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Rhizomucor miehei Lipase Immobilized on Macroporous Resin and its Application in Biodiesel Synthesis

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Abstract

In order to achieve the industrialization of lipase-catalyzed biodiesel production, economic and effective carriers for immobilized lipase need to be exploited. In this study, *Rhizomucor miehei* lipase (RML) was immobilized on different kinds of macroporous resins and NKA was found to be the best carrier. The immobilized RML was then employed to catalyze biodiesel production from soybean oil. 8 different organic solvents were used as media in the trans-esterification reactions. Isooctane was proved to be the optimal one. Subsequently, immobilization conditions and reaction conditions were further optimized through single factorial experiments and response surface methodology (RSM). Results were as follows: the recovery activity of the immobilized RML was up to 1531.7%; the corresponding specific activity could reach 73761.99 U/g protein, almost 15 fold than that of the free RML; the biodiesel yield attained 99.2% under the optimal conditions in isooctane system; the immobilized lipase can yield 64% product after being run for 4 cycles, much better than the commercial immobilized lipase, Lipozyme RM IM (remains only 31%). In conclusion, NKA is a promising carrier for the immobilization of RML and exhibits a good performance in biodiesel production compared with Lipozyme RM IM.

Keywords: *Rhizomucor miehei* lipase; NKA; Immobilization; Lipozyme RM IM; Biodiesel yield

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Introduction

Lipase from *Rhizomucor miehei* can catalyze reactions of hydrolysis [1,2], acidolysis [2,3], esterification [4,5] and trans-esterification [6]. It has been extensively applied in food industry [7], organic synthesis, pharmaceuticals production [8] and biodiesel fields [6]. Among these applications, biodiesel is an effective substitute to conventional fossil fuel owing to its environmental friendliness, safety, renewability, superior combustion efficiency and biodegradability. It can be easily produced by trans-esterification between vegetable oils or animal fats and short chain alcohols, such as methanol and ethanol. Nowadays, biodiesel preparation via lipase catalysis has drawn particular attention [9] due to its convenient pretreatment on feedstock, environmental friendliness, nontoxicity, reuse of catalyst and mild operating conditions compared with chemical catalysis method or physical method [10]. In addition, compared with free lipase, immobilized lipase has a good reusability and stability, resulting in production cost saving.

So far, different kind of materials are used for the immobilization of lipases, such as silica nanoparticles [11], nanozeolites [12], carbon nanotubes [13], silica gel particles [14], hybrid nanospheres [15], octyl-glyoxyl agarose beads [16], chitosan microspheres [17] and even cells [18]. Among them, macroporous resins have been proved to be an efficient carrier for lipases used in biodiesel production [19-21]. For example, Lipozyme RM IM (*R. miehei* lipase immobilized in macroporous anion exchange resins) was reported in the synthesis of conventional biodiesel [22] though its recyclability was not good. Moreover, with the rapid development of new materials technologies, there appear

hundreds of new resins. It can be assumed that RML may perform better when immobilized on some new supports used for biodiesel synthesis. It has been reported that using hydrophobic carriers for immobilized lipase obtained better catalytic properties than using hydrophilic ones [23,24]. The surface of the hydrophobic carrier has a similar properties compared with the substrates of lipase and that will cause interfacial activation of the lipase. The hydrophobic carriers are helpful to expose the hydrophobic regions of the catalytic center through interfacial activation. Simultaneously, hydrophobic groups on the carriers contribute to increase the concentration of lipophilic substrate around the enzyme molecule and that is beneficial to improve catalytic activity of lipase [25,26].

In this work, different kinds of macroporous resins and ion exchange resins were examined to compare the immobilization efficiency, specific activity and activity recovery. After getting the optimal carrier, the immobilization conditions were further optimized. Then, the immobilized lipase was used in biodiesel production. Different organic solutions were selected as reaction media, and the reaction conditions were also optimized. Furthermore, the immobilized RML were compared with the commercial Lipozyme RM IM in different reaction systems.

