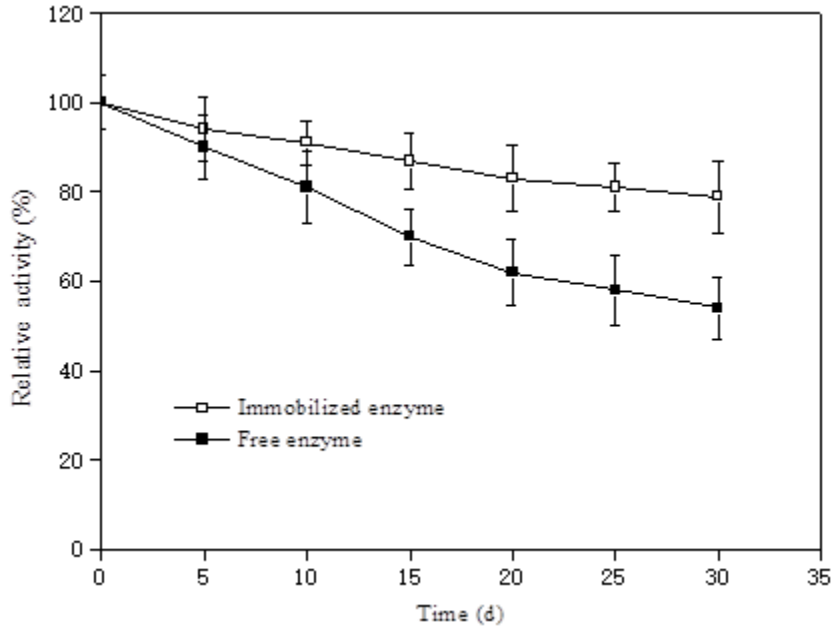

Figure 4. Effect of storage time on free and immobilized lipase activity. All reactions were carried out at room temperature. The relative activity of 100% for free lipase and immobilized lipase was 19.8 U/mL and 4.1 U/mg, respectively.

denatured [24], but only a few natural enzymes have been discovered stable in the presence of organic solvents. Therefore, the immobilization method for stabilizing enzyme in the presence of organic solvents was developed [25]. The activity of lipase was assayed after stored in different organic solvents varying from polar to non-polar for 1 h. As shown in Figure 5, among the five

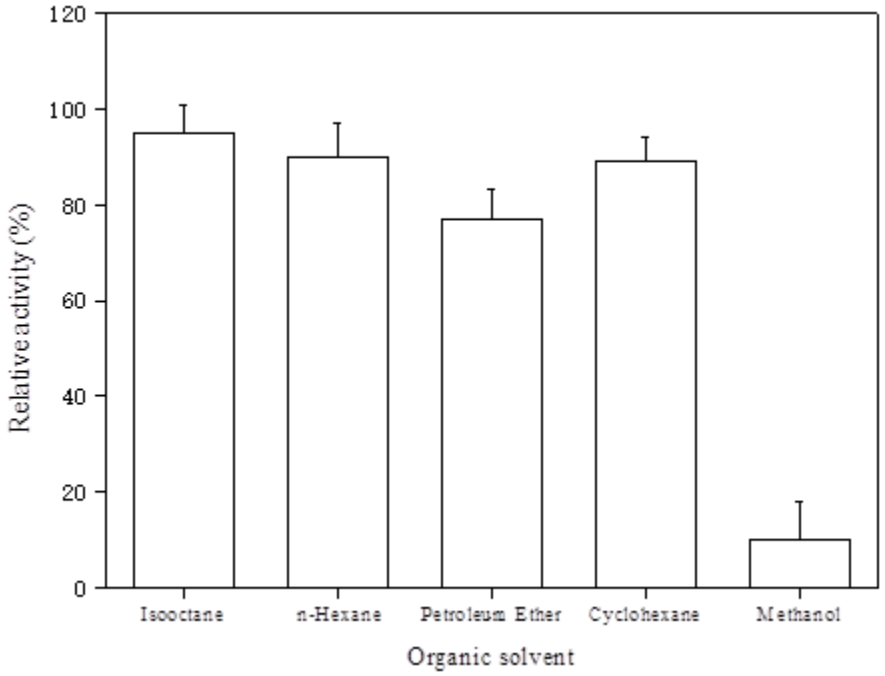

Figure 5. Effect of organic solvent on immobilized lipase activity. All reactions were carried out for 1 h at room temperature, using 1 g of immobilized lipase, and 10 mL of organic solvent. The relative activity of 100% for immobilized lipase was 4.1 U/mg.

solvents, immobilized lipase has a high tolerance for non-polar organic solvent such as isooctane, for which the activity is maintained 95% during 1 h incubation. However, the presence of polar organic solvent, methanol, led to the loss of the catalytic activity, which was only 10% of its initial activity. Due to the nature of non-polar organic solvent tolerance, it is expected that immobilized lipase can be used as catalysts for reactions in non-polar organic phase and has huge potential in industrial application.

(4) Reusability:

The reusability of the immobilized lipase was an important feature that distinguishes it from the free lipase. It is also vital for cost-effective usage and the recovery of product from the reaction system in industrial applications. As shown in Figure 6, the activity of the immobilized lipases showed a slow decline with reuses in accordance with findings reported by Tang, *et al*. [26]. The immobilized lipase retained 70% of its initial activity after successively repeated use for 10 cycles. The results indicated that this immobilized lipase had high operational stability and could be suitable for being used in batch systems with high-speed agitation. Likewise, biocatalyst performance in continuous systems, for instance, as catalyst of a packed bed reactor, can significantly reduce the operation cost in practical application and will be a subject for future study.

