



## Immobilized recombinant *Rhizopus oryzae* lipase for the production of biodiesel in solvent free system

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### ABSTRACT

Different kinds of macroporous and anion resins were applied to immobilize recombinant *Rhizopus oryzae* lipase in the initial screening test. Anion exchange resin Amberlite IRA-93 was the most proper support to immobilize *R. oryzae* lipase by covalent binding technology. The transesterification reaction catalyzed by the immobilized lipase was investigated in a solvent free system. It was found that the anion resins used as the immobilized carriers per se contributed to the promotion of acyl migration in the methanolysis reactions and improved biodiesel yield by about 4–8%. The highest biodiesel yield of 90.5% was achieved under the optimum conditions (enzyme dosage 24 U/g<sub>oil</sub>, methanol to oil molar ratio 4.8:1, water content 60% by weight of oil, temperature 37 °C). There was no obvious loss in immobilized lipase activity after being consecutively used for 7 cycles in the transesterification reactions.

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## 1. Introduction

Biodiesel, a natural substitute of diesel fuel, has attracted the attentions all over the world in recent years for its benign characteristics such as biodegradability, reproducibility and non-toxicity. Biodiesel fuel (fatty acid methyl esters) can be obtained by the transesterification reactions of vegetable oils and animal fats with methanol or ethanol in the presence of catalysts.

The conventional biodiesel technology is usually catalyzed by inorganic base and acid, which makes the separation of catalysts from products be difficult and consequently increases the cost of production. Recently, lipase-catalyzed methanolysis method has become more attractive than the traditional chemical means in biodiesel production, since the recovery of glycerol is easy and transesterification of oil with high fatty acid content is available [1].

Lipases, triacylglycerol hydrolases, which belong to  $\alpha$ / $\beta$ -hydrolases, are widely used in food, detergent and pharmaceutical industries. The molecular structure of lipase permits that it can catalyze various reactions at the oil/water interface in the reaction

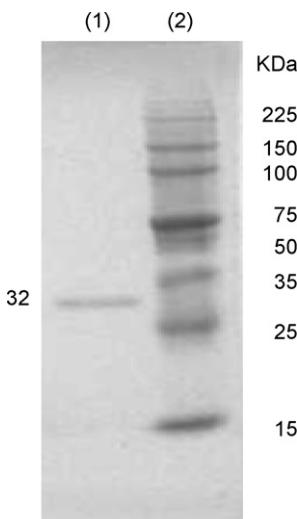
media [2]. *Rhizopus oryzae* lipase, which is a 1,3-position specific regiospecific enzyme used for the hydrolysis and synthesis of various ester compounds, has been commonly used in the production of biodiesel in recent decades. It has been demonstrated that the acyl migration, which would increase biodiesel yield, happened in the process of transesterification reactions catalyzed by 1,3-position specific enzymes such as *R. oryzae* lipase and lipozyme TL [3–5].

Nowadays, one of the main hurdles for the production of biodiesel by lipase is the cost of enzyme. The technology of immobilization is a good method to solve this problem when enzyme can be used repeatedly in the reactions. The properties of support materials and the immobilization strategies are the two key factors that should be considered firstly in the process of immobilization since they determine the stabilization of immobilized enzyme [6]. Support materials used for the immobilization of enzyme should be readily available, cheap, and non-toxic. It was reported that lipases could be immobilized to many kinds of carriers such as macroporous resin by adsorption, hydrophobic sol–gel by entrapment, alginate by encapsulation and chitosan by covalent binding for the enzymatic biodiesel production [7–10].

Ionic exchange resins such as DOWEX and Amberlite IRA are polystyrene polymers whose matrix structures can be altered with the cross-linking agent divinylbenzene. They have been used in the purification, concentration and fractionation unit operations in laboratory and industry for a long time [11]. Some

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**Fig. 1.** SDS/PAGE analysis of expressed lipase. SDS-PAGE of protein was performed in a 12% (w/v) acrylamide gel. Broad range protein molecular weight markers (Promega Corp.) were used as the standard of proteins for the determination of molecular weights. Lane 1: crude extract of KM71H transformed with pPICZ $\alpha$ A-ROL induced at 30 °C; lane 2: protein marker.

researchers also used ionic exchange resins to immobilize lipase for the production of structured lipid and found that the half-life of the immobilized lipase reached 370 d [12]. Besides that, the various functional groups on the surface of ionic exchange resins may make it possible to immobilize enzymes onto resins firmly by covalent attachment technique. It was also found that resins had an accelerating action on the acyl migration in the transesterification and consequently increased the conversion of biodiesel [13,14]. Therefore, resin may become a proper support for the immobilization of *R. oryzae* lipase in biodiesel industry.

