

An Efficient and Stable Magnetic Nano-Biocatalyst for Biodiesel Synthesis in Recyclable Ionic Liquids

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Abstract

Transesterification of jatropha oil obtaining process was studied for biodiesel production. *Rhizomucor miehei* lipase (RML) immobilized on 3-aminopropyltriethoxysilane functionalized magnetic Fe_3O_4 was employed as a biocatalyst. The immobilized lipase was confirmed by scanning electron microscopy (SEM), Fourier transform infrared spectra (FT-IR), thermogravimetric analysis (TGA), vibrating sample magnetometer (VSM) and confocal laser scanning microscopy (CLSM) techniques. The efficient biodiesel synthesis in ionic liquids using immobilized RML was demonstrated. Fifteen kinds of ionic liquids based on different alkyl chain lengths of the methyl imidazolium cation ($[\text{C}_2\text{MIM}]$, $[\text{C}_4\text{MIM}]$, $[\text{C}_8\text{MIM}]$, $[\text{C}_{12}\text{MIM}]$ and $[\text{C}_{16}\text{MIM}]$) combined with $[\text{NTf}_2]$, $[\text{N}(\text{CN})_2]$, $[\text{PF}_6]$ or $[\text{BF}_4]$ anions were assayed as reaction media for RML producing biodiesel. The highest synthetic activity of immobilized RML appeared in $[\text{BMIM}][\text{PF}_6]$, which is more than five times higher than the free enzyme. The enzyme catalytic activity remained 60% even after the magnetic nano-biocatalyst and ionic liquid had been reused 5 times in a 48-hour reaction cycle. In addition, the immobilized enzyme exhibited excellent storage stability with almost no decrease in catalytic activity after storing at -20°C for 98 days.

1. Introduction

Rapidly increasing demand for energy, the declining stockpile of nonrenewable fuel source, environmental contamination, and global warming caused by the emission of greenhouse gases are the notable decisive factors which are activating to search for alternative energy sources (Demirbas A 2008; Stamenkovic OS et al. 2008). Biodiesel is one of the most significant substitute fuels comprised of fatty acid methyl esters, which can be acquired from renewable sources through catalytic transesterification with primary aliphatic alcohol (Ding H et al. 2018). Noticeably, the transesterification process can be carried out variously using alkali catalysts, acid catalysts or biocatalysts (Lozano P et al. 2013), among which, biocatalysts have attracted more attention due to their mild reaction conditions and environmental friendliness (Jayaraman J et al. 2020). However, the high cost of enzymes is one of the general disadvantages of enzyme-based approaches, making it urgent to develop reusable biocatalyst derivatives that possess high operational stability (Badoei-dalfard A et al. 2019).

Immobilization is fairly imperative to enhance the operational effectiveness of enzymes in industrial processes, especially when using enzymes in non-aqueous media (Badoei-dalfard A et al. 2019). For decades, there have been different approaches for enzyme immobilization onto solid supports widely developed, in pursuance of providing active, easy-recycling and stable heterogeneous biocatalyst derivatives suitable for long-term operation processes. By using advanced materials, such as metal-organic frameworks (MOFs) (Hu YL et al. 2018), mesoporous silica (Popat A et al. 2011), magnetic nanoparticles (Miao CL et al. 2018), graphene oxide (Xie WL and Huang MY 2018), or PVA-alginate-sulfate beads (Matinja AI et al. 2019), it has been possible to acquire more active and stable immobilized lipase derivatives for biodiesel synthesis. Magnetic Fe_3O_4 nanoparticles, which become a novel material used as the support of immobilized lipase, have unique properties including the large surface area for

functionalization, large enzyme loading capacity, low mass transfer resistance and good reusability, etc (Thangaraj B and Solomon PR 2019). Combining magnetic nanoparticles with cross-linked enzyme aggregates (CLEAs) is considered as an excellent immobilization approach since the immobilized enzyme can be recycled by easily separated from the reaction media with the use of an external magnetic field (Mehde AA et al. 2018).

Although enzymes are regarded as the highly efficient green tools that provide noteworthy selectivity for catalytic processes, the common use of volatile organic solvents as media in biodiesel synthesis is not environment-friendly (Taher H and Al-Zuhair S 2017). Consequently, it is urgent to search for green solvents that are easy to recover and recycle. In the past decade, ionic liquids (ILs) (Lozano P et al. 2013), deep eutectic solvent (DES) (Wu XL et al. 2019) and supercritical carbon dioxide (scCO₂) (Ciftci ON and Temelli F 2011) have been developed as environment-friendly non-aqueous solvent alternatives for biocatalytic biodiesel synthesis.

Ionic liquids (ILs), as the reaction media, possess excellent properties which are less volatile, less flammable, low toxic, and uniquely soluble in both organic and inorganic materials (Elgharbawy AA et al. 2018). Over the decades, ILs have attracted research interests in non-aqueous reaction media for biotransformation due to their negligible vapor pressure and their exceptional ability to stabilize enzymes (Taher H and Al-Zuhair S 2017). In addition, two-phase reaction systems that can efficiently recover products are able to be possibly designed (Solhtalab M et al. 2015). It has been demonstrated that various enzymatic reactions of organic synthesis can be carried out with the use of ILs as solvents or co-solvents, such as hydrolases, oxidoreductases, lyase, and even whole cells (Itoh T 2017). In this context, the ionic liquids (ILs) as the reaction media develop new opportunities for enzymatic biodiesel production.

In the recent study, we have investigated immobilized lipase catalyzing biodiesel production in ionic liquids. Although the biodiesel prepared by immobilized lipase in ILs has been reported recently (Taher H and Al-Zuhair S 2017), in which magnetic cross-linked enzyme aggregates are rare. In this article, we directly separated the products utilizing the properties of appropriate ionic liquids. Both the ILs and the immobilized enzyme can be recycled for another round. The [BMIM][PF₆] was regarded as an excellent reaction system for biodiesel production catalyzed by immobilized RML. Noticeably, the magnetic RML CLEAs exhibited high activity towards five different oils in catalyzing biodiesel production. Moreover, the spent IL and the spent immobilized enzyme exhibited favorable recyclability in the FAME production, the enzyme catalytic activity remained 60%, even after the magnetic nano-biocatalyst and ionic liquid had been reused 5 times in a 48-hour reaction cycle. Notably, the influence of several reaction conditions was studied, such as the amount of catalyst and oil feedstock, reaction temperature, reaction time, methanol-to-oil molar ratio, IL-to-substrate weight ratio.