Materials and Methods

Materials

Palatase 20000 L, bovine serum albumin (BSA), aliphatic ester standards and coomassie brilliant blue G250 were bought from SigmaAldrich (St. Louis, MO, USA). Lipozyme RM IM (lipase from *R. miehei*) were purchased from Nov Norvisk (Bagsvaerd, Denmark). Macroporous resin (including D4020, D3520, AB-8, NKA), macroporous weakly acidic acrylic cationic exchange resin (including D152H, D151H, D113), macroporous weakly basic cinnamic anion exchange resin (including D380, D301R, D311), and macroporous weakly basic styrene type chelating resin (including D401, D418) were got from Tianjin Nankai Science & Technology Co. Ltd. (Tianjing, China). (Their detailed information was listed in **Table 1**). Soybean oil was bought from the local supermarket. Other reagents, such as medicinal alcohol, lauric acid, laurinol, tetrahydrofuran, tert-Butanol, cyclohexane, n-hexane, n-heptane, petroleum ether, octane, isooctane, NaOH, hydrochloric acid, phenolphthalein, ethanol, acetone, K_2HPO_4, KH_2PO_4 were of analytical grade and got from the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Methods

Lipase immobilization: RML was immobilized on different kinds of macroporous resins through physical adsorption and ion exchange interaction. Macroporous resins were screened and one of them named NKA was found to be the best carrier. The RML solution and carriers were dispersed in phosphate buffer. During the optimization of the immobilization conditions, the enzyme loading (0.2 mL/g RML-support to 2.0 mL/g RML-support), immobilization time (10-60 min), immobilization temperature (20-45°C), pH value (pH 6-8, see **Table 2**) on the effect of immobilization efficiency, specific activity and activity recovery of the immobilized lipase were examined. After immobilization, the immobilized lipase was washed by the same buffer. All of the solutions were collected to get the residual protein content in the supernatants via the method of Bradford using bovine serum albumin (BSA) as the standard protein [27]. The immobilized lipase (RML-NKA) was dried in a vacuum drier and stored at 4°C for later use.

Enzyme assays: The esterification reaction between lauric acid and 1-dodecanol was used to measure the enzyme activity of the free lipase and the immobilized lipase. A brief depiction is presented in the Supporting Information.

Measurement: The FT-IR spectra of RML solution, NKA and RML-NKA were obtained in transmission mode on a Fourier-transform infrared spectroscopy (Bruker, VERTEX 70, Germany) using the KBr pellet technique.

Biodiesel production and GC analysis: Biodiesel production and GC analysis methods were described in our previous work [20,28,29]. The methods of biodiesel production, preparation of fatty acid alkyl esters (biodiesel) samples for GC analysis and calculation of biodiesel yield are presented in the Supporting Information. The samples were measured in the GC-9790 gas chromatograph (Fuli Analytical Instrument Co. Ltd., Wenlin, China). The column is an Agilent INNOWAX capillary column (30 m × 0.25 mm i.d. × 0.25 μm, J and W Scientific, Folsom, CA).

Statistical analysis: All experiments were conducted in three parallel replicates. The data were analyzed using SAS 9.2, and the graphs were plotted using Origin 9.0.

Results

Effect of immobilization carriers

12 different kinds of carriers on the effect of the immobilized RML were selected and macroporous resin NKA was found to be the best one. The immobilization efficiency, specific activity and activity recovery were 94.96%, 46977.15 U/g protein and 970.50%. According to the results of **Figure 1**, the immobilization efficiencies of 4 kinds of macroporous resins were more than 90%. The other resins were between 73%-87%. As for the specific activity and activity recovery, NKA, D3520, D301R showed better results, but D113, D311, D401 and D418 did not show very high esterification activities. The good performance of NKA is ascribed to the hydrophobic properties, relatively larger average pore diameter (≥ 20 nm) and higher specific surface area [19,30].

Characterization of RML solution, NKA and RML-NKA

The FT-IR spectra of RML solution, NKA and RML-NKA are shown in **Figure 2**. Spectra of both NKA and RML-NKA contained characteristic absorption bands at 2850 and 2927 cm^{-1} due to the aliphatic C–H stretching [31], bands at 3025 and 3058 cm^{-1} due to the aromatic C–H stretching, bands at 1450-1600 cm^{-1} owing to the aromatic C=C stretching, and bands at 700-890 cm^{-1} assigned to aromatic out-plane bending [32]. Compared with the spectra of NKA, the peak at 1650 cm^{-1} was for amide I of RML-NKA and RML solution, indicating the presence of enzyme in NKA [13].

Table 1 The specific information about different macroporous resins.