In this present study, various macroporous and anion resins were used as the supports to immobilize *R. oryzae* lipase in order to tailor an immobilized biocatalyst for the synthesis of biodiesel and the procedure of immobilization was optimized. The effects of various factors such as reaction temperature, water content, methanol concentration, methanol stepwise addition means and additives were investigated in the process of transesterification reactions.

## 2. Materials and methods

### 2.1. Materials

Crude recombinant *R. oryzae* lipase LY6 (83 U/mg), a mutant from *R. oryzae* DSM 853 by ultraviolet radiation, was prepared by our laboratory (the culture conditions for the lipase are as follows: microorganism recombinant *Pichia pastoris* yeast KM71H transformed with pPICZ $\alpha$ -*R. oryzae* lipase, agitation 250 rpm, aeration pH 6.0, temperature 30 °C). The subunits of *R. oryzae* lipase showed a molecular mass of about 32 kDa on 12% SDS-PAGE. The protein (83 U/mg) was judged to be homogeneous by SDS-gel electrophoresis as shown in Fig. 1.

The fatty acid composition of soybean oil is as follows: 11.6% palmitic (C16:0), 3.4% stearic (C18:0), 22.8% oleic (C18:1), 54% linoleic (C18:2), 6.6% linolenic (C18:3) and 1.6% other fatty acids. Macroporous resins were provided by Bonchem Co. (Hebei, China). Anion resins were obtained from Bengbu Co. (Anhui, China). The standard methyl esters used for preparing calibration curves and heptadecanoic acid methyl ester used as internal standard were purchased from Sigma

Co. (Beijing, China). All other chemicals were of analytical grade.

### 2.2. Preparation of immobilized lipase

Macroporous resins were firstly treated with 95% ethanol for 4 h and rinsed with deionized water, and then equilibrated with sodium phosphate buffer (0.2 mol/L, pH 7.5). Macroporous resins (0.5 g) and lipase (5 mL) were mixed together at 28 °C for 4 h with a rotational velocity of 160 rpm. These particles above were filtered under reduced pressure and rinsed with phosphate buffer, and then deposited at 4 °C until use.

Anion resins were firstly treated with 50 °C deionized water for 30 min, and then dealt with 1 mol/L NaOH and HCl alternately for three times and equilibrated with sodium phosphate buffer (0.2 mol/L, pH 7.5) in the end. Anion resins (0.5 g) and lipase (5 mL) were mixed together at 28 °C for 4 h with a rotational velocity of 160 rpm. These particles above were filtered under reduced pressure, and then rinsed with phosphate buffer. After the preliminary adsorption, the particles (0.5 g) above were mixed with fresh lipase (5 mL) and diverse amounts of glutaraldehyde (GA, 25%) at 28 °C and shaken at intervals in 20 min. The immobilized lipase was rinsed thrice with 15 mL phosphate buffer, and then deposited at 4 °C until use [2].

### 2.3. Measurement of hydrolytic activity and quantities of protein

The activity of immobilized lipase was determined by a standard olive oil emulsion method as described previously [9]. One unit of activity was defined as the amount of enzyme that liberated 1  $\mu$ mol of free fatty acid in 1 min at 35 °C with pH of 7.5. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard [15]. The apparent enzyme recovery was given by the following equation: apparent enzyme recovery (%) = (apparent activity of immobilized lipase)/(total activity of lipase used)  $\times$  100%.

### 2.4. Enzymatic production of biodiesel

The enzymatic transesterification reactions were carried out in a 50 mL shaking screw-caped bottle at a rate of 160 rpm. Methanol was added to the reaction system in batches and 12 h for one batch. To study the role of water content on methanolysis, the deionized water (0–120% (w/w) by weight of oil) was added to the reaction mixtures. In order to investigate the effect of temperature on enzymatic transesterification, the reactions were conducted at 30, 35, 40, 45 and 50 °C, respectively. Samples (100  $\mu$ L) were taken from the reaction mixture and centrifuged at 9184  $\times$  g for 5 min to obtain the upper layer. Heptadecanoic acid methyl ester which served as the internal standard and an aliquot of the upper layer were precisely measured and mixed thoroughly for GC analysis to determine the contents of fatty acid methyl esters. Each experiment conditions were repeated at least once.

### 2.5. Analysis of fatty acid methyl esters by GC and GC-MS

Reaction products were monitored by gas chromatography/mass spectrometry (GC/MS) and quantitatively determined by capillary column GC. The methyl ester contents in the reaction mixture were quantified using a GC7890A gas chromatograph (Agilent Corp., California, America) connected to a PEG-20M capillary column (0.32 mm  $\times$  30 m  $\times$  0.25  $\mu$ m) and a flame ionizing detector (FID). The injector and detector temperatures were adjusted to 250 and 260 °C, respectively. Nitrogen was used as the carrier gas. The column temperature was maintained at 180 °C for 2 min, and then

increased to 230 °C at the rate of 5 °C/min, and maintained at 230 °C for 20 min.