2. Experimental Section

2.1. Materials

Methyl esters of the major fatty acids and lipase from *Rhizomucor miehei* (solution) were purchased from Sigma-Aldrich. Jatropa oil was purchased from Yanyuan County, China. Olive oil, soybean oil, canola oil, castor oil and Rhodamine B were purchased from Aladdin (shanghai, China). All other reagents were of analytical grade and used without further purification. Double distilled water was employed throughout the experiments.

2.2. Preparation of magnetic RML cross-linked enzyme aggregates

The magnetic RML CLEAs (Cross-Linked Enzyme Aggregates) were prepared using a typical method as reported (Badoei-dalfard A et al. 2019). Briefly, 10 mg of APTES-Fe₃O₄ nanoparticles (see Supplemental Information for details) were added into 1 mL of RML solution (20mM, PBS buffer), and ultrasonicated for 1 min. After this, 9 mL of precipitant was added and stirred at 4°C for 45 min. Then the glutaraldehyde was added gradually to the ultimate concentration of 1.6% v/v with stirring at 30°C for 180 min. After cross-linking, the resulting precipitates were separated by centrifugation (11000 rpm, 8 min), and were repeatedly washed with PBS buffer and water, respectively. Then the precipitates were lyophilized under vacuum at -70°C and stored at -20°C.

2.3. Preparation of magnetic CLEAs of rhodamine B-RML

The enzyme tagging (1 mg mL⁻¹ in 20 mM, pH 9.0 carbonate buffer) was carried out with Rhodamine B (1 mg mL⁻¹ in acetonitrile), the mixture was stirred for 24 h at room temperature shielded from light (Cui CX et al. 2020). The free Rhodamine B was removed by dialysis against double distilled water. The Rhodamine B-labeled lipase was then immobilized on APTES-Fe₃O₄ nanoparticles. All subsequent steps were the same as the preparation of RML magnetic CLEAs.

2.4. Characterization

Fourier-transform infrared (FT-IR) spectra were collected on a Shimadzu FTIR-4200 spectrometer from 400 to 4000 cm⁻¹ using the KBr pellet technique. The structural characterizations of the particles were viewed by a scanning electron microscope (SEM, JSM 7500F, JEOL, Tokyo, Japan). The magnetic properties were measured using a magnetometer (VSM, LakeShore7404, USA) at room temperature. Thermogravimetric analyses (Shimadzu DTG-60H Instruments) were obtained in nitrogen (10°C/min). The crystal structure of the magnetic nanoparticles was identified by powder X-ray diffraction (XRD) using a Shimadzu XRD-6100 diffractometer. The scanned area was $2\theta = 5^\circ - 80^\circ$, operated at 40 kV and 30 mA using Cu-K α radiation. Confocal laser scanning microscopy (CLSM) observation was performed with a Leica TCS SP5 microscope (LSM 780, Germany). The samples were excited at 543 nm and Rhodamine B fluorescence was detected between 548 nm and 703 nm.

2.5. Biodiesel production

In order to investigate the catalytic effectiveness of Jatropa oil bio-conversion catalyzed by immobilized lipase, the reactions of transesterification were performed in a 10 cm⁻³ screw-capped on a shaking

incubator. A typical reaction mixture consisted of 0.25 mg *Jatropha* oil and anhydrous methanol with the presence of an appropriate amount of the magnetic nano-biocatalyst. All transesterification reactions were carried out in dried ILs. Specimens (100 μ L) of the reaction mixture were detached at different times throughout the whole reaction process. Then the amount of FAME was analyzed by GC under predefined chromatographic conditions. Considerably, the effects of solvents, reaction temperatures and other reaction parameters on the FAME yield were investigated by single-factor experiment design.

After the transesterification reaction completed, equal amount of deionized water was added. After 1 hour of stirring, use a magnet to attract enzymes and rest for three hours. The mixture has three layers, biodiesel on the top, aqueous solution of glycerol and methanol in the middle, ILs and enzymes at the bottom. After separating biodiesel and aqueous solution, enzyme and ILs can be recycled after freeze-drying at -70 $^{\circ}$ C. Taking the nano-biocatalyst activity of the first cycle as 100 %, and the activity after each cycle was calculated by remaining activity.

2.6. Analytical methods

The content of biodiesel from the reaction mixture was measured by a Fuli 9790 Gas Chromatograph (Fuli, China) equipped with a Flame Ionization Detector (FID). N-hexane and methyl pentadecanoate were used as the solvent and internal standard, respectively. Nitrogen was used as the carrier gas and the constant flow is 1.5 mL/min. The capillary column was an AT.SE-54 column (0.33 μ m \times 0.25 mm \times 30 m), and the column temperature was held at 160° C for 2 min and raised at a rate of 2° C /min to 240° C which was then held for 10 min. The total content of FAME in the reaction mixture can be calculated by comparing the retention times and peak areas of standard fatty acid methyl ester peaks.

3. Results And Discussion

3.1. Characteristic analysis of magnetic nanoparticles and immobilized RML

In this study, an immobilization strategy for magnetic RML CLEAs was developed and studied. As shown in Fig. 1a, APTES- Fe_3O_4 can be easily obtained and then used for the immobilization of RML.

The magnetic characterization was shown in Fig. 2a. The magnetization hysteresis curves of magnetic RML CLEAs indicates the superparamagnetic character, and such a decrease compared to the Fe_3O_4 attributes to enzyme-loading. Moreover, the XRD patterns show that the incorporation of APTES and enzyme had no effect on the crystal structure of Fe_3O_4 , and the main reflections completely match with pure Fe_3O_4 (Fig. 2b). TGA curves provide information on the thermal stability of Fe_3O_4 , APTES- Fe_3O_4 and magnetic RML CLEAs (Fig. 2c). The TGA curve of Fe_3O_4 nanoparticles shows an initial weight loss (6%) corresponding to the removal of water molecules and unreacted ligand on the surface within the range of 200 – 300° C. The APTES- Fe_3O_4 exhibits two weight losses from room temperature to 200° C and 220 to 450° C, because of the removal of free water and the decomposition of the APTES, respectively (Li Q et al.

