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Pickering emulsion stabilized by lipase-containing periodic mesoporous organosilica particles: A robust biocatalyst system for biodiesel production

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HIGHLIGHTS

- Pickering emulsion stabilized by lipase-containing PMO was constructed firstly.
- The Pickering emulsion system was used as biocatalyst for biodiesel production.
- The Pickering emulsion showed outstanding activity, mechanical and storage stability.
- Applications of this Pickering emulsion system in biocatalysis field are promising.

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ABSTRACT

A novel catalytic system of Pickering emulsion stabilized by lipase-containing periodic mesoporous organosilica was constructed (named LP@PE) and used as biocatalyst for biodiesel production. The reaction parameters were optimized and the optimum conditions were as follows: the water fraction 0.65%, molar ratio of ethanol to oleic acid 2:1, immobilized lipase particles 150 mg, phosphate buffer pH 7.0 and temperature 30 °C. Under these conditions, the maximum biodiesel yield obtained via esterification of oleic acid with ethanol could reach 95.8%. The biodiesel yield could maintain 88.6% after LP@PE was used 15 times. The LP@PE was also used in the synthesis of biodiesel from *Jatropha curcas* oil. The highest yield could reach 87.1% and the yield was 73.0% after 10 cycles. All these results demonstrated that Pickering emulsion system stabilized by immobilized enzyme may possess much potential in many enzymatic industrial applications.

1. Introduction

Biodiesel, which refers to fatty acid alkyl esters, has attracted considerable attention as an alternative fuel for diesel engines during the last decade, due to its biodegradable, non-toxic, a low emission profile and renewable (Juan et al., 2011; Kiss and Bildea, 2012). Many processes for biodiesel production have been developed, which can be broadly classified into two categories: chemical and enzymatic. The drawbacks, such as high energy requirement, difficulties in the recovery of catalyst and glycerol and potential pollution to the environment, have limited the chemical processes for biodiesel production (Abbaszaadeh et al., 2012). Enzymatic biodiesel production using lipase (EC 3.1.1.3), has become an interesting alternative for biodiesel production, since the problems mentioned above can be circumvented (Hama and Kondo, 2013). The industrialization of this enzymatic system is often hampered by high cost, low operational stability and the difficulties in recovery and recycling of native lipase (Zhou et al., 2011). These drawbacks can generally be overcome by the immobilization of lipase. Therefore, using robust immobilized lipase for biodiesel production will be a promising and efficient approach compared to the chemical method (Sheldon and van Pelt, 2013).

Periodic mesoporous organosilica (PMO) materials are promising candidates for lipase immobilization due to their large surface area, narrow pore size distribution, well defined pore geometry, outstanding thermal and mechanical stability (Wang et al., 2010). Using PMO as support for the lipase immobilization, not only the mesoporous channels can promote protein refolding (Wang et al., 2007) but also the functional groups regularly distributed within the framework can facilitate lipase loading capacities, prevent lipase leakage and enhance enzymatic activity and stability (Jin et al., 2011; Van Der Voort et al., 2013; Zhou et al., 2012, 2011).

Recently, Pickering emulsions, a kind of oil/water or water/oil emulsions stabilized by solid nanoparticles, have attracted a surge of interest for enzyme immobilization. Compared to the traditional

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surfactant-stabilized emulsion, Pickering emulsions have many advantages such as no detrimental effects on enzyme activity, higher stability, non-pollution and easy purification when they are used in biocatalytic systems (Chen et al., 2011; Fletcher et al., 2002). Up to now, there are only a few reports attempting to apply Pickering emulsions to immobilize enzymes. All these reports demonstrated that the catalytic performances of the enzymes were considerably enhanced (Wang et al., 2012; Wu et al., 2011a). Nevertheless, until now, no study that regarding Pickering emulsions stabilized with lipase-containing PMO has been reported.