For GC-MS analysis, sample was injected into a HP-5M capillary column for the determination of methyl ester (ME), free fatty acid (FFA) and monoglyceride (MG) content in the reaction mixture. The temperatures of the injector and ion source were adjusted at 250 and 230 °C, respectively. The column temperature was maintained at 160 °C for 2 min, and raised to 200 °C at the rate of 5 °C/min, then to 210 °C at the rate of 0.5 °C/min, and then to 280 °C at the rate of 5 °C/min in the end, with holding at this temperature for 5 min.

Biodiesel qualitative yield is defined as the percentage of fatty acid methyl esters produced in the reaction mixture versus the total amount of oil used at the beginning of reaction.

#### 2.6. Thin-layer chromatograph analysis for the process of methanolysis

During the lipase-catalyzed methanolysis for biodiesel production, the upper layer of samples were dissolved in hexane and spotted onto a TLC plate to study the process of trans-esterification reaction (Silica Gel F254, China). The plate was developed with hexane/ethyl acetate/acetic acid (90:10:1, v/v). After development, the plate was dried thoroughly, sprayed with a mixture of sulfuric acid and methanol (1:1, by weight) and dried again in the end. The plate was put into an iodine bottle to visualize the spots. At the same time, 1, 2-(2, 3-) and 1,3-diglyceride (DG) intermediates were also analyzed with the plate developed with chloroform/acetone (96:4, v/v)

and dried thoroughly. The spots were visualized as described above.

### 3. Result and discussion

#### 3.1. Methanolysis activity of *R. oryzae* lipase immobilized on different resins

Macroporous and anion resins have been widely used as carriers for the immobilization of enzymes in recent years [7,11,12]. To find a suitable matrix for *R. oryzae* lipase, the immobilized efficiencies of various resins with different immobilized technologies are compared in Table 1. The activities of the immobilized lipases onto macroporous resins by physical adsorption force decreased gradually in the repeated cycles, and moreover, non-polar and weakly polar resins had better effect than polar one. Biodiesel yield declined sharply in the repeated reactions when the lipase was immobilized onto ionomer resins only by chemical adsorption which mainly involves an electrostatic or ionic interaction without the covalent binding of glutaraldehyde. These phenomena may be resulted from the fact that the physical and chemical adsorption force may not couple the resins with *R. oryzae* lipase firmly.

Compared with the immobilized lipases obtained by physical and chemical adsorption force, different conditions happened to immobilized enzyme prepared by covalent binding method. As shown in Table 1, the immobilized lipase onto Amberlite IRA-93 resin obtained higher activity than those onto other anion resins by covalent binding technology using glutaraldehyde as coupling agent. Besides that, this immobilized lipase maintained its activity during three successive batches, which indicated that the enzyme