2020). For the magnetic RML CLEAs composite, a first weight loss similar to APTES-Fe₃O₄ nanoparticles was observed, related to free water molecules. When the temperature reached 200°C, a rapid loss started to occur as a result of the decomposition of the enzyme, structural water, and other guest molecules until 700°C (Lin CP et al. 2019).

FT-IR spectra of Fe₃O₄, APTES-Fe₃O₄ and magnetic RML CLEAs are shown in Fig. 2d. Presence of surface functional groups, binding of enzyme onto magnetic nanoparticles were vindicated by FT-IR spectroscopy. The peak near 540–560 cm⁻¹ is consistent with the stretching vibration of Fe-O in Fe₃O₄, indicating the presence of Fe₃O₄ in the microspheres, which proves the successful preparation of magnetic RML CLEAs nanoparticles. In addition, compared to the black curve, new peaks at 2969 cm⁻¹, 2923 cm⁻¹ and 2883 cm⁻¹ appear in the red curve, which correspond to the C-H vibrations on the aliphatic chain in APTES, confirming the modification of APTES on Fe₃O₄ microspheres. The peaks at 3351 cm⁻¹ and 3307 cm⁻¹ in the blue curve correspond to the stretching vibration of the N-H bond in the enzyme molecules, while peaks at 1646 cm⁻¹ and 1600 cm⁻¹ were detected corresponding to the C = O bond vibration in -CONH₂, confirming the successful immobilization of the enzyme on the APTES-modified magnetic nanoparticles (Miao CL et al. 2018). The acquired peaks matched those in previous reports well (Xie WL and Ma N 2009).

Figure 3 shows the surface morphology of the magnetic nanoparticles in scanning electron microscopy (SEM) images. As shown in Fig. 3A, the average diameters of the bare Fe₃O₄ particles range from 10 to 20 nm. An increase of 30 nm in the size of Fe₃O₄ can be found after the modification of APTES. Notably, compared to bare Fe₃O₄, the APTES-Fe₃O₄ particles showed a looser surface structure. The agglomeration of the magnetic nanoparticles can be weakened by modifying APTES on the surface of bare Fe₃O₄ nanoparticles, which was beneficial to improve the efficiency of immobilized enzyme. It is noteworthy that the formed APTES-Fe₃O₄ nanoparticles not only displayed magnetic behaviors but also had a broad active surface available for lipase immobilization, which is essential and important in term of protein immobilization (Miao CL et al. 2018).

To further confirm that lipase was immobilized on the support, fluorescein isothiocyanate (Rhodamine B)-labelled lipase was prepared and used for detecting the process of immobilization. The confocal laser scanning microscopy (CLSM) images of the Rhodamine B-labelled RML@Fe₃O₄ (Fig. 3d, single-channel mode of CLSM) showed red particles, and the confocal microscope image revealed that the labeled enzymes (red) were distributed uniformly on the support(Fig. 3f).

3.2. Catalyst performance of the magnetic RML CLEAs

In order to study the properties of the immobilized enzyme, the oil/IL quality ratio, temperature, reaction time, catalyst amount and the methanol/oil molar ratio were explored.

To determine the optimal solvent, the biocatalytic synthesis of biodiesel was carried out through methanolysis of jatropha oil in these fifteen different ILs as reaction media at 50°C, using a 1:5 molar

ratio of oil and methanol as a substrate (Table 1). Transesterification reaction cannot be performed in the ILs used in this work in the absence of lipase. The catalytic efficiency of the immobilized lipase (magnetic RML CLEAs) on the methanolysis reaction was then checked in media containing methyl imidazolium ionic liquids (MIM) of different cation chain lengths (C_2 , C_4 , C_8 , C_{12} and C_{16}) and in combination with four different anions ($[BF_4]$, $[PF_6]$, $[N(CN)_2]$ and $[NTf_2]$).

Table 1
Effect of different ionic liquids on biodiesel production ^a.

Cation	Biodiesel yield (%) ^b			
	Anion			
	$[NTf_2]$	$[BF_4]$	$[PF_6]$	$[N(CN)_2]$
[EMIM]	23.5 ± 3.5	2.5 ± 2.1	24.5 ± 4.1	3.1 ± 2.4
[BMIM]	24.8 ± 2.8	8.6 ± 3.1	31.3 ± 2.9	2.2 ± 1.8
[OMIM]	23.7 ± 3.7	17.6 ± 2.6	22.3 ± 5.4	1.7 ± 1.1
$[C_{12}$ MIM]	15.3 ± 2.7	14.0 ± 3.7		
$[C_{16}$ MIM]	12.9 ± 2.9			
Solvent free	3.0 ± 1.5			
^a The reaction conditions: time 48h, oil/IL weight ratio 1: 2.0, immobilized lipase 4 wt% and methanol/oil molar ratio 5:1.				
^b Determined by GC analysis.				

When the anion was changed, the FAME conversion of ILs based on the $[PF_6]$ and $[NTf_2]$ anions was higher than that of ILs based on the $[BF_4]$ and $[N(CN)_2]$ anions. This may be due to the ILs with $[BF_4]$ and $[N(CN)_2]$ anions are more nucleophilic than ILs with $[PF_6]$ and $[NTf_2]$ anions. Therefore, they can promote the change of the secondary structure of the protein, resulting in a loss of activity (Zhao J et al. 2015).