(5) Esterification capacity:

Methanol has strong inhibition against the activity which can inactivate lipase by destroying the structure of proteins, so the methanol was appended in the reaction system for several times [27]. The transesterification reached equilibrium after 10 h, and the same amount of methanol was added to the reaction system in order to break the reaction equilibrium and continue to react. Figure 7 showed that there was no detectable increase in the yield of biodiesel after methanol was appended thirty times, indicating that this reaction ended up with 81% conversion of biodiesel. The result also showed that the immobilized lipase achieved a high esterification power, as high as those reported in other study [27]. Therefore, the results provided

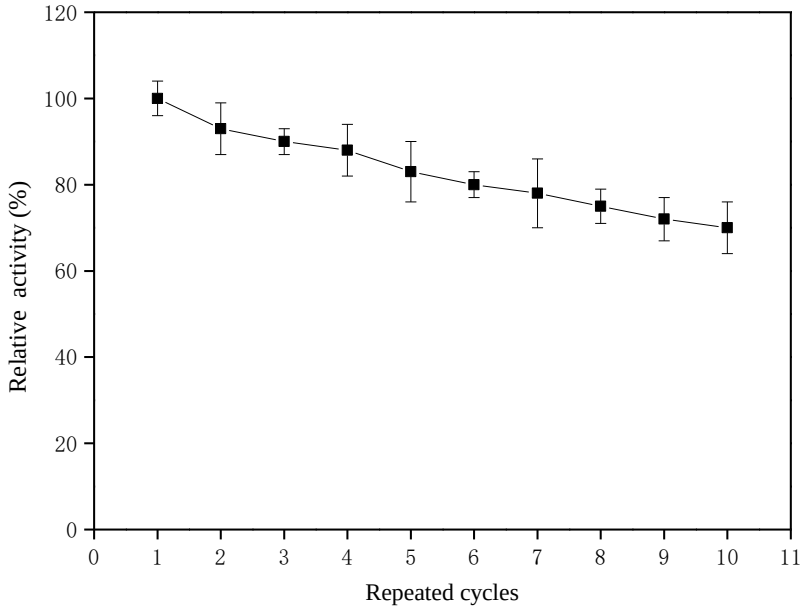

Figure 6. Effect of repeated cycle on immobilized lipase activity. All reactions were carried out for 15 min at 40℃, using 4 mL of olive oil emulsion, 5 mL of phosphate buffer (pH 7.5, 0.2 M). The relative activity of 100% for immobilized lipase was 4.1 U/mg.

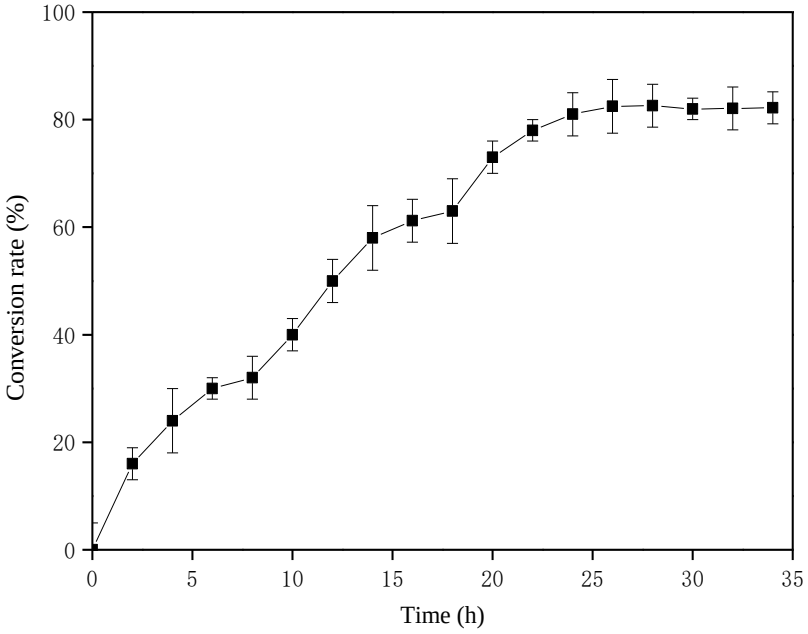

Figure 7. Esterification capacity of immobilized lipase was carried out for 34 h at 40℃, using 6 g of refined oil, 400 mg of immobilized lipase, 3 mL of hexane, and 420 µL of methanol added in three steps.

the evidence for the industrialization application of lipase-catalyzed esterification in organic media.

Conclusion

In this study, a macroporous resin modified by chitosan was successfully prepared in order to be

used as support for the immobilization of lipases. SEM and FT-IR demonstrated lipase was immobilized on the macroporous resin coated with chitosan by covalent binding. Comparing to free lipase, the immobilized lipase exhibited significant improvement in storage, operation, and thermal stability. Comparing to the other current exist methods, the strength of the immobilized enzyme was greatly improved by using resin as substrate, which made it possible to use the immobilized enzyme in column bioreactor for continuous reaction. Immobilized lipase in non-polar solvent exhibited a higher stability than that in polar organic solvent. The synthesis of biodiesel catalyzed by the immobilized lipase in organic media provided the basis for the industrial applications. The integrated strategy proposed in this study would be a promising modified approach for large-scale preparation of immobilized lipases for industrial applications.

Acknowledgements

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