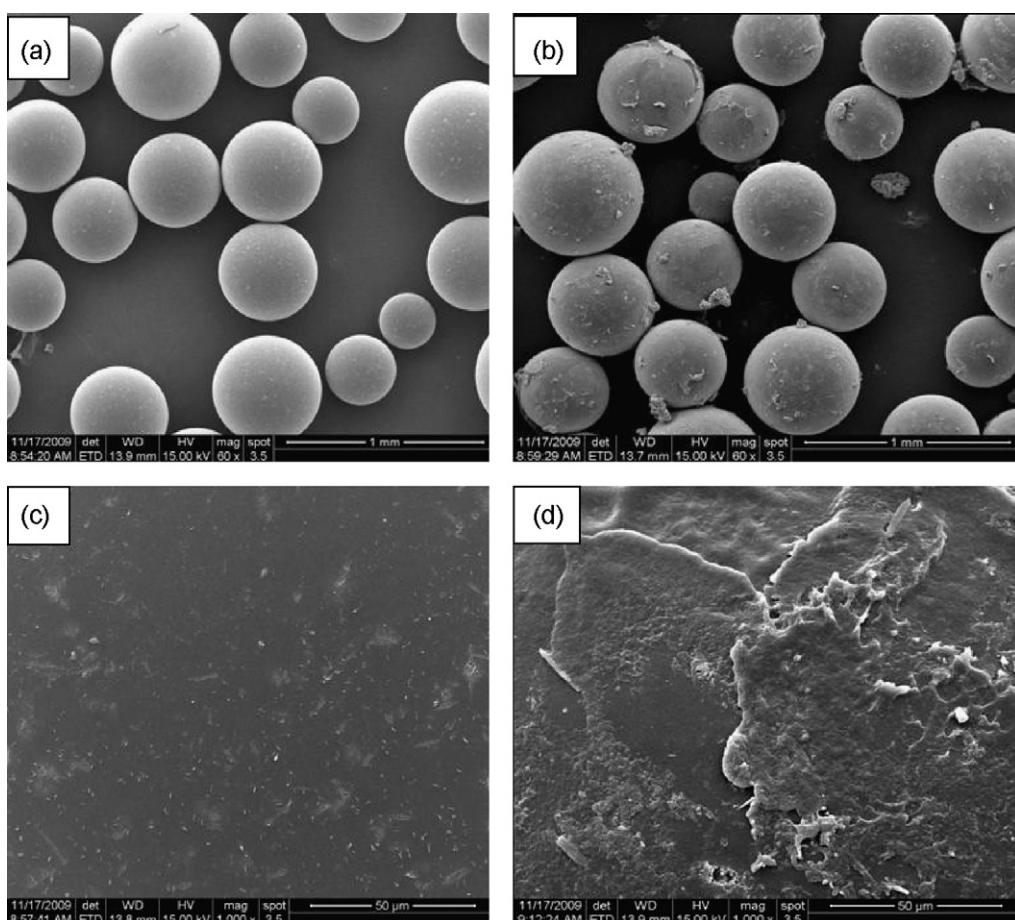


Fig. 2. Photomicrographs of resin Amberlite IRA-93 particles (a and c) before and (b and d) after the immobilization of ROL.

**Table 1**

Methanolysis activity of *Rhizopus oryzae* lipase immobilized on different supports.<sup>a</sup>

Support	Biodiesel yield (%) <sup>a</sup>		
	First cycle	Second cycle	Third cycle
Amberlite IRA-68 <sup>b</sup>	58.1	52.3	38.0
Amberlite IRA-93 <sup>b</sup>	75.3	76.5	76.2
DUoliteA-30B(330) <sup>b</sup>	60.9	54.6	44.2
DUoliteA-30B(339) <sup>b</sup>	52.3	44.4	32.5
Amberlite IRA-68 <sup>c</sup>	30.5	1.8	—
Amberlite IRA-93 <sup>c</sup>	37.2	2.4	—
ADS-7 <sup>d</sup>	38.6	—	—
XAD-2 <sup>d</sup>	74	71.3	66.5
ADS-17 <sup>d</sup>	78.7	76.2	70.5

<sup>a</sup> Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4:1, enzyme dosage 24 U/g, 40% water (pH 7.5) by weight of oil, 35 °C and 180 rpm for 48 h. Methanol was added into the system at four-stepwise addition means.

<sup>b</sup> Lipase was immobilized onto resin by covalent bidding technology.

<sup>c</sup> Lipase was immobilized onto resin by chemical adsorption technology.

<sup>d</sup> Lipase was immobilized onto resin by physical adsorption technology.

**Table 2**

Effect of glutaraldehyde concentration on the immobilization.<sup>a</sup>

Glutaraldehyde concentration (v/v%)	Biodiesel yield <sup>a</sup> (%)	Hydrolytic activity (U/g)
0.25	68.5	129
0.5	72.7	144
1.0	70.4	123
1.5	66.5	112
2.0	60.4	108
2.5	55.5	107

<sup>a</sup> Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4:1, enzyme dosage 24 U/g, 50% water (pH 7.5) by weight of oil, 35 °C and 180 rpm for 48 h. Methanol was added into the system at four-stepwise addition means.

was firmly immobilized onto support by covalent attachment. On the contrary, the immobilized lipases onto cation resins by covalent attachment lost their activity gradually in the consecutive cycles. This may be attributed to the fact that different functional groups and skeleton structures of diverse ionomer resins lead to different characters of immobilized lipases. Therefore, Amberlite IRA-93 resin was the most proper carrier to immobilize *R. oryzae* lipase in the screening test. Fig. 2 shows the SEM micrographs of Amberlite IRA-93 resin before and after immobilization.

### 3.2. Optimization of the immobilization procedure

The covalent attachment between anion resin Amberlite IRA-93 and *R. oryzae* lipase was carried out after the preliminary chemical adsorption between the carrier and enzyme. Glutaraldehyde (GA) which can react with amino, phenol, thiol and imidazole groups of proteins has been used as a coupling agent by many authors in the immobilization of enzymes onto many matrixes [10,16,17]. Therefore, glutaraldehyde was used to connect lipase with anion resin in the process of covalent binding in this paper. Proper amount of GA is vital to the efficiency of immobilization. Inadequate amount of GA cannot attach all the lipase onto carrier, whereas excessive amount of GA may inactivate the lipase. As shown in Table 2, the highest biodiesel yield was obtained at the GA concentration of 0.5% based on lipase volume. GA concentration higher or lower than 0.5% would decrease the activity of immobilized lipases. Therefore, GA concentration of 0.5% was suitable for the covalent binding process between lipase and anion resin. It is worth noting that the duration of immobilization is of equal importance. The fatty acid methyl esters yield and hydrolytic activity of lipase increased quickly while immobilized time ranged from 10 to 20 min and decreased gradually when the duration was over 20 min in Table 3. The optimal enzymatic activity of immobilized lipase was 168 U/g

**Table 3**

Effect of glutaraldehyde covalent binding time on the immobilization.<sup>a</sup>

Covalent binding time (min)	Biodiesel yield <sup>a</sup> (%)	Hydrolytic activity (U/g)
10	60.4	121
15	70.3	155
20	72.5	168
25	69.1	158
30	67.0	148
35	66.6	141
40	62.2	140
45	60.4	137

<sup>a</sup> Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4:1, enzyme dosage 24 U/g, 40% water (pH 7.5) by weight of oil, 35 °C and 180 rpm for 48 h. Methanol was added into the system at four-stepwise addition means.

under the optimal conditions and the apparent enzyme recovery was 21%.