Thus, in the current study, hydrophobic PMO with suitable pore size were designed for the lipase immobilization and then the lipase-containing PMO particles were used to prepare Pickering emulsions. The assembly of the PMO nanoparticles can both stabilize the emulsions and retain the surface availability of the lipase for catalytic reaction (Samanta et al., 2009). Then this system was used to catalyze esterification and transesterification reactions for biodiesel production. The catalytic conditions were optimized and the stability of this system was also investigated.

2. Methods

2.1. Materials

Triblockpoly(ethyleneoxide)-block-poly(propyleneoxide)-block-poly(ethyleneoxde) copolymer (P123), Bis(triethoxysilyl)ethane (BTEE), 1,3,5-trimethylbenzen (TMB) and 4-nitrophenyl palmitate were purchased from Tokyo Chemical Industry Co., Ltd. (TCI). Lipase from *Candida* sp. 99–125 (the protein concentration of the crude lipase is 113 mg protein g^{-1} determined by UV detector at the wavelength of 280 nm) was obtained from Beijing CAT New Century Biotechnology Co., Ltd. (China). *Jatropha curcas* oil was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other reagents were of analytical grade.

2.2. Preparation of Pickering emulsions

The PMO particles were synthesized by using BTEE as a precursor, P123 as a template, and TMB as a pore expander. Then the lipase from *Candida* sp. 99–125 was immobilized onto the PMO particles by adsorption and named LP@PMO. The preparation procedures were shown in Supporting Information.

100 mg of LP@PMO particles was dispersed in 5 mL of toluene by oscillator for 1 min. An amount of phosphate buffer was added into the dispersion, and the mixture was emulsified using ultrasonic cell disruptor (BILON-250Y) operating at 40 w for 12 s with 1 s interval every 2 s. Thus, the Pickering emulsion stabilized by LP@PMO particles was obtained and named LP@PE.

2.3. Application in esterification and transesterification for biodiesel production

The esterification was carried out in a lab scale reactor with a volume of 25 ml. An amount of oleic acid mixed with ethanol was added and toluene was used as co-solvent. The reaction was started with the addition of desired biocatalyst (LP@PE, LP@PMO or native lipase) with equal protein content and the reactor was kept in thermostatic water bath with shaking (200 r/min) for a certain time.

Effect of various parameters including the water fraction, molar ratio of ethanol to oleic acid, the dosage of LP@PMO, pH of phosphate buffer, and the temperature on the biodiesel production was studied. The yield of fatty acid ethyl ester was quantitatively analyzed by using gas chromatograph (GC) (see Supporting Information). One unit of lipase activity (U) was defined as 1 μmol product produced within 1 min. The specific activity was related to the amount of the enzyme (U mg^{-1}).

After each round of reaction, 95% of the toluene phase was carefully removed, followed by adding fresh toluene for several times until no GC signals of substrates and products were detected. Then, a certain amount of fresh substrates (oleic acid and ethanol) as in the first round was added into the system to start the reaction again. The storage and mechanical stability of LP@PE were evaluated via optical microscopy by monitoring the morphology changing with storage and shaking time increasing. The diameter distributions of LP@PE were determined using a Malvern Mastersizer 2000.

LP@PE was also applied in catalyzing transesterification of *J. Curcas* oil for biodiesel production and the reusability of LP@PE was investigated. The yield of biodiesel was quantitatively analyzed by using GC (see Supporting Information). All the experiments data were an average of three parallel replicates.