Type	Structure	Functional group	Bead size (mm)	Surface area (m^2/g)	Pore diameter (Å)
D4020	Cross linked -Polystyrene	—	0.3-1.25	540-580	100-105
D3520	Cross-linked -Polystyrene	—	0.3~1.25	480-520	85-90
AB-8	Cross-linked -Polystyrene	—	0.3~1.25	480-520	130-140
NKA	cross-linked -Polystyrene	—	0.3~1.0	570-590	200-220
D152H	Acrylic-DVB	-COOH	0.315~1.25(≥ 95%)	—	—
D151H	Acrylic-DVB	-COOH	0.315~1.25(≥ 95%)	—	—
D113	Acrylic-DVB	-COOH	0.315~1.25(≥ 95%)	—	—
D380	Styrene-DVB	$-NH_2$	0.315~1.25(≥ 95%)	—	—
D301R	Styrene-DVB	$-N(CH_3)_2$	0.315~1.25(≥ 95%)	—	—
D311	Acrylic-DVB	$-NHCH_3$	0.315~1.25(≥ 95%)	—	—
D401	Styrene-DVB	$-N(CH_2COONa)_2$	0.315~1.25(≥ 95%)	—	—
D418	Styrene-DVB	$-NHCH_2PO_3Na_2$	0.315~1.25(≥ 95%)	—	—

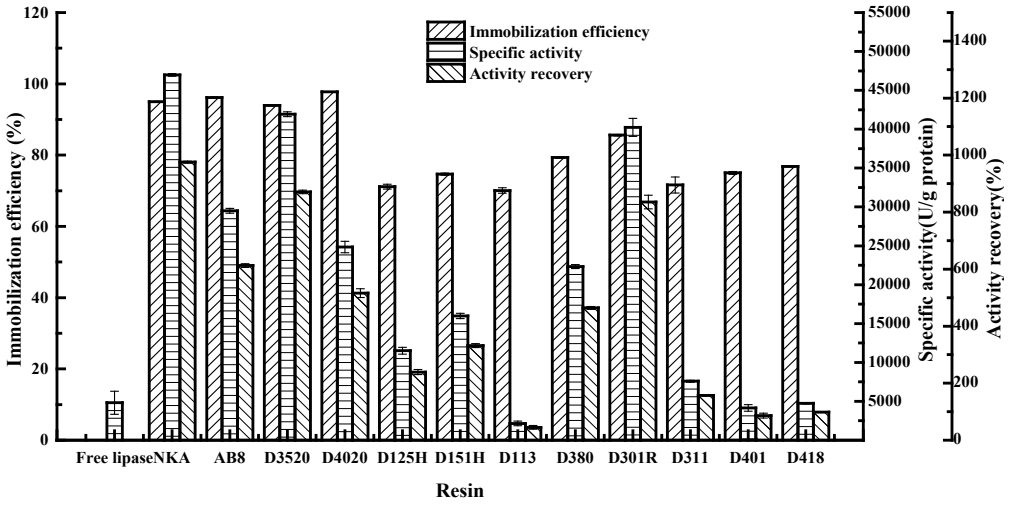

Figure 1 Effect of different kinds of supports on immobilization efficiency, specific activity and activity recovery (conditions: enzyme loading, 0.6 mL/g RML-support; immobilization time, 60 min; buffer, pH 7; immobilization temperature, 35°C; stirring speed, 200 rpm).

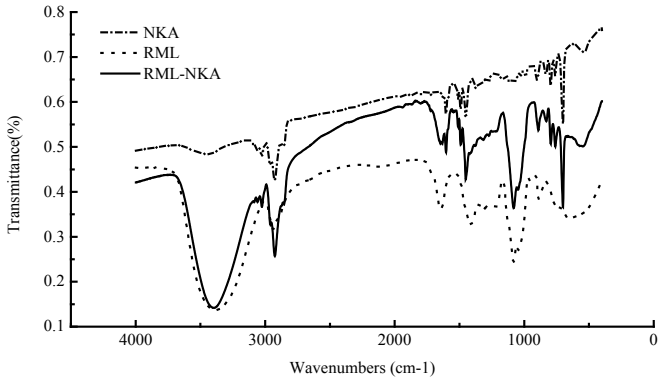

Figure 2 The FT-IR spectra of RML solution, NKA and RML-NKA.

Effect of immobilization conditions

In this part, 4 main factors of the immobilization conditions were selected to test their effects on the immobilized lipase, RML-NKA. The factors were enzyme loading, immobilization time, temperature and pH value of the immobilization buffer.

Effect of enzyme loading: The effect of enzyme loading was shown in **Figure 3a**. It is observed that the optimal enzyme loading was 0.6 mL/g RML-NKA, at which adding amount the specific activity and activity recovery were the highest. When adding more enzyme solution into the immobilization system, the immobilization efficiency declined but the specific activity and activity recovery first reached to 57470.41 U/g protein and 1193.40% and then dropped down which is owning to the diffusion limitation [33]. The hydrophobic support NKA had a very good performance of adsorption which could led to the multilayer adsorption of RML. Multilayer adsorption increased diffusion limitation due to the reduction of porous diameter of NKA [34,35].