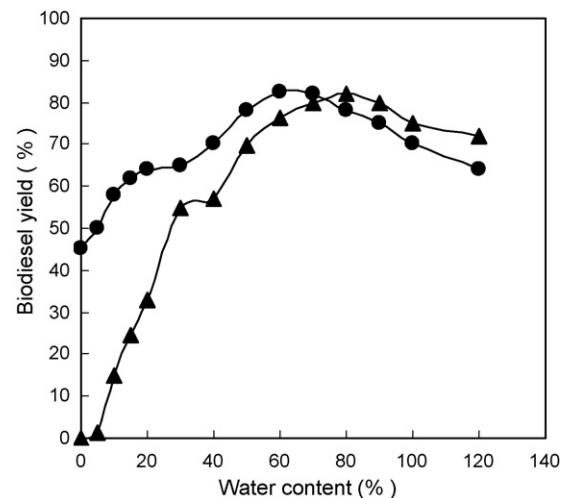
### 3.3. Optimization of transesterification reaction

#### 3.3.1. Effect of added water on biodiesel yield

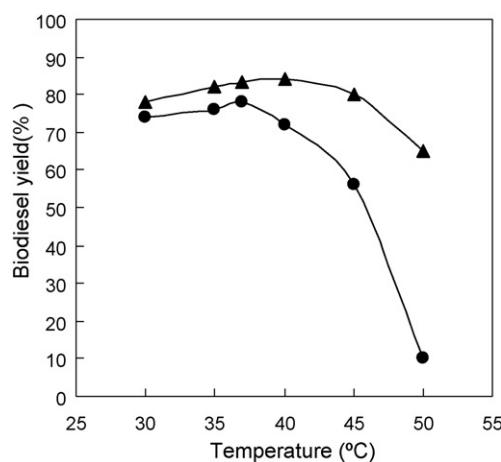
Lipase is one kind of special enzymes acting at the interface between the aqueous and organic phase. The activation of lipase includes unmasking and restructuring the active site of enzyme molecule, which requires the presence of oil–water interface [18]. The water content in the reaction mixture is vital to the activation of enzyme, which will ultimately affect the activity of lipase. Only proper amount of water in the reaction medium could maximize the catalytic activity of lipase.

Results presented in Fig. 3 show that the free lipase activity was very poor at low water content in the transesterification reaction, which might be resulted from the fact that a small amount of water in the system did not activate the enzyme completely. The conversion of biodiesel increased considerably with the increase of water content, which indicated that the free lipase was greatly activated by the added water. The maximum biodiesel yield was achieved at the water content of 80% by weight of oil when the free lipase was used in the reactions. This result was similar to those of the previous researches [4,19]. However, there was a gradual decrease in the yield of methyl esters when water content exceeded 80%, which may be attributed to the effect of hydrolysis reaction.

The immobilized lipase had the homothetic catalytic trend compared with the free one in the water range of 10–120% and got its



**Fig. 3.** Effect of added water (pH 7.5) on biodiesel yield, Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4:1, enzyme dosage 24 U/g, 35 °C and 180 rpm for 48 h. Methanol was added into the system at four-stepwise addition means. (●) Immobilized lipase; (▲) free lipase.



**Fig. 4.** Effect of temperature on the immobilized and the free lipase activities. Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4:1, enzyme dosage 24 U/g, 60% water (pH 7.5) by weight of oil and 180 rpm for 48 h. Methanol was added into the system at four-stepwise addition means. (▲) Immobilized lipase; (●) free lipase.

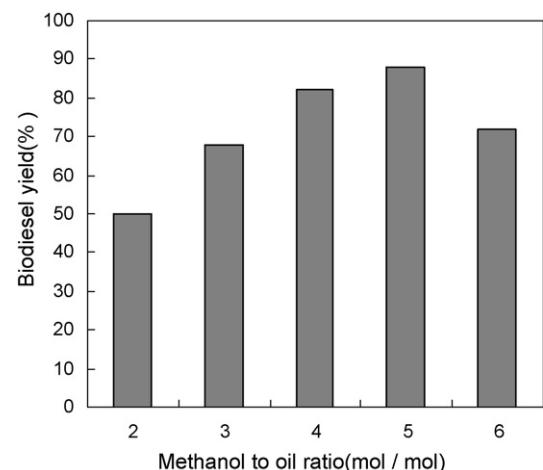
optimal activity at water content of 60%. It should be illustrated that the immobilized lipase used in the methanolysis contains a little water (2.5–5% by weight of oil), which may affect the water content in the reaction system and result to the alternation of reaction equilibrium. Therefore, the fatty acid methyl esters in the products still reached about 45% without any added water to the reaction system in Fig. 3.