3. Results and discussion

3.1. Study of LP@PMO and LP@PE

To immobilize lipase *Candida* sp. 99–125 efficiently, the pore sizes were tailored by varying the mass ratio of TMB to P123. Finally, the ratio of 0.25 was selected and the average pore size calculated by the Barrett–Joyner–Halenda (BJH) method was around 6.37 nm (Fig. S1a). The SEM image revealed that the PMO typically consisted of spheroidal particles with a diameter of 1.5 ± 0.2 μm (Fig. S1b). In order to specify whether the lipase was adsorbed onto PMO, the FT-IR spectra of native lipase, PMO and LP@PMO were studied. As shown in Fig. S1c, the amide I band at approximately 1600–1700 cm^{-1} was attributed to C=O stretching vibration coupled with an out-of-phase C—N stretching and C—C—N deformation of the peptide backbone. The amide II band centered around 1550 cm^{-1} was attributed to the in-plane N–H bending and C–N stretching vibrations (Zhou et al., 2012). The amide I and amide II bands presented in the spectrum of LP@PMO could roughly confirm that lipase had been immobilized onto PMO successfully.

For ease of morphological observation, the aqueous core of the LP@PE was solidified by addition of agarose (1.5 wt%) prior to emulsification. The jellified droplets containing lipase were visualized via scanning electron microscopy. The SEM image revealed the enclosure of the jellified droplets by random, closely packed, LP@PMO shells (Fig. S2). In order to observe the behaviors of LP@PMO in LP@PE and confirm the successful immobilization of lipase, the lipase was labeled with Rhodamine B. The LP@PE was observed as round spherical droplets by confocal laser scanning microscopy (CLSM) and the droplet size was in the range of 30 ± 10 μm. LP@PMO formed the interface between water and toluene, which were observed as green dots (Fig. S3).

3.2. Optimization of esterification reactions catalyzed by LP@PE

The synthesis of biodiesel through esterification of a commercial oleic acid and ethanol was selected as the model reaction. In order to increase the catalytic efficiency of LP@PE and the yield of biodiesel, the conditions of the esterification reactions were optimized.

3.2.1. Effect of water fraction

To obtain stable Pickering emulsions and improve the biodiesel yield, the effect of phosphate buffer amount (water phase) was investigated as a function of the water fraction (% v/wt., ml/100 mg LP@PMO). Various water fractions over a range from 0.45% to 0.95% were performed to prepare LP@PE for catalyzing