Effect of immobilization time: As shown in **Figure 3b**, the immobilization time of 30 min was chosen as the optimal time, because as the immobilization became longer, the immobilization efficiency increased but the specific activity and the activity

recovery decreased. When immobilization process operated for 30 min, almost 90% of the protein inside the solution was adsorbed on NKA, and the specific activity and activity recovery decreased very little. The overall activities of RML-NKA at 10, 20, 30, 40, 60 min were 52067.93, 52350.65, 52798.29, 51852.26 and 51528.76 U/g protein, respectively and that of 30 min had the highest value. The loss of specific activity and activity recovery was caused by RML's instability in aqueous phase [20]. Although some other literatures reported that the reaction duration was around 1 h [33,36], we chose 30 min as the optimal immobilization time to get a balance between activity and immobilization efficiency.

Effect of immobilization temperature: The temperature changing from 20°C-45°C on the performance of RML-NKA was investigated. When the system was tested at 25°C, the specific activity and activity recovery reached to 73503.01 U/g proteins and 1526.32% as shown in **Figure 3c**. According to the general trend of activity variation, when increasing the immobilization temperature, thermal motion between NKA and protein accelerated which led to the increase of immobilization efficiency. But low temperature was better for maintaining the right conformation of RML. So, a compromise was needed and 25°C was set as the optimal immobilization temperature.

Effect of the pH value of the immobilization buffer: When the RML solution and NKA mixed with the phosphate buffer solution of different pH (from 6.0-8.0), the immobilization efficiency did not change too much. **Figure 3d** showed that the specific activity and activity recovery were respectively 73736.29 U/g proteins and 1531.17% when the pH was 7. These results showed that the microenvironment provided by the buffer had a great influence on the activity of RML-NKA and a neutral pH condition seemed to benefit RML when immobilized onto hydrophobic macroporous materials [33].

Optimization of the immobilization conditions through RSM: As shown in **Figure 3b**, the immobilization time has a negative effect on specific activity and activity recovery. So enzyme loading, immobilization temperature and pH value of buffer were selected as three independent factors. SAS 9.2 was employed to analyze the data using Box-Behnken design. The experimental design, results of activity recoveries and analysis of variance are provided in the "Supporting Information" in details.

The optimal levels for the three independent factors calculated from the regression model were: immobilization temperature, 24.11°C, enzyme loading, 0.58 mL/g RML-NKA, pH value of buffer, 6.90. The predicted activity recovery of the RML-NKA was 1516.20% under the optimal immobilization conditions. In order to verify the validation of the model, experiments were performed in triplicate to test the activity recovery. The average activity recovery was 1531.71%, which coincided with the predicted value, and the associated specific activity was 73761.99 U/g proteins.

Effect of different solvent on the biodiesel yield

The effects of 8 different solvents on the biodiesel yield of RML-NKA were studied in the trans-esterification reactions. The results are shown in **Table 2.**

According to **Table 3**, the biodiesel yields were totally different. There were no clear relationship between biodiesel yield and log *P*. But it could still be seen that most of the hydrophobic solvents were better than the hydrophilic ones. Among all of the 8 solvents, only octane and isooctane were better than the solvent free system. Isooctane was selected as the solvent used in trans-esterification reactions of soybean oil and ethanol.

Effect of reaction conditions on the biodiesel yield

Effect of acyl acceptors on the biodiesel yield: Methanol and ethanol were used as acyl acceptors respectively. When adding methanol in one step, there was even no activity in the trans-esterification reaction of RMK-NKA. The result of ethanol was shown in **Figure 4** and **Figure 5a**. When increasing the adding steps, the biodiesel yield was improved, too. So, ethanol added at 3 steps was used in the later experiment. As for the molar ratio of ethanol/oil, the result was shown in **Figure 5a**. It could be seen that 4:1 had the highest biodiesel yield. Increasing the amount of short chain alcohol could accelerate the reaction rate, at the same time, it also inactivated the lipase. Consequently, a slight excess of ethanol was necessary to achieve a satisfactory biodiesel yield [28].

Effect of immobilization lipase dosage on biodiesel yield: In order to investigate the immobilization lipase dosage on the effect of biodiesel yield, 10 mg-400 mg of RML-NKA (protein content is 0.86%) were respectively added into 2.19 g soybean oil in isooctane system. 0.58 mL ethanol were used as acyl acceptors, added at 3 steps. The results were presented in **Figure 5b**. Biodiesel yield became stable when the immobilization lipase dosage was more than 9.13 wt% (based on oil weight). Through integrated into account, 9.13 wt% was considered as the best one which could get a biodiesel yield of 97.24%.