### 3.3.2. Effect of temperature on biodiesel yield

Experiments were performed to examine the effect of temperature on the catalytic activities of the free and the immobilized lipases in the methanolysis reactions of soybean oil with methanol. It was observed that the immobilized lipase had wide temperature adaptability compared with the free one during the reaction temperature between 30 and 50 °C (Fig. 4). Although the optimum temperature for the free and the immobilized lipases were very close, 35 °C for the free lipase and 37 °C for the immobilized one, respectively, the thermal stability at high temperature was apparently different. Particularly, the free lipase only retained 10% of its activity when the transesterification reaction was carried out at 50 °C for 60 h. On the contrary, the immobilized lipase still maintained about 77% of its activity under the same conditions. Therefore, the method of immobilization seems to improve the thermal stability of lipase at high temperature. Considering the catalysis efficiency and thermal stability of lipase, 37 °C was chosen as the optimal reaction temperature for the transesterification catalyzed by immobilized lipase.

### 3.3.3. Effect of methanol concentration and stepwise addition manners on biodiesel yield

The effect of methanol to oil molar ratio on biodiesel conversion was investigated since it is a vital parameter in the enzymatic transesterification reaction. Although three molar equivalent of methanol are required for the complete conversion of soybean oil to its corresponding fatty acid methyl esters, excessive methanol are needed to promote the reaction equilibrium towards products in practice. Experiments were performed at different molar ratios of methanol to oil ranging from 3:1 to 6:1. The biodiesel yield increased from 68% to 88% at the molar ratio changing from 3:1 to 5:1 and decreased to 72% at the molar ratio of 6:1 in Fig. 5. It was found that the biodiesel yield reached maximum level at methanol to oil molar ratio of 4.8:1 in the further experiments (data



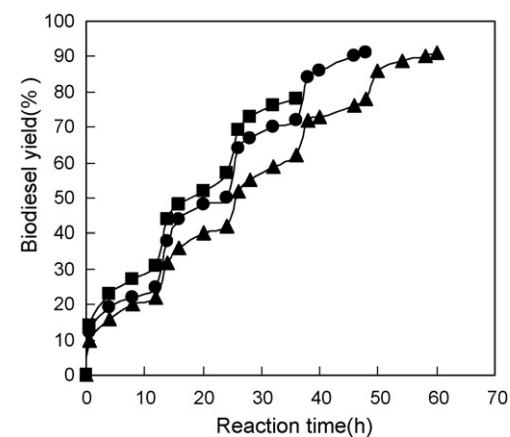
**Fig. 5.** Effect of methanol concentration on biodiesel yield. Reaction conditions: 5 g soybean oil, enzyme dosage 24 U/g, 60% water (pH 7.5) by weight of oil and 180 rpm for 48 h. Methanol was added into the system at five-stepwise addition means.

not shown in the paper). Therefore, the molar ratio of 4.8:1 was considered as optimum.

Because of the poor solubility of methanol in vegetable oils, which may lead to the loss of enzyme activity, methanol should be added to the reaction system in batches [20]. Different kinds of methanol additional manners were compared for the sake of decreasing the negative effect of methanol to lipase and shortening the transesterification reaction time. Compared with the four-stepwise additional means, the three-stepwise one resulted in a higher initial reaction velocity but a lower biodiesel yield, which could be found in Fig. 6. This phenomenon may be attributed to the fact that excessive insoluble methanol inhibit the activity of immobilized lipase at the three-stepwise means. On the other hand, the four-stepwise addition manner had the same catalysis effect as the five-stepwise one and shortened the reaction time compared with the latter. Therefore, the four-stepwise technology was used as the methanol addition method for further investigations. The highest biodiesel yield was 90.5% at the four-stepwise addition manner.

### 3.3.4. Effect of additives on biodiesel yield

Because of the significant effect of acyl migration on biodiesel yield in the transesterification catalyzed by *R. oryzae* lipase, some



**Fig. 6.** Effect of methanol additional manner on biodiesel yield. Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4.8:1, enzyme dosage 24 U/g, 60% water (pH 7.5) by weight of oil, 37 °C and 180 rpm. Methanol was added into the system at three-, four-, and five-stepwise addition means, respectively. (■) Three-stepwise means; (●) four-stepwise means; (▲) five-stepwise means.