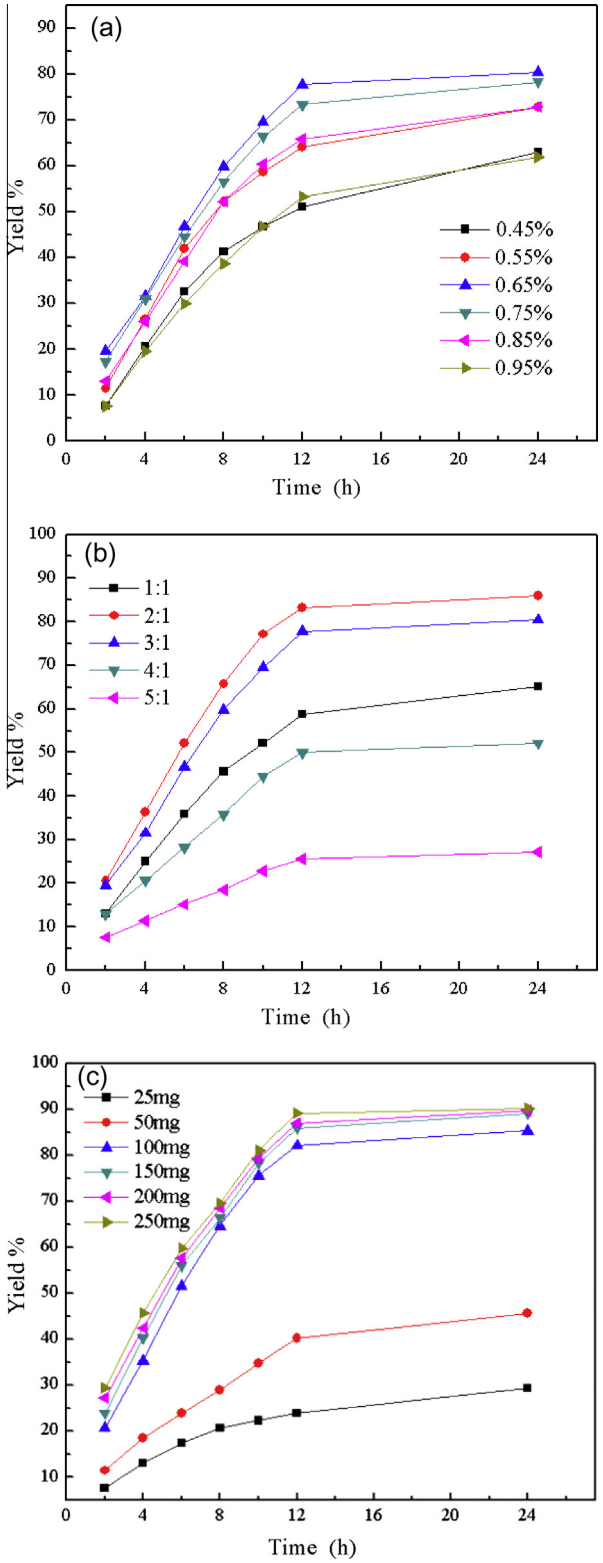

Fig. 1. (a) Effect of the water fraction on yield of biodiesel (The reaction conditions: oleic acid 0.42 g, molar ratio of ethanol to oleic acid 3:1, LP@PMO 100 mg, phosphate buffer pH 8.0, 35 °C and 200 r/min); (b) effect of the molar ratio of ethanol to oleic acid on yield of biodiesel (The reaction conditions: water fraction 0.65%, oleic acid 0.42 g, LP@PMO 100 mg, phosphate buffer pH 8.0, 35 °C and 200 r/min); (c) effect of the dosage of LP@PMO on yield of biodiesel (The reaction conditions: water fraction 0.65%, oleic acid 0.42 g, molar ratio of ethanol to oleic acid 2:1, phosphate buffer pH 8.0, 35 °C and 200 r/min).

biodiesel production. The results were presented in Fig. 1a. The biodiesel yield increased gradually from 63.0% to 80.4% with the water fraction increasing from 0.45% to 0.65%. With the water fraction further increased to 0.95%, the biodiesel yield decreased to 62.0%. These results can be explained as follows: (1) when the water fraction was below 0.65%, there was not enough water to form the stable emulsions with all the LP@PMO particles. Additionally, the water content could not meet lipase conformation requirement to maintain its catalytic activity in continuous reactions (Jung et al., 2012). With the water increasing, more and more LP@PE drops containing activated lipase were formed and would participate in catalytic reactions, which were beneficial for the biodiesel production. (2) When the water fraction was above 0.65%, insufficient LP@PMO particles were available to stabilize the LP@PE. Thus, the coalescence of the emulsion drops would be occurred and decreased the interfacial area, which had negative effect on the catalytic performance of LP@PE and final yield (Arditty et al., 2003; Binks and Whitby, 2004). Therefore, the water fraction of 0.65% was adopted in the subsequent experiments.

3.2.2. Effect of molar ratio of ethanol to oleic acid

The molar ratio of ethanol to oil is one of the most important parameters in biodiesel production and the optimal value depends on the properties of oil and the type of lipases. As can be seen in Fig. 1b, the yield of biodiesel increased with increasing molar ratio of ethanol to oleic acid firstly and then decreased significantly. When the molar ratio was 2:1, the highest yield (85.9%) was obtained. Shifting the ratio above or below the optimum value would decrease the biodiesel yield. These results could be explained as follows: Usually, high concentrations of ethanol are toxic to lipase and decrease the enzymatic activity (You et al., 2013). Nevertheless, low ethanol concentrations might not enough to meet the requirement of oleic acid and also could affect the mass transfer leading to the decrease of reaction rate and biodiesel yield (Gu et al., 2011; Jiang et al., 2012). Thus the molar ratio of 2:1 was adopted in the subsequent experiments.