The activity and stability of enzymes can be substantially affected by the alkyl chain length of cation in ILs (Itoh T 2017). The results showed that with the increase in alkyl chain length of the corresponding imidazolium cation, the synthetic activity increased first and then decreased. The initial increase of synthetic activity results from the higher solubility of the oil substrate in the more hydrophobic ILs, given that the hydrophobicity of the IL increased with the alkyl chain length on the cation (Diego TD et al. 2011). Nevertheless, as for the same anion, the viscosity of the IL increases with the increasing length of the alkyl chain substituted on the imidazolium ring. Regarding the $[C_n$ MIM] cations, the viscosity increases in this order with the anion type: $[PF_6] > [BF_4] > [NTf_2]$ (Diego TD et al. 2011). The mass transfer resistance increases due to the increase of viscosity, which negatively affects the operating process. The

highest production of FAME was obtained in [BMIM][PF₆] and its production was over ten times higher than in a solvent-free system. Such a low yield in the solvent-free system may be due to the low solubility of methanol in triglycerides or to the inactivation of lipase by methanol (Lozano P et al. 2013; Rafiei S et al. 2018).

The influence of the oil-to-ionic liquid (oil/IL) weight ratio on the immobilized-RML-catalyzed biodiesel synthesis was studied after 48 h of reaction at 50°C. The oil: ionic liquid weight ratio shown in Fig. 4a, and in all cases, the initial system for the transesterification reaction was a homogenous medium. For the case of [BMIM][PF₆], the yield of FAME increased when the oil: IL ratio decreased, due to the higher concentration of methanol in the reaction mixture the greater lipase inactivation (Rafiei S et al. 2018).

These results may also be due to the special protection of ILs on lipases in non-aqueous environments, which prevents lipase from deactivating by certain organic solvents (Sheldon RA 2016).

As the temperature is a crucial factor to be considered in the enzymatic synthesis of biodiesel, the influence of reaction temperature on the yield of FAME was studied by carrying out the reaction in [BMIM][PF₆] at different temperatures. As can be seen from Fig. 4B, the immobilized RML was more active than free RML in the range of 20–60°C. The optimum temperature interval of RML CLEAs was found to be 30–60°C and when the temperature was higher than 37°C its activity decreased slightly. It was indicated that immobilization could effectively protect lipases from thermal denaturation (Otari SV et al. 2020). Based on the results, 37°C was selected for further studies.

The effect of reaction time on the preparation of biodiesel was studied from 12 h to 72 h. According to Fig. 4c, the longer reaction time, the higher yield, and the yield tended to be a constant after reaction time was longer than 48 h. Especially, the yield of FAME was 51% after 48 h while the yield reached only 58% after 72 h. Therefore, the comparatively optimal reaction time is 48h.

In this study, methanol was used as a substrate for transesterification reaction with jatropha oil. Excess methanol can drive the chemical equilibrium to biodiesel formation, but it was harmful to the enzymes (Xie WL and Wan F 2019). The reaction rate and the degree of transesterification can be increased by adding a proper amount of methanol into the reaction mixture. The influence of the methanol/oil molar ratio on the production of biodiesel is shown in Fig. 4d, the biodiesel yield increased with the methanol to oil ratio from 3:1 to 7:1, the highest biodiesel yield (60%) could be acquired at the ratio of 4:1. However, the yield of biodiesel reached the maximum and further increased in the alcohol concentration resulted in a dramatic decrease in conversion due to the inactivation of the enzyme. Consequently, the appropriate methanol/oil molar ratio is 4:1.

The lipase dosage has a direct impact on the reaction rate (Sun S et al. 2015). The effect of catalyst dosage on the conversion of jatropha oil into biodiesel was studied by varying its dosage in the range of 0–12 wt% (as referred to the oil mass). Figure 5a indicates that the FAME yield increased significantly as the lipase dosage increased. The high yield was obtained at 10 wt%. Further increase in catalyst concentration did not enhance the conversion yield. In fact, increasing the dosage of catalyst by more

than 10 wt% caused a decrease in biodiesel yield. This result indicated that the excess magnetic nanoparticles tended to form enzyme aggregates, which reduced the accessibility of lipase to substrates (Palomo JM et al. 2003)

In many European countries, the most commonly used vegetable oils in the production of biocatalytic biodiesel are obtained from soybean seeds and sunflower seeds. Countries can use different types of raw materials based on the abundance or availability of the region, such as palm oil and coconut oil in tropical countries (Christopher LP et al. 2014; Rahimi V and Shafiei M 2019).

The influence of oil feedstock on the initial yield of biodiesel was analyzed in [BMIM][PF₆]. In the present work, four different vegetable oils were used (soybean oil, olive oil, canola oil, and castor oil) in addition to jatropha oil (Fig. 5b). Notably, compared to free RML, the magnetic RML CLEAs exhibited better activity towards five different oils in catalyzing biodiesel production. Especially, the catalytic effect of free enzyme for five oils are poor, and the mostly yield are less than 10 %. There was no significant difference for the different oils when free RML was used, but slightly higher for jatropha oil.

3.3. The reusability and storage of the magnetic RML CLEAs

No matter the reusability of the IL and the enzyme, it is always an important advantage for the commercial viability of a process (Nadar SS et al. 2020). We further demonstrated the recyclable use of the enzyme in this solvent system (Fig. 6a). Surprisingly, the spent IL and immobilized enzyme exhibited favorable recyclability in the FAME production, and the catalytic activity of immobilized RML remained 60 % even after 5 cycles. This indicated that the lipase-IL had great reusability, which was satisfying for reducing the cost-effectiveness in the enzymatic synthesis of biodiesel. Furthermore, the nano-biocatalyst exhibited excellent reusability in biodiesel production might be due to: (i) The protection of hydrophobic ILs. (ii) The suitability of the proposed methodology to extract biodiesel and glycerol by an easy and sustainable approach that permits full recovery and reuse of enzyme-IL systems (Elgharbawy AA et al. 2018). In addition, the slight decrease of catalytic activity after several times of recycling may be caused by some loss of enzyme during the cycle or residual products in the IL accumulate and inactivate the enzyme.

Storage stability is one of the most important aspects in the development of reliable biocatalyst for commercial-scale application (Cui JD et al. 2017). Hence, a time-course study on the storage stability of immobilized RML was carried out by leaving the composite at -20°C for a period of time. As shown in Fig. 6b, the activity of the immobilized RML was almost no decrease after storage of the magnetic RML CLEAs for 98 days. This result showed that nano-biocatalyst performed well in long-term storage, which can be explained by the covalent binding of the enzyme on the carrier, thus increasing the strength and stability of lipase by effectively decreasing its denaturation (Talekar S et al. 2012).