**Table 4**Effect of additives affecting biodiesel contents in the products.<sup>a</sup>

Additive/(0.10 g/g <sub>oil</sub> )	Characteristics	Biodiesel yield <sup>a</sup> (%)
Crude lipase		82.2
Silica gel for thin-layer chromatography	Solid additive	2.5
Amberlite IRA-68	Anion resin	89.8
Amberlite IRA-93	Anion resin	86.4
DUoliteA-30B	Cation resin	83.5
DUoliteA	Cation resin	90.2
ADS-7	Polar resin	83.2
XAD-2	Weakly polar resin	87.4
ADS-17	Non-polar resin	88.8

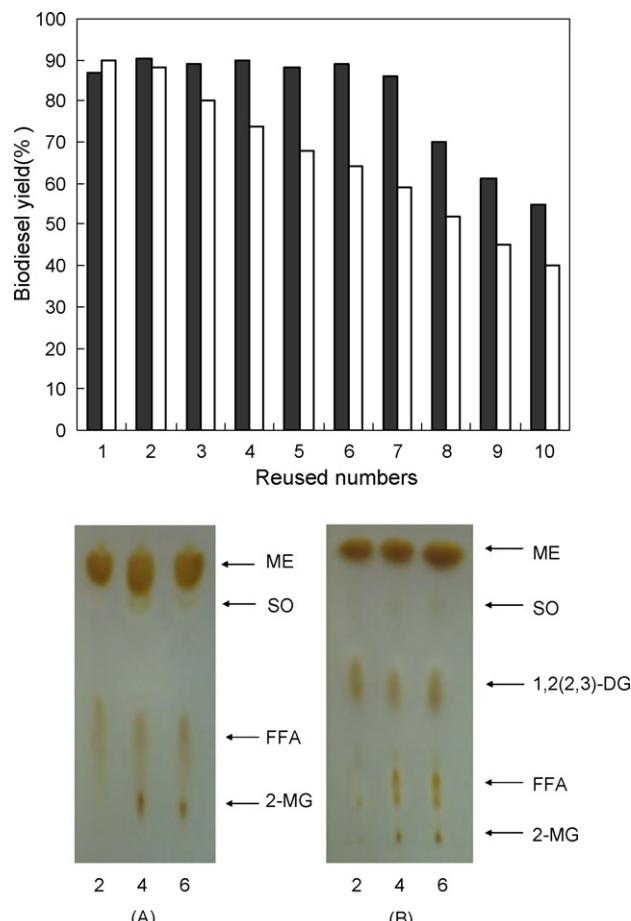
<sup>a</sup> Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4.8:1, 3 mL free ROL (240 U), 0.5 g additives, 35 °C and 180 rpm for 48 h. Methanol was added into the system at four-stepwise addition means.

additives may capable of enhancing the acyl migration were investigated. It has been reported that the acyl migration can be affected by some additives such as resin and silica [13]. Wang et al. studied the effect of different kinds of additives on acyl migration and found that triethylamine (TEA), resins, quartz sand and silica gel had a positive impact on acyl migration [14]. Du et al. also reported that 6% silica gel added into the reaction improved about 24% biodiesel yield when 4% lipozyme TL was used to catalyze the methanolysis reaction [5].

Therefore, different kinds of additives were tested for their effects on the acyl migration in the lipase catalyzed transesterification of soybean oil. In this experiment, the concentration of additives was used with 0.1 g/g<sub>oil</sub>. As shown in Table 4, different additives added to the reaction had different effects on biodiesel yield. Unlike the conclusion reported by Du et al., silica gel had a negative effect on biodiesel conversion, which might be attributed to the hygroscopicity of it, thereby decreasing the promotion of acyl migration. However, all of the resins used as additives had a positive effect on biodiesel yield and improved about 1–8% yield of biodiesel. Since anion resin Amberlite IRA-93 which was used as the immobilized support for *R. oryzae* lipase per se had a positive effect on the conversion of biodiesel, it would not need any other additives added into the reaction system to improve the acyl migration.

#### 3.4. Reusability of immobilized lipase in solvent free system

The reusability of immobilized lipase is very important to the enzymatic transesterification in biodiesel production. Therefore, the reusability of two kinds of immobilized lipases is compared in Fig. 7. The immobilized lipase after each batch was washed with phosphate buffer and used in the next batch with fresh substrate. It was found that the immobilized lipase onto anion resin Amberlite IRA-93 almost maintained its full activity during 7 consecutive batch reactions, each lasting for 48 h at 37 °C. The TLC analysis of the transesterification catalyzed by immobilized *R. oryzae* lipase also indicated that most of the oils were transferred into fatty acid methyl esters and the immobilized lipase kept its activity in the reaction cycles. However, under the same conditions, the immobilized enzyme onto macroporous resin lost its activity gradually. This may be resulted from the fact that some lipase dropped out of macroporous resin in the repeated experiments, while lipase onto anion resin Amberlite IRA-93 was firmly fixed by covalent binding technology. Therefore, it was indicated that the covalent binding technology used in the immobilization of *R. oryzae* lipase was more effective than the physical or chemical adsorption method.