3.2.3. Effect of the dosage of LP@PMO

The dosage of enzyme used is a crucial factor for successful industrial applications (Kim et al., 2004) and the particles concentration has an influence on the average emulsion drop size and stability (Aveyard et al., 2003; Chevalier and Bolzinger, 2013; Frelichowska et al., 2009). Therefore, the effect of LP@PMO dosage ranging from 25 to 250 mg on the yield of biodiesel was examined and the results were shown in Fig. 1c. The biodiesel yield increased with the increasing of LP@PMO dosage and the highest yield was about 89.1% after 24 h when 150 mg of LP@PMO was used. Further increase of lipase dosage resulted in slightly increase of the yield. These could be explained as follows: when LP@PMO dosage was below 150 mg, increase the amount of available LP@PMO would allow for more substrate molecules to be absorbed by the lipase active center, and then improved of the reaction rate and yield (You et al., 2013). When the LP@PMO dosage was above 150 mg, further increase in the lipase quantity did not have much effect on biodiesel yield. The leveling-off behavior at higher lipase quantity was typical and the results were consistent with the previous reports (Lu et al., 2007; Thimmaraju et al., 2003). Thus, 150 mg of LP@PMO was the best amount for biodiesel synthesis in terms of maximizing economics and process efficiency.

3.2.4. Effect of the pH of phosphate buffer

Usually, pH can not only influence the stability of Pickering emulsions by changing the wettability of the particles (Binks and Lumsdon, 2000; Wiese et al., 2012), but also influence the stability and activity of lipase (Szczesnaantczak et al., 2009). To determine the optimum pH of phosphate buffer, the reactions were

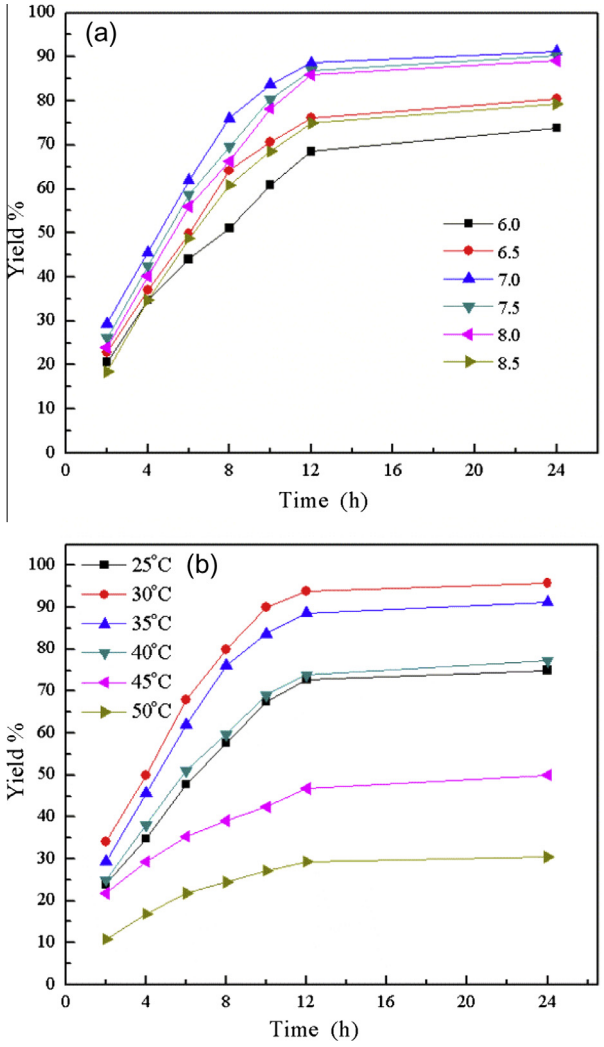

Fig. 2. (a) Effect of the phosphate buffer pH on yield of biodiesel (The reaction conditions: water fraction 0.65%, oleic acid 0.42 g, molar ratio of ethanol to oleic acid 2:1, LP@PMO 150 mg, 35 °C and 200 rpm); (b) effect of reaction temperature on yield of biodiesel (The reaction conditions: water fraction 0.65%, oleic acid 0.42 g, molar ratio of ethanol to oleic acid 2:1, LP@PMO 150 mg, phosphate buffer pH 7.0 and 200 r/min).

