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ARTICLE

Immobilized lipase on porous ceramic monoliths for the production of sugar-derived oil gelling agent

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Abstract: In this study, lipase from *Candida sp*. 99-125 was immobilized on glutaraldehyde-activated APTES-functionalized or epoxy-functionalized porous ceramic monoliths. The activity and stability of the immobilized lipases were investigated. The results indicated that the stabilities of immobilized lipases were improved significantly compared to native lipase. The immobilized lipases were used as catalysts for the production of oil gelling agent by using mannitol and fatty acid vinyl ester as substrates. Take Man-8 as an example, the yield of this oil gelling agent was more than 78% after 48 h reaction that catalyzed by the immobilized lipases. After the 5th batch reaction, more than 50% of yield can be maintained. Furthermore, the results of gelation tests indicated that the oil gelling agent can gel various hydrocarbons and oils. When the oil gelling agent was used to recover diesel from a biphasic mixture, more than 90% of the initial diesel can be recovered.

Introduction

Marine oil spills including crude oil, refined petroleum products, heavier fuels and any waste oil can cause irrecoverable damage to the marine ecosystem and environment. The oil spills incident, such as the incident of Gulf of Mexico in 2010 and ConocoPhillips in 2011, not only represents a profound waste of energy, but also contaminates the environment that cannot be recovered in short time [1]. Several methods, such as mechanical recovery, use of sorbents, dispersants and solidifiers, have been developed for the oil spill treatment [2, 3]. Practically, most of the current methods have limitations, both in containing the oil spread and in allowing recovery of the oil spill. For instance, it is difficult to blend the solidifiers with viscous oils, and the recovery of oil from the gels is cumbersome. So, there is clearly a need for developing a novel material to recover the oil spills.

Oil gelling agents, the effective solidifiers, can gel oil to form a solidified mass through hydrogen bonds, van der Waals and/or π–π stacking interactions and have been used as model compounds for the study of oil spill recovery [4-6]. Currently, amino acids, chitosan, leather fiber, starch and sugar-derived oil gelling agents have been synthesized by base-catalyzed or acid-catalyzed reactions [4, 7-9]. However, these base or acid-catalyzed reactions are energy-intensive, equipment corrosion, and require tedious separation/purification steps. For example, mannitol 1,6-diesters can function as phase-selective gelators of the oil phase from a mixture of oil and water. However, conventional methods for the synthesis of mannitol 1,6-diesters are limited by the drawbacks of high energy consumption and the producing of numerous byproducts [10]. This can be easily overcome by using biocatalysts because of their excellent properties of low energy consumption, high catalytic efficiency, and high selectivity, which lead to high purity of products and reduced post processing operations and costs [2, 10, 11]. However, until now, only one work has attempted to use the immobilized lipase from *Candida antarctica* (Novozyme 435) as the catalyst for the synthesis of the oil gelling agent[2]. The literature concerning the application of the other enzymes for oil gelling agent synthesis is very scarce and further studies are indeed required. Compared to Novozyme 435, lipase from *Candida*

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sp. 99-125 is a low cost enzyme with high activity and stability [12]. Therefore, the employment of the lipase from *Candida sp.* 99-125 as catalyst may be a potentially powerful approach for producing sugar-derived oil gelling agent.

For a successful industrial application of lipases, these biocatalysts are usually immobilized, since immobilization can improve the performance of the biocatalysts and permits their simple reuses. Up to now, numerous methods and supports have been developed for enzyme immobilization [13-18]. Recently, the development of monolithic technologies for use in the fields of enzyme immobilization and biological engineering has attracted considerable attention because of their potential applications in industrial catalysis [19]. The properties of ease fabrication and surface modification, improved mass transfer kinetics and separation performance make the monolithic materials highly attractive carriers for enzyme immobilization [20]. Krenkova et al. reviewed the progress of immobilizing enzymes on monolithic supports and pointed out that the monolithic materials will enjoy high popularity in the future since they can offer many advantages not achievable by other means [21]. For instance, porous ceramic monoliths (PCM) that combined the large specific surface areas with high liquid flow permeability has been prepared and demonstrated to be an excellent platform for laccase immobilization [20]. This approach, however, has never been extended to immobilize other enzymes.

Thus, in this study, PCM was prepared in a fast and facile eco-friendly two-step process according to Klein's report with minor modification [20]. Lipase from *Candida sp.* 99-125 was immobilized on the functionalized PCM and the immobilizing conditions were optimized. The thermal stabilities of the obtained biocatalysts in water, acetone, and isooctane were investigated. Then, the biocatalysts were used to catalyze the production of sugar-derived oil gelling agent. Overall, the objective of this study was to demonstrate the feasibility of immobilizing lipase onto PCM and utilizing the immobilized lipase in novel oil gelling agent production.

2. Experimental

2.1 Materials

Silica nanoparticles (5±5 nm) and α-alumina (0.20 μm) were purchased from Shanghai Pure Biochemical Technology Co., Ltd. (China). Sodium alginate and Tri-sodium citrate dihydrate were purchased from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd. 3-Aminopropyltriethoxysilane (APTES) and (3-glycidyloxypropyl) trimethoxysilane (GPTMS) were purchased from Beijing Shenda Fine Chemical Co., Ltd. Lipase from *Candida sp.* 99-125 (native lipase in the powder form) was purchased from Beijing CAT New Century Biotechnology Co., Ltd. (China). Mannitol was purchased from Sigma. Novozyme