**Fig. 7.** The reusability of the immobilized lipases. Reaction conditions: 5 g soybean oil, methanol to oil molar ratio 4.8:1, enzyme dosage 24 U/g, 60% water (pH 7.5) by weight of oil, 37 °C and 180 rpm for 48 h. Methanol was added into the system at four-stepwise addition means. TLC analysis: (A) the plate was developed with hexane/ethyl acetate/acetic acid (90:10:1, v/v); (B) the plate was developed with chloroform/acetone (96:4, v/v). Lanes 2, 4, and 6 stand for the second, fourth and sixth cycles of transesterification, respectively. (■) Amberlite IRA-93; (□) ADS-17.

#### 4. Conclusion

The present study demonstrated the successful immobilization of *R. oryzae* lipase onto anion resin Amberlite IRA-93 by covalent binding technology. The immobilized material, anion resin Amberlite IRA-93 had an accelerating effect on the acyl migration and improved biodiesel yield of 4.2%. This immobilized lipase was used to catalyze transesterification reaction in biodiesel production. The maximum biodiesel yield of 90.5% could be achieved with 24 U/g immobilized lipase for the methanolysis. The immobilization of lipase increased its thermal stability and reusability. The reusability characteristic shows that the immobilized enzyme remains its activity after being used repeatedly for 7 consecutive batches with each of 48 h. These results suggest that this method for immobilization of *R. oryzae* lipase has potential in industrial applications for biodiesel production.

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## References

- [1] N. Dizge, B. Keskinler, *Biomass Bioenergy* 32 (2008) 1274–1278.
- [2] A. Salis, M.S. Bhattacharyya, M. Monduzzi, V. Solinas, *J. Mol. Catal. B: Enzym.* 57 (2009) 262–269.
- [3] K. Ban, M. Kaieda, T. Matsumoto, A. Kondo, H. Fukuda, *Biochem. Eng. J.* 8 (2001) 39–43.
- [4] M. Kaieda, T. Samukawa, T. Matsumoto, K. Ban, A. Kondo, Y. Shimada, H. Noda, F. Nomoto, K. Ohtsuka, E. Izumoto, H. Fukuda, *J. Biosci. Bioeng.* 88 (1999) 627–631.
- [5] W. Du, Y.Y. Xu, D.H. Liu, Z.B. Li, *J. Mol. Catal. B: Enzym.* 37 (2005) 68–71.
- [6] N. Dizge, C. Aydiner, D.Y. Imer, M. Bayramoglu, A. Tanriseven, B. Keskinler, *Biore-sour. Technol.* 100 (2009) 1983–1991.
- [7] Y. Gao, T.W. Tan, K.L. Nie, F. Wang, *Chin. J. Biotechnol.* 22 (2006) 114–118.
- [8] O. Orcaire, P. Buisson, A.C. Pierre, *J. Mol. Catal. B: Enzym.* 42 (2006) 106–113.
- [9] Z. Knezevic, S. Bobic, A. Milutinovic, B. Obradovic, L. Mojovic, B. Bugarski, *Proc. Biochem.* 38 (2002) 313–318.
- [10] N. Dizge, B. Keskinler, A. Tanriseven, *Colloids Surf. B* 66 (2008) 34–38.
- [11] E.J. Tomotani, M. Vitolo, *Proc. Biochem.* 41 (2006) 1325–1331.
- [12] T. Nagao, Y. Shimada, A. Sugihara, A. Murata, S. Komemushi, Y. Tominaga, *JAOCs* 78 (2001) 167–172.
- [13] X.B. Xu, *Eur. J. Lipid Sci. Technol.* 102 (2000) 287–303.
- [14] Y. Wang, H. Wu, M.H. Zong, *Bioresour. Technol.* 99 (2008) 7232–7237.
- [15] A. Salis, M. Pinna, M. Monduzzi, V. Solinas, *J. Mol. Catal. B: Enzym.* 54 (2008) 19–26.
- [16] N. Dizge, B. Keskinler, A. Tanriseven, *Biochem. Eng. J.* 44 (2009) 220–225.
- [17] M.L. Foresti, M.L. Ferreira, *Enzyme Microb. Technol.* 40 (2007) 769–777.
- [18] H. Noureddini, X. Gao, R.S. Philkana, *Bioresour. Technol.* 96 (2005) 769–777.
- [19] G.Y. Chen, M. Ying, W.Z. Li, *Appl. Biochem. Biotechnol.* 129 (2006) 911–912.
- [20] S. Hama, H. Yamaji, T. Fukumizu, T. Numata, S. Tamalampudi, A. Kondo, H. Noda, H. Fukud, *Biochem. Eng. J.* 34 (2007) 273–278.