conducted with pH ranging from 6.0 to 8.5. As can be seen in Fig. 2a, the biodiesel yield increased with the pH increasing and the maximum yield of 91.3% was obtained at pH 7.0. When pH was above 7.0, the yield began to decrease. Shifting the pH below or above 7.0, the wettability and charge of the LP@PMO might be changed and led to the destabilization of the emulsions (Binks and Lumsdon, 1999). In addition, deviating from pH 7.0, the ionization of important functional groups of lipase was also changed, which might induce the decrease of lipase activity and final yield (Stergiou et al., 2013). Thus, the optimum pH of the phosphate buffer in this study was 7.0.

3.2.5. Effect of reaction temperature

Each enzyme has its own optimum temperature. Thus, the effect of temperatures from 25 to 50 °C on the yield of biodiesel was studied. As can be seen in Fig. 2b, the biodiesel yield increased with the ascending of the temperature from 25 to 30 °C and the highest biodiesel yield of 95.8% was obtained. This was attributed to the increased collisions of lipase with substrates and the increased reaction rate with temperature rising (Jung et al., 2012). However, with the further ascending of temperature from 30 to 50 °C, the yield was decreased, which was due to the deactivation of lipase caused by the high temperatures (Stergiou et al., 2013). Thus, the temperature of 30 °C was adopted in this study.

3.3. Reusability of the LP@PE in biodiesel production

The reusability is one of the important factors to heterogeneous catalysts for industry application. Therefore, the reusability of LP@PE was investigated in the esterification reactions of oleic acid with ethanol. In this LP@PE catalyzed system, most of the unreacted ethanol would be presented in the water phase. Thus, to ensure the ethanol–oleic acid ratio to be 2:1, the amount of ethanol required in the next cycle should be adjusted by mass balance law. This means the amount of ethanol required should be equal to the total of ethanol consumed in reaction and the residual ethanol in toluene. Amount of residual ethanol in toluene phase could be measured by GC.

As can be seen in Fig. 3a, the maximum yields of biodiesel (based on oleic acid) were 95.8%, 96% and 81% for the first run that catalyzed by LP@PE, LP@PMO and native lipase, respectively. After recycling 15 times, the yield of biodiesel that catalyzed by LP@PE was still maintained at 88.6%. Nevertheless, the yield catalyzed by LP@PMO and native lipase decreased dramatically to 45.8% and 20.5%, respectively. This phenomenon indicated that LP@PE