Effect of reaction time on biodiesel yield: Time course curve **(Figure 5c)** showed the general information of biodiesel yield within 72 h. Over 98.72% of the fatty acid ethyl esters (FAEEs) formed within 12 h, and then tended towards stability. Ethanol was added into the system at 3 steps at the same interval. Reaction time longer than 12 h did not improve the biodiesel yield on account of the formation of a relative equilibrium conversion.

Effect of isooctane amount on biodiesel yield: The highest biodiesel yield was achieved when adding 0.50 ml/g oil of the isooctane into the reaction system which could be seen in **Figure 5d**. When increasing the isooctane amount, the biodiesel yield started to decrease gradually. Isooctane in the reaction system can increase the solubility of FAEEs and triglyceride. That is why isooctane system is better than solvent free system (seen in **Table 2**) [28]. However, when adding too much isooctane into the reaction system, the concentration of substrates would decline which led to the decrease of biodiesel yield.

Effect of water content on biodiesel yield: The influence of water content on the biodiesel yield was shown in **Figure 5e**. It could be seen when water content changing from 0 wt%-40 wt% (based on oil weight), the biodiesel yield stayed around 85%. The result is likely to be caused by the special properties of the carrier. The

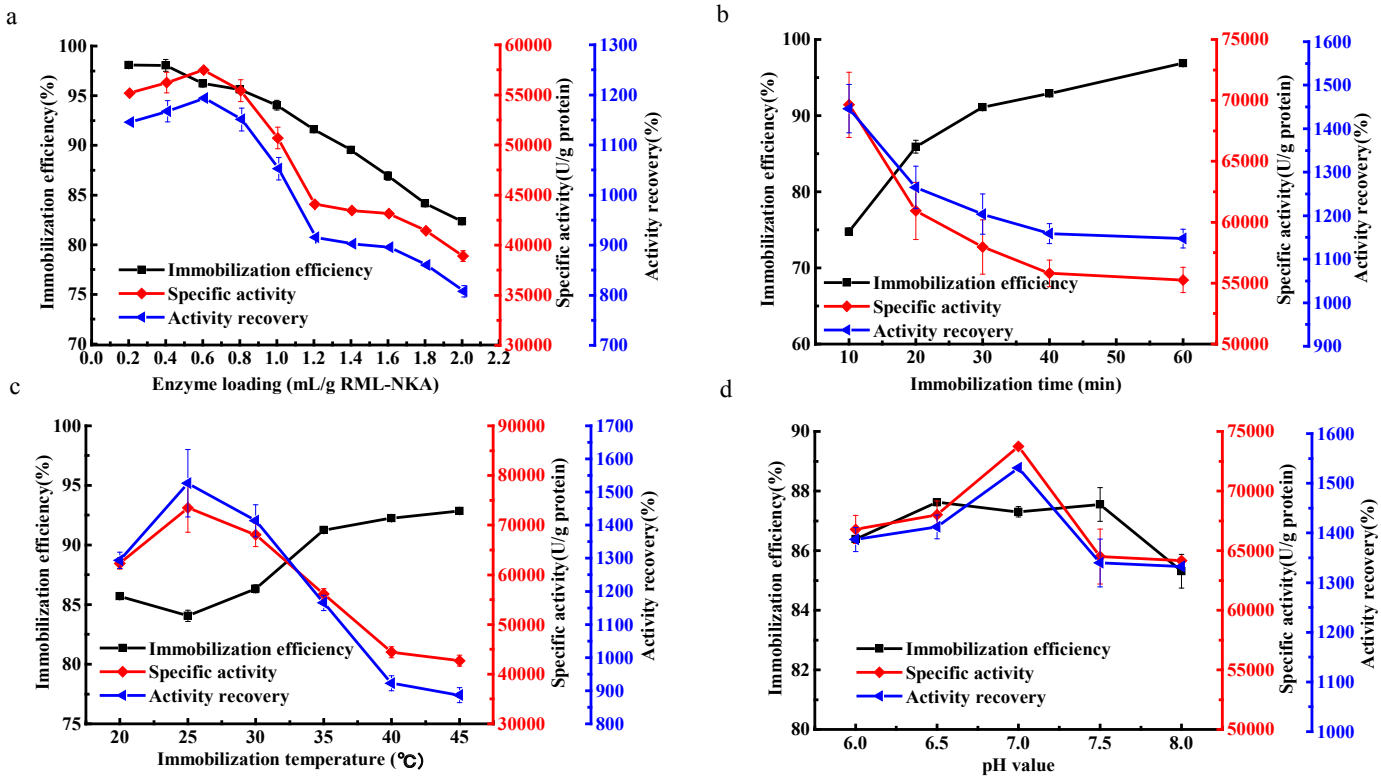

Figure 3 Effect of different immobilization conditions on immobilization efficiency, specific activity and activity recovery. a. Effect of enzyme loading; b. Effect of immobilization time; c. Effect of immobilization temperature; d. Effect of pH value.