4. Conclusions

In conclusion, the lipase was successfully immobilized to amino-functionalized magnetic nanoparticles using glutaraldehyde as a linker. Furthermore, we investigated the lipase-catalyzed jatropha oil transesterification with methanol to produce biodiesel in ionic liquids and demonstrate that it is possible to repeatedly use the enzyme and the ionic liquid. Further investigation of the scope and limitations of this reaction, several reaction parameters were optimized, such as the amount of catalyst and oil feedstock, reaction temperature, reaction time, methanol-to-oil molar ratio, IL-to-substrate weight ratio. The highest FAME production after 48h was obtained in [BMIM][PF₆] with 98% yield. The immobilized enzyme exhibited excellent storage stability with almost no decrease in catalytic activity after stored at -20°C for 98 days.

Abbreviations

RML

Rhizomucor miehei lipase; APTES:3-aminopropyltriethoxysilane; SEM:scanning electron microcopy; FT-IR:Fourier transform infrared spectra; TGA:thermogravimetric analysis; VSM:vibrating sample magnetometer; CLSM:confocal laser scanning microscopy; CLEAs:cross-linked enzyme aggregates; FAME:fatty acid methyl esters.

Declarations

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Abbreviations

RML: *Rhizomucor miehei* lipase; APTES: 3-aminopropyltriethoxysilane; SEM: scanning electron microcopy; FT-IR: Fourier transform infrared spectra; TGA: thermogravimetric analysis; VSM: vibrating sample magnetometer; CLSM: confocal laser scanning microscopy; CLEAs: cross-linked enzyme aggregates; FAME: fatty acid methyl esters.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors' contributions

The experimental objectives were designed by Xiu Xing and Na Wang, and Xiu Xing performed all the sample preparation and characterizations. Zi-Wen Zhou and Yun-Jie Wei helped with the experiment. Xiu Xing, Yun-Jie Wei, Kun Li, Na Wang and Xiao-Qi Yu contributed in the discussion, revision, and editing of the manuscript. All authors read and approved the final manuscript.

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Supplementary Materials

The Supplementary materials are available online.

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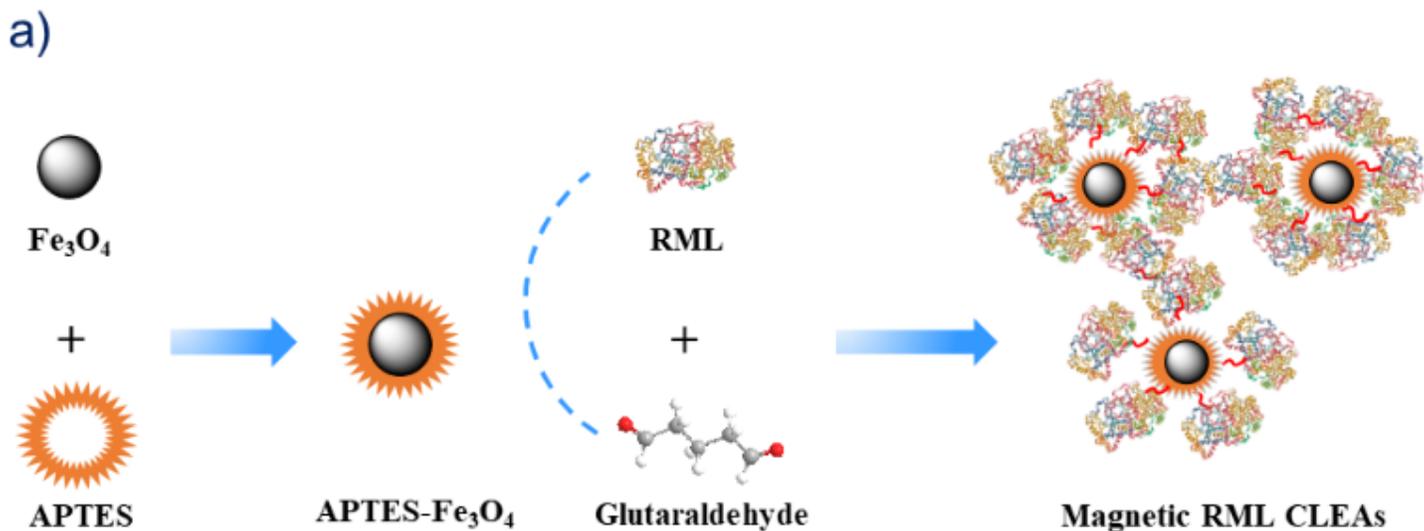
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Figures



b)

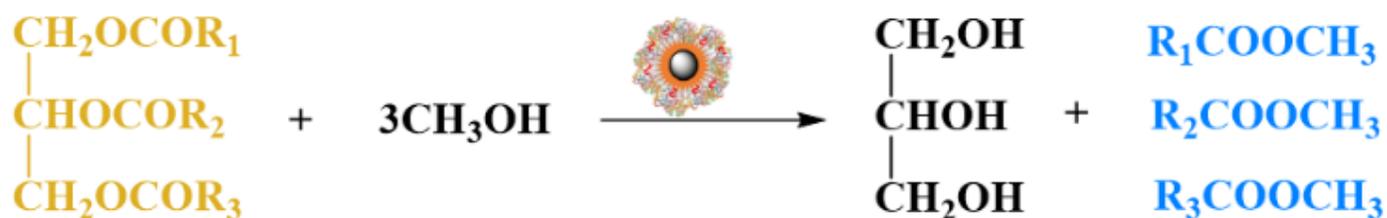


Figure 1

Preparation of magnetic RML CLEAs (a) and Its application for biodiesel production by using jatropha oil(b).