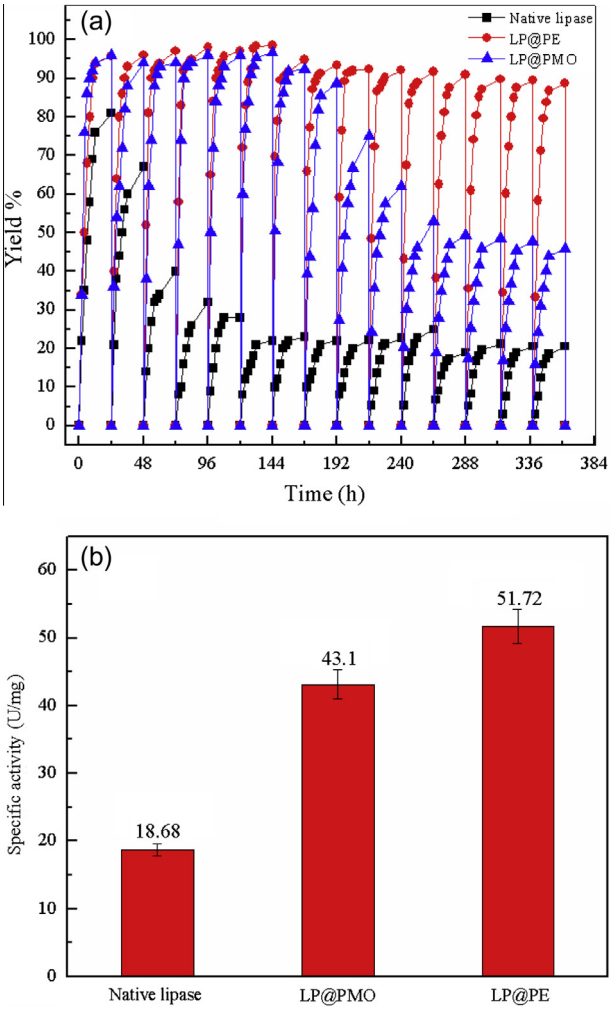

Fig. 3. (a)The catalytic performance of LP@PE, LP@PMO and native lipase in esterification reactions (The reaction conditions: water fraction 0.65%, oleic acid 0.42 g, molar ratio of ethanol to oleic acid 2:1, LP@PMO 150 mg, phosphate buffer pH 7.0, 30 °C and 200 r/min). (b) The highest specific activity of LP@PE, LP@PMO and native lipase in esterification reactions.

owned better reusability than LP@PMO and native lipase. The decreased yield of biodiesel that catalyzed by LP@PE might be due to the lipase deactivation resulting from ethanol accumulation in the water phase with cycle increasing. For measuring the operational stability of LP@PE, the morphologies were observed via optical microscope. The LP@PE had the expected spherical morphology as that in first cycle after 15 cycles (Fig. S4). The increased size and darker coating color of LP@PE were due to unavoidable drops coalescence in continuous operations. However, as can be seen Figs. S5 and S6, the morphology and size distributions of LP@PE had little change after 5-month's static storage, which indicated that the storage stability of LP@PE was excellent.

Additionally, as can be seen in Fig. 3b, the highest specific activity of LP@PE (51.72 U/mg) was 1.2 times higher than that of LP@PMO (43.1 U/mg) and 2.8 times than that of native lipase (18.68 U/mg). This result indicated that the Pickering emulsion system was more favorable for improving the activity of lipase *Candida* sp. 99–125. Since it had been proposed that Pickering emulsion could not affect the reaction mechanism of lipase (Wu et al., 2011a), the significant enhanced activity might be due to the considerably increased interfacial area of the Pickering emulsion droplets, which can improve the mass transfer of reactants and the accessibility of enzyme in the reaction system (Wang et al., 2012).