Table 2 Detailed information for different pH of phosphate buffer.

pH	K_2HPO_4 (1 mol/L)/mL	KH_2PO_4 (1 mol/L)/mL
6.0	13.2	86.8
6.5	32.9	67.1
7.0	61.5	38.5
7.5	83.4	16.6
8.0	94.0	6.0

Table 3 Effect of different solvents on the biodiesel yield of RML-NKA.

S No	Solvent	log *P*	Biodiesel yield (%)
1	solvent free	-	65.56 ± 0.16
2	tetrahydrofuran	0.45	4.40 ± 0.09
3	tert-Butanol	0.8	40.11 ± 0.31
4	cyclohexane	3.2	64.10 ± 0.31
5	n-hexane	3.5	64.89 ± 1.74
6	n-heptane	4	62.41 ± 0.97
7	petroleum ether	4	63.33 ± 0.32
8	octane	4.5	70.60 ± 0.59
9	isooctane	4.7	74.68 ± 2.27

structure of NKA is crosslinked-polystyrene. Hydrophobic groups such as phenyl group prevent hydrone from getting inside the resin which reduce the effects of water molecules on the lipase.

Effect of reaction temperature on biodiesel yield of RML-NKA and Lipozyme RM IM: The reaction temperatures of RML-NKA and Lipozyme RM IM on biodiesel yield were tested and the results were shown in **Figure 5f**. It could be seen that RML-NKA had better thermo stability compared with Lipozyme RM IM.

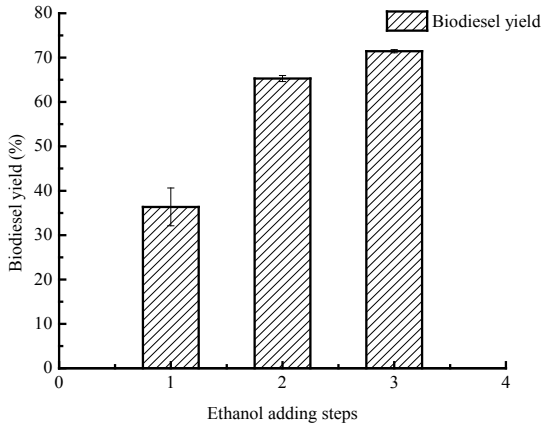

Figure 4 Effect of ethanol adding approach (1. one-step methanolysis; 2. two-step methanolysis (interval time was 6 h); 3. three-step methanolysis (interval time was 4h)).

RML-NKA both in the water-free system and in 10% water system presented better results than Lipozyme RM IM. Lipozyme RM IM even had no activities in 10% water system. RML-NKA had the best results in the water-free system and the biodiesel yield could reach 97.43% at 50°C.

Optimization of the reaction conditions for biodiesel synthesis through RSM: As shown in **Figures 5b** and **5c**, biodiesel yield could not be further improved by increasing immobilization lipase dosage and reaction time. Water content has an unobvious