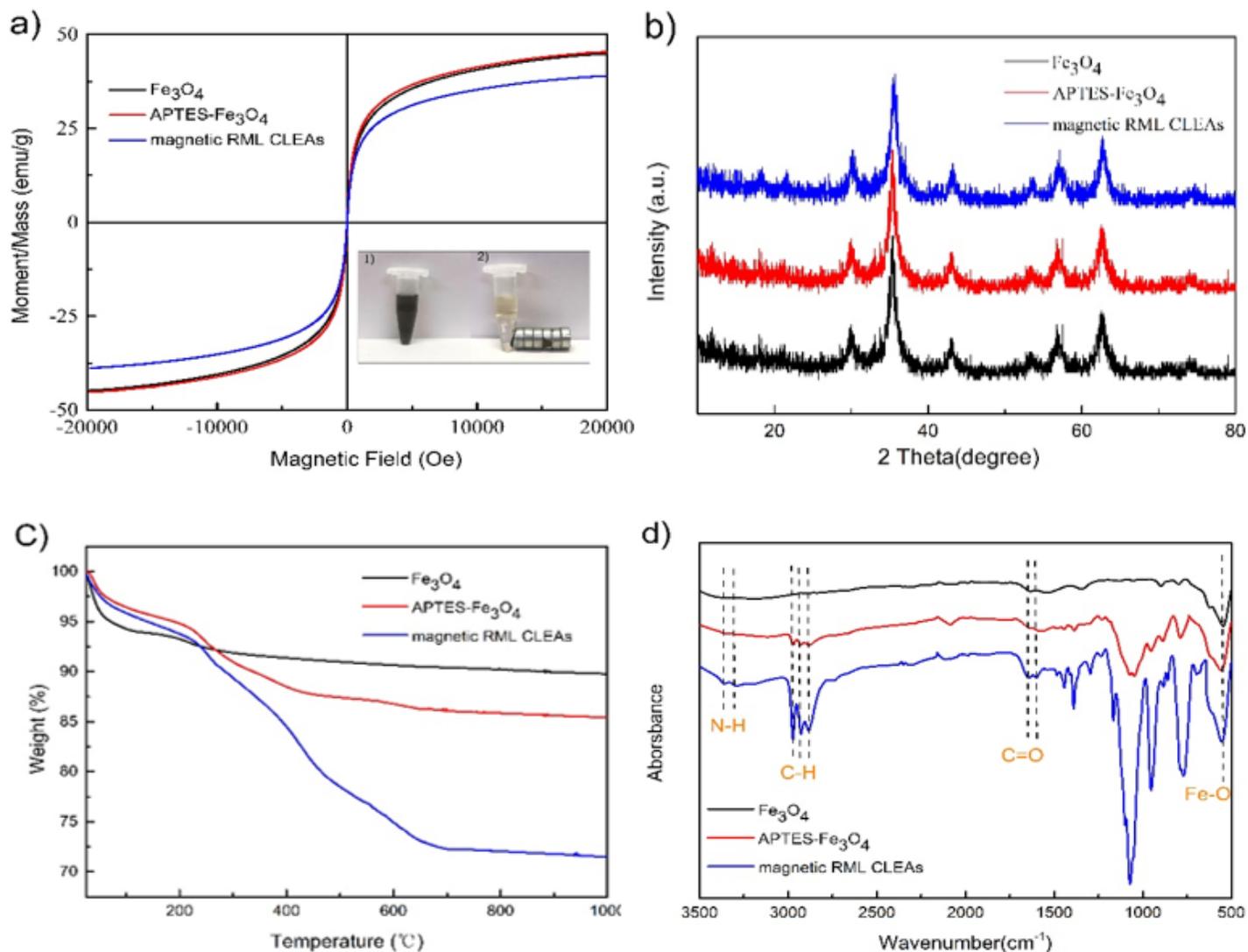


Figure 2

Characterization of Fe₃O₄, APTES-Fe₃O₄ and magnetic RML CLEAs: (a) magnetic hysteresis loops, (b) XRD pattern, (c) thermogravimetric (TG) curve, (d) FT-IR spectra. The insert picture shows the image of the magnetic RML CLEAs that are dispersed in the reaction mixture (1) and the magnetic RML CLEAs after a magnet was placed aside (2).