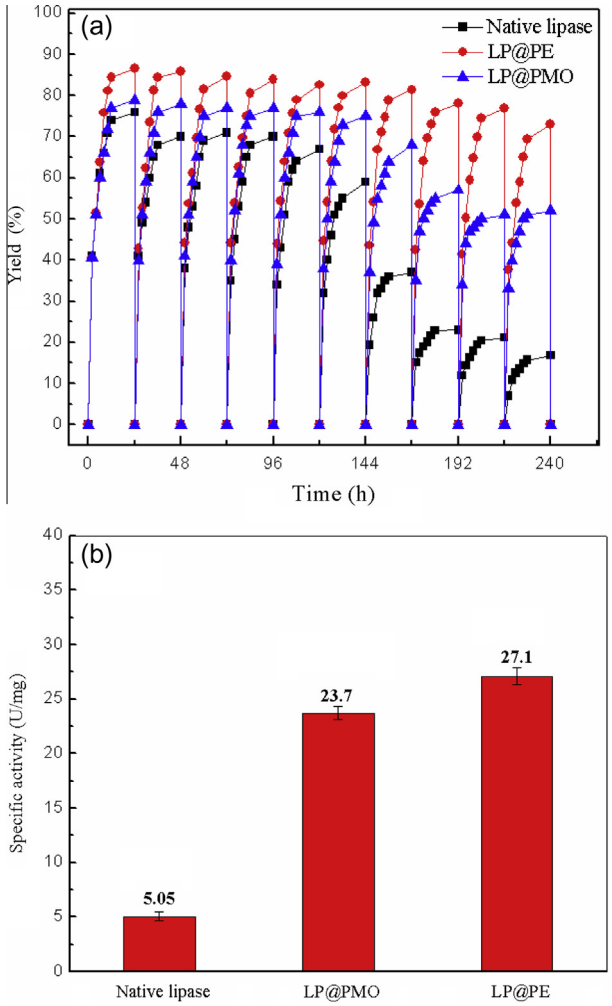

Fig. 4. (a)The catalytic performance of LP@PE, LP@PMO and native lipase in transesterification reactions (The reaction conditions: water fraction 0.65%, *Jatropha curcas* oil 0.42 g, molar ratio of ethanol to oil 4:1, LP@PMO 200 mg, phosphate buffer pH 7.0, 30 °C and 200 r/min). (b) The specific activity of LP@PE, LP@PMO and native lipase in transesterification reactions after using 10 times.

3.4. Application and reusability of the LP@PE in transesterification for biodiesel production

J. curcas oil, a non-edible plant oil, was selected for biodiesel production via transesterification because of its rich natural resource and high oil content. Transesterification reactions of *J. curcas* oil with ethanol that catalyzed by LP@PE, LP@PMO and native lipase were investigated and the conditions were set as follows: water fraction 0.65%, *J. curcas* oil 0.42 g, molar ratio of ethanol to oil 4:1, LP@PMO 200 mg, 30 °C, and phosphate buffer pH 7.0. To ensure the ethanol–oil ratio to be 4:1, the amount of ethanol required in the new cycle should be adjusted by mass balance law. In the first reaction cycle, the highest biodiesel yield (based on *J. curcas* oil) that catalyzed by LP@PE, LP@PMO and native lipase was 87.1%, 79.8% and 76%, respectively (Fig. 4a). After 10 batches operations, the biodiesel yield was about 73.0% for LP@PE, which was better than that of LP@PMO (52.1%) and native lipase (16.8%). The specific activity of LP@PE (27.1 U/mg) was also higher than that of LP@PMO (23.7 U/mg) and native lipase (5.05 U/mg) (Fig. 4b). The improved catalytic performance of LP@PE was probably attributed to the dramatically enlarged area of the water/oil interface where the biocatalytic reaction occurred (Wang et al., 2012; Wiese et al., 2012; Wu et al., 2011a, 2011b; You et al., 2013). In the continuous operations, the decreased activity of lipase might be ascribed to the effect of enzyme inhibitors (i.e., glycerol and ethanol) in the reaction media (Jiang et al., 2012). In addition, another obvious superiority when carrying out enzymatic reactions in Pickering emulsion was the ease of biocatalyst separation from substrates and products in the organic phase. Upon the completion of one batch, the LP@PE could be precipitated rapidly after standing for several seconds, which makes the recovery easily. These advantages clearly showed that the Pickering emulsion stabilized by lipase-containing PMO particles (LP@PE) had considerable potential for the design of green and sustainable biotransformation.

4. Conclusion

The successful construction of Pickering emulsion stabilized by lipase-containing PMO particles (LP@PE) for application in biodiesel production has been demonstrated firstly. Compared to native lipase and LP@PMO, the activity, stability and reusability of LP@PE were considerably enhanced. Although some notable issues (i.e. the exact mechanism of the activity enhancement, the effect of water phase on reaction equilibrium, how to avoid reunited phenomena) deserve further investigation, the present enzyme immobilization system can be expected as an important technique for various biotechnological applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.12.001.

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