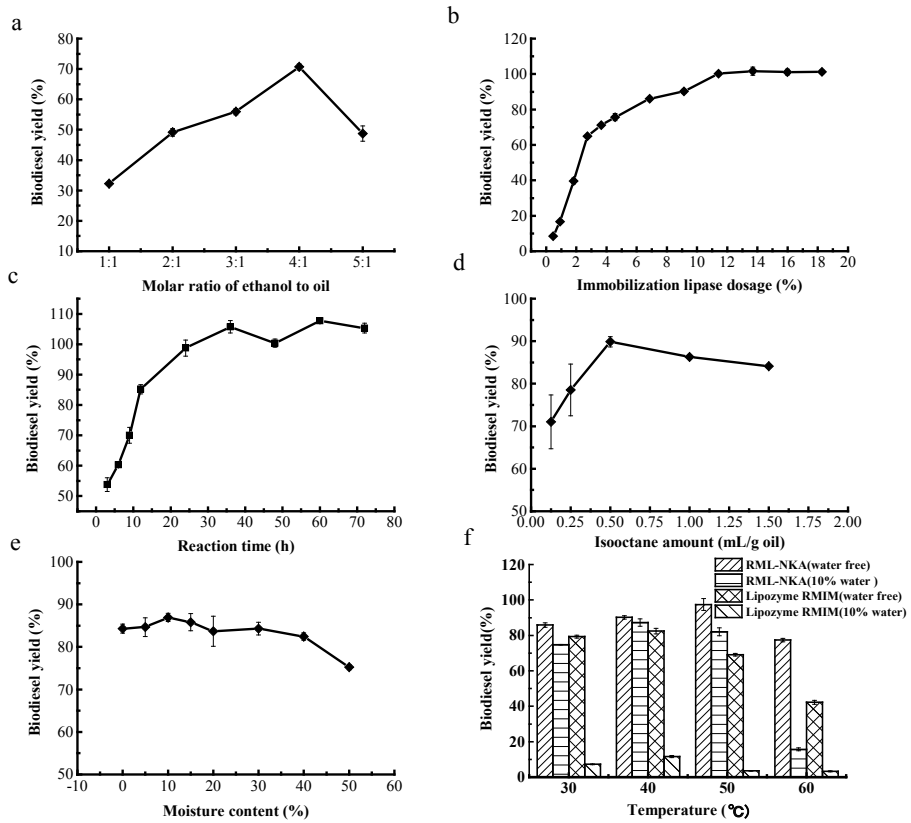

Figure 5 Optimization of biodiesel synthesis. a. Influence of ethanol/oil molar ratio; b. Influence of immobilization lipase dosage; c. Influence of reaction time; d. Influence of isooctane adding amount; e. Influence of water adding amount; f. Influence of reaction temperature.

effect on the biodiesel yield in the range of 0 wt%-40 wt% **(Figure 5e)**. So, molar ratio of ethanol/oil, isooctane amount and reaction temperature were selected as three independent factors. SAS 9.2 was employed to analyze the data using Box-Behnken design. The detailed experimental design, results of biodiesel yield and analysis of variance for biodiesel yield are also provided in the "Supporting Information".

The optimal levels for the three independent factors calculated from the regression model were: molar ratio of ethanol/oil 3.9:1, isooctane amount 0.56 ml/g oil and reaction temperature 47.5°C. The predicted biodiesel yield was 98.47% under the optimal reaction conditions. In order to verify the validation of the model, experiments were performed in triplicates to test the biodiesel yield. The average biodiesel yield was 99.23%, which closely coincided with the predicted value.

Reusability of RML-NKA and lipozyme RM IM in biodiesel synthesis: Reusability of RML-NKA and Lipozyme RM IM used in biodiesel production were investigated. Both of the immobilized RML were operated under their optimal reaction conditions **(Figure 6)**. The operational stability of RML-NKA was better than Lipozyme RM IM in isooctane system. RML-NKA could remain 64% conversion yield after being run for 4 cycles while Lipozyme RM IM remain only 31% and lost 52% of the conversion yield on the second operational cycle. These results arise from the hydrophobic/hydrophilic properties of the carriers. The macroporous anion exchange resins of Lipozyme RM IM is easier to contact with agglomerate of ethanol compared with NKA, which inactivates the lipase and results in bad operational stability or lower optimal reaction temperature **(Figure 5f)**.

Discussion

This study found an economic and effective method for industrialization of lipase-catalyzed biodiesel production. Different kinds of macroporous resins were selected as the immobilization carriers. These resins were cheap and effective when they were used in the immobilization of lipase [20,28,36]. We selected these resins and found that NKA had the best performance **(Figure 1)**. NKA is a kind of cross-linked - polystyrene resin. It has the highest surface area and pore diameter (listed in **Table 1**). This characteristic is good for lipase immobilization [37]. The hydrophobic groups (phenyl group) on the surface and inside of NKA have a positive effect on the activation of the lipase [20]. Many studies reported that immobilization on hydrophobic carrier can enhance stability and activity of the lipases, especially when macroporous resin NKA was used as support [21]. The successful immobilization was verified in FT-IR spectra **(Figure 2)** which existed a peak at 1650 cm^{-1} for amide I of RML-NKA and RML solution.

The optimization part is consisting of immobilization conditions and reaction conditions. We used both single factorial experiments and response surface methodology (RSM) to complete the two parts of optimization in order to get a good catalyst used in biodiesel production. The results of RSM are provided in the "Supporting Information" in details.