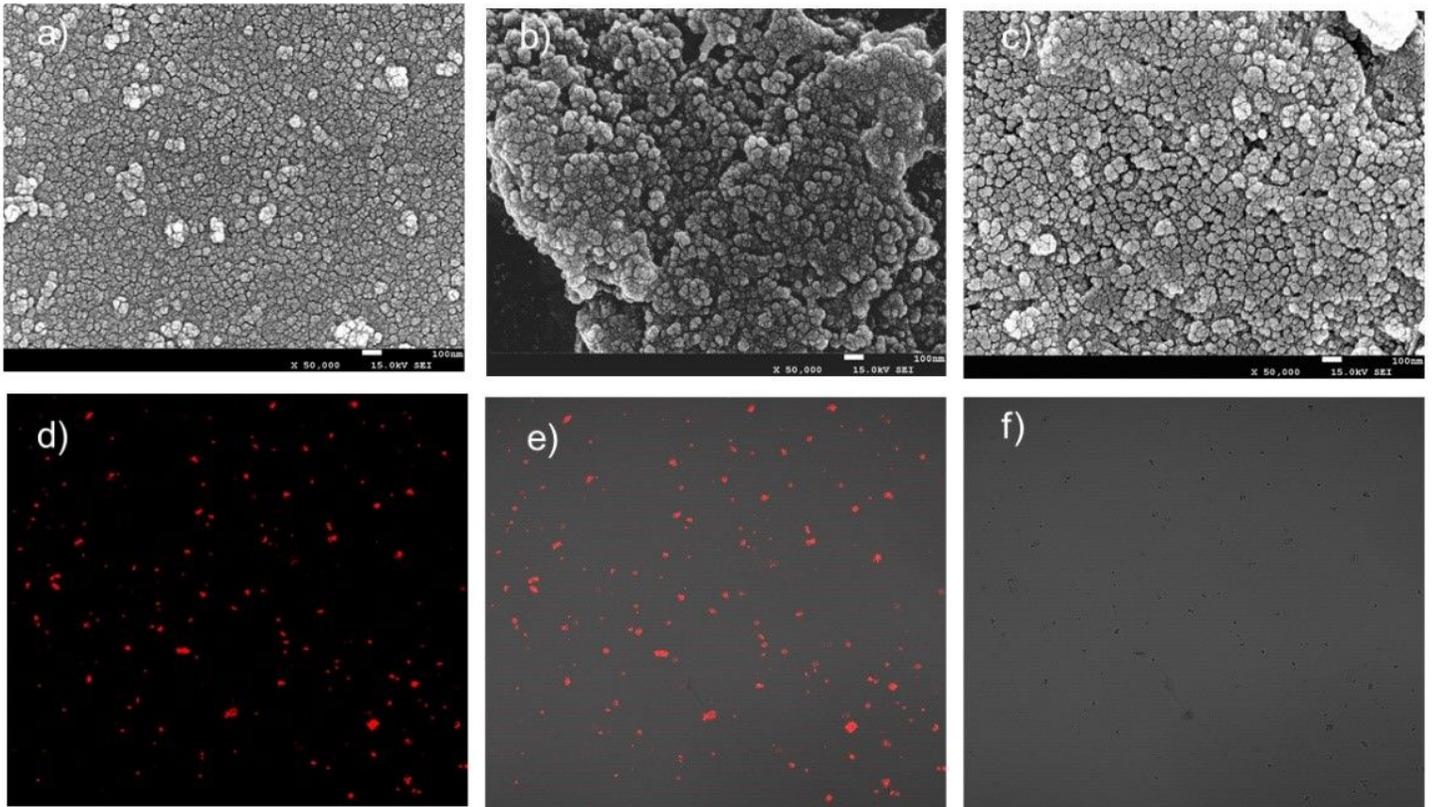


Figure 3

SEM images of (a) Fe₃O₄, (b) APTES-Fe₃O₄, (c) magnetic RML CLEAs; and confocal microscope images of the Rhodamine B-labelled RML@ Fe₃O₄ (d-f).

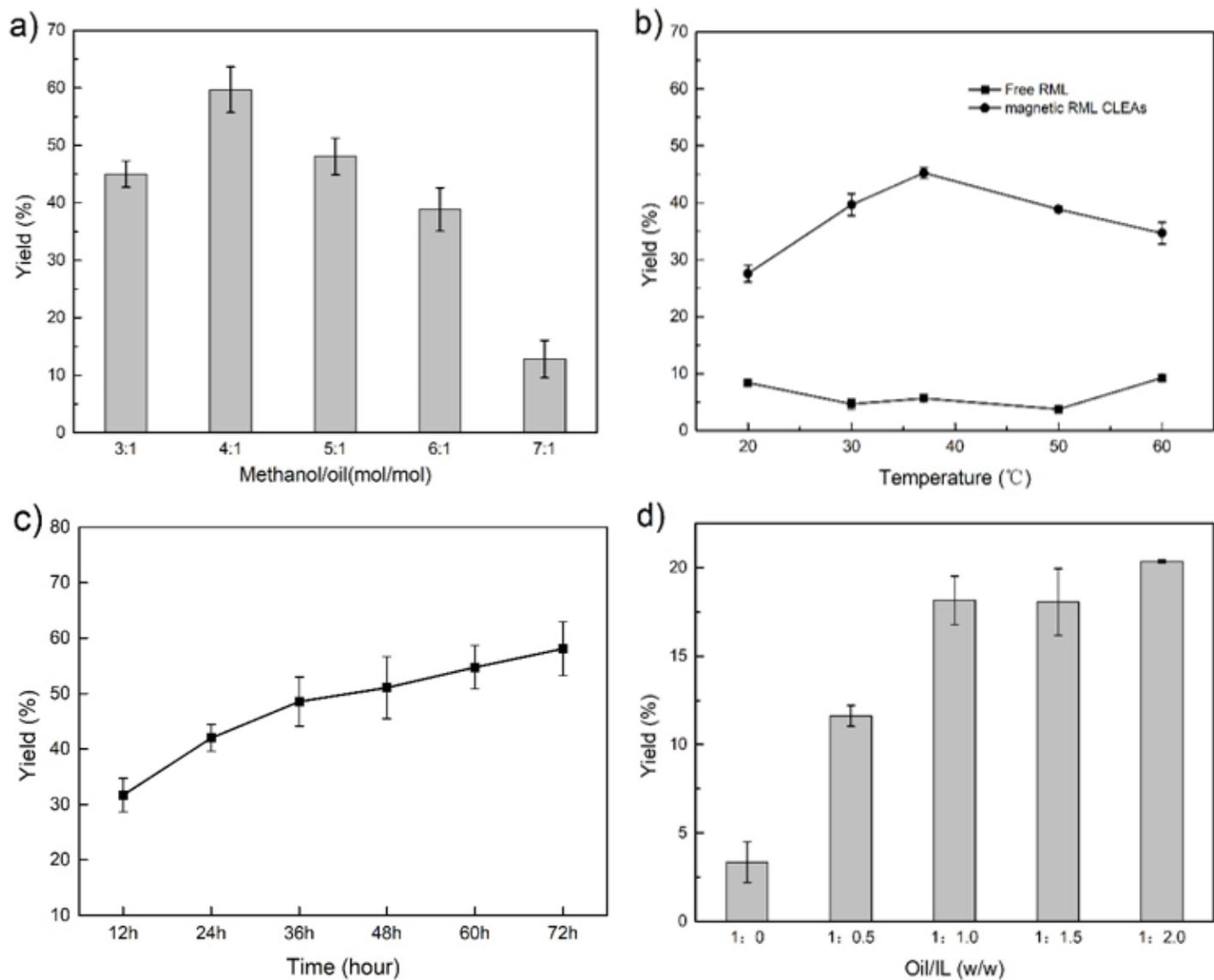


Figure 4

Effect of (a) oil/IL weight ratio, (b) temperature, (c) reaction time and (d) methanol/oil molar ratio on biodiesel production. The reaction conditions were: (a) time 48h, temperature 50 °C, immobilized lipase 2 wt% and methanol/oil molar ratio 5:1. (b) time 48h, oil/IL weight ratio 1: 2.0, immobilized lipase 4 wt% and methanol/oil molar ratio 5:1 (c) temperature 37 °C, oil/IL weight ratio 1: 2.0, immobilized lipase 4 wt% and methanol/oil molar ratio 5:1. (d) time 48h, temperature 37 °C, immobilized lipase 4 wt% and oil/IL weight ratio 1: 2.0.

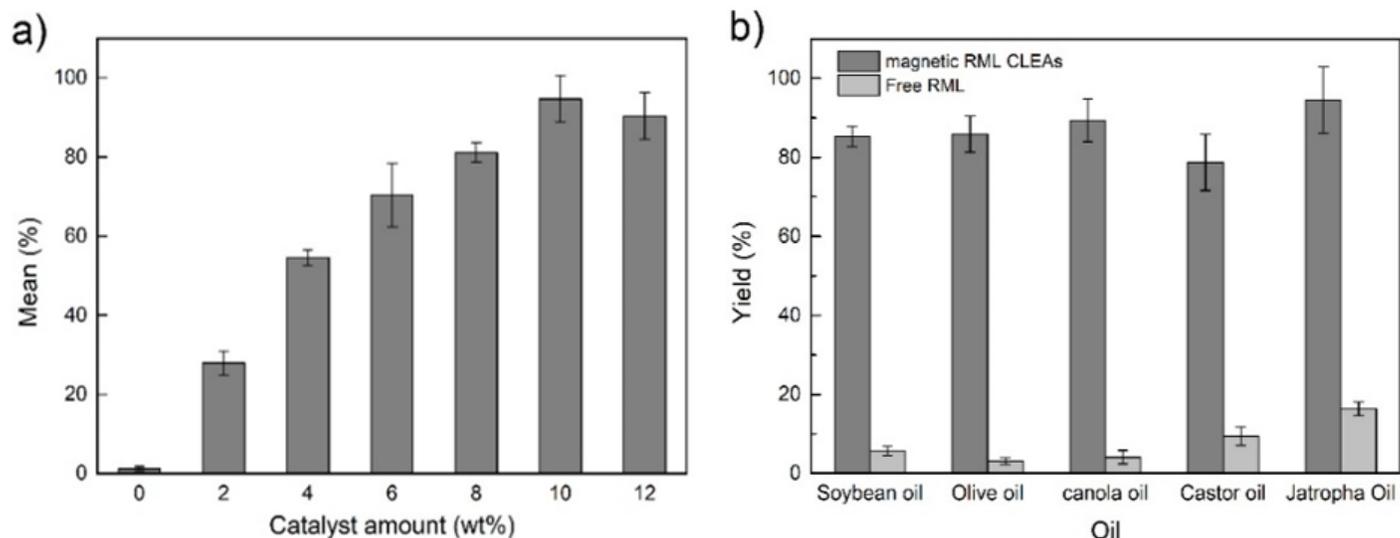


Figure 5

Effect of (a) catalyst content and (b) oil type on biodiesel production. The reaction conditions were: (a) time 48 h, temperature 37 °C, oil/IL weight ratio 1: 2.0 and methanol/oil molar ratio 4:1; (b) time 48 h, temperature 37 °C, oil/IL weight ratio 1: 2.0, immobilized lipase 10 wt% and methanol/oil molar ratio 4:1.

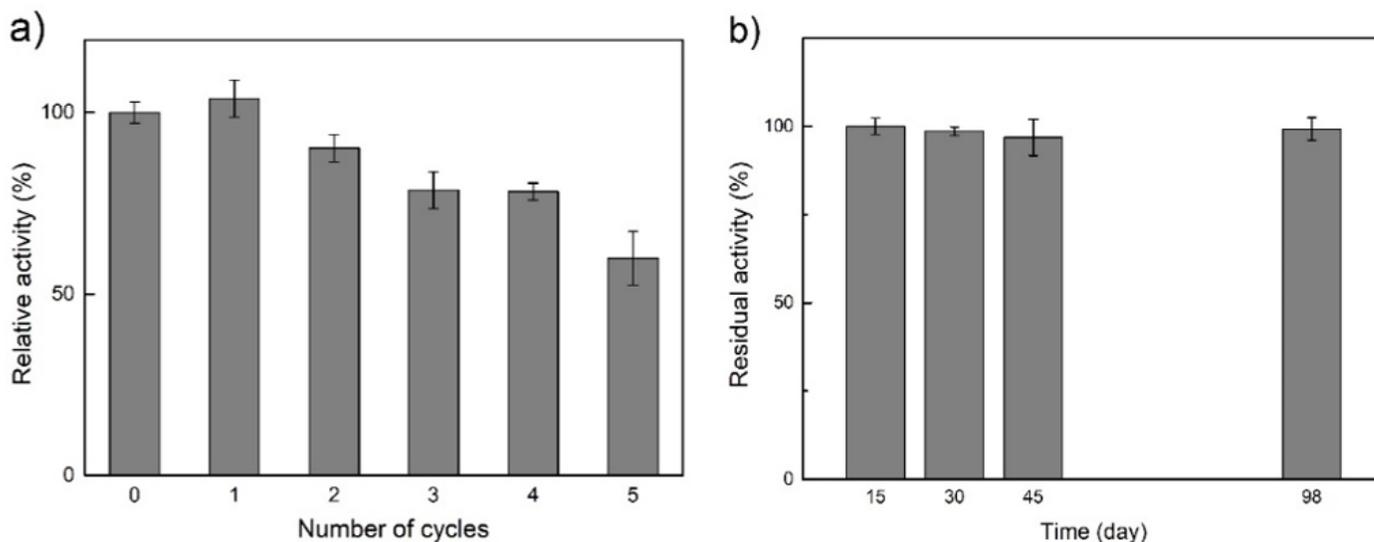


Figure 6

(a) The reusability of the IL and the immobilized RML for 5 cycles; and (b) storage stability. The reaction conditions were: (a) time 48 h, temperature 37 °C, oil/IL weight ratio 1: 2.0, immobilized lipase 4wt% and methanol/oil molar ratio 4:1 (b) time 48 h, temperature 37 °C, oil/IL weight ratio 1: 2.0, immobilized lipase 4wt% and methanol/oil molar ratio 4:1.

Supplementary Files

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