In the optimization of immobilization conditions, enzyme loading, immobilization time, temperature and pH value of the immobilization buffer were selected as 4 main factors [20,21,28,33,36]. According to the results listed in **Figure 3**, enzyme loading, temperature and pH value of the immobilization buffer were easy to be confirmed. Diffusion limitation must be taken into account in the enzyme loading part; too high concentration of protein content in the immobilization system will lead to multilayer adsorption which has a negative effect on the immobilized RML. Leakage of protein on or inside NKA may exist during the immobilization, reaction, especially reusability of RML-NKA [28]. Immobilization temperature and pH value of the immobilization buffer are related to feature of RML [13]. Low temperature is good for maintaining the lipase activity. A neutral pH condition not only can help keep the suitable microenvironment of RML, but also maintain its favorable structure during freeze drying [21,33]. As for the immobilization time, we use the overall activity of RML-NKA to evaluate the effect of immobilization time. The specific activity and activity recovery both declined when the immobilization duration changed from 10 to 60 min. It was caused by RML's instability in phosphate buffer.

Before the optimization of reaction conditions, 8 different organic solvents were used in the biodiesel production. Those solvents can increase the immiscibility of oil and ethanol and protect RML from the activity loss and structure change caused by short chain alcohol. Isooctane system reaches the highest biodiesel yield under the same conditions. Liu et al. [33] found the immobilized *Burkholderia cenocepacia* lipase (BCL) could be used in isooctane system for 50 cycles (400 h) showing significant advantages over tert-butanol and solvent free systems. Ethanol addition approaches can also affect the biodiesel yield **(Figure 4)**. Ethanol can replace the water which exists in microenvironment of RML, leading to conformational change and activity loss. Ethanol added in three steps can decrease the concentration of the ethanol in the hybrid system and protect immobilized RML. Molar ratio of ethanol to oil, immobilization lipase dosage, reaction time, isooctane amount, water content and reaction temperature were further optimized **(Figures 5a-5f)**. The results and analysis were listed in the results session. In the reaction temperature part of **Figure 5f** and reusability session of **Figure 6**, commercial immobilized RML, Lipozyme RM IM, were compared with RML-NKA in water-free system and 10% water system. RML-NKA presented better results under 30-60°C and better operational reusability than Lipozyme RM IM under the optimum reaction conditions. The results proved that RML-NKA was better than Lipozyme RM IM in biodiesel production. The water-free system was more stable than 10% water system, especially in higher temperature. These results indicate that the step of freeze drying is unable to remove all of the water inside the RML-NKA, necessary water existing in microenvironment of RML and NKA can keep the suitable conformation of lipase and provide a buffer area for the trans-esterification between soybean oil and ethanol [10]. This study demonstrated that RML-NKA catalyzed trans-esterification for biodiesel production in the isooctane system is the optimum choice and immobilization strategy makes it economically viable in industrial application.

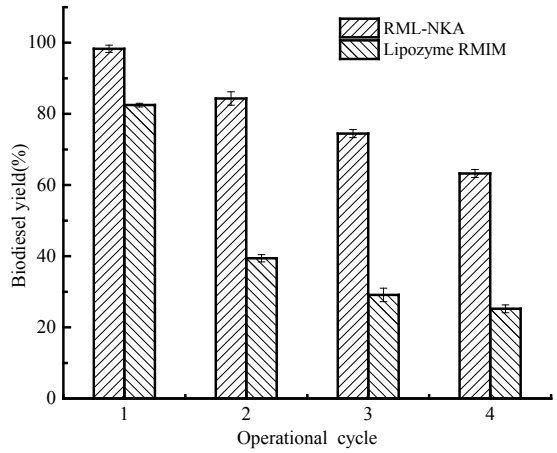

Figure 6 Reuse of RML-NKA and Lipozyme RM IM (RML-NKA: molar ratio of ethanol/oil 3.9:1, immobilization lipase dosage 9.13 wt%, reaction time 12 h, the interval time of adding ethanol 4 h, isooctane amount 0.56 ml/g oil, water content 0 wt% and reaction temperature 47.5°C; Lipozyme RM IM: molar ratio of ethanol/oil 4:1, immobilization lipase dosage 9.13 wt%, reaction time 12 h, the interval time of adding ethanol 4 h, isooctane amount 0.55 ml/g oil, water content 0 wt% and reaction temperature 40°C).

Conclusion

Lipase from *R. Miehei* immobilized on macroporous resin NKA exhibited the best result in the specific activity and activity recovery. The RML, NKA and RML-NKA were characterized by FT-IR, and the immobilization conditions for the RML-NKA were further optimized. The specific activity and the activity recovery of the immobilized lipase were almost 15-fold than those of the free RML. The immobilized lipase, RML-NKA, was then used in biodiesel production. Different organic solvents were tested in trans-esterification reactions and hydrophobic solvent isooctane was found to be the best one. The reaction conditions were also optimized, and the highest yield attained 99.23% under the optimal conditions. The RML-NKA exhibited higher biodiesel yield, better thermo stability and operational reusability than Lipozyme RM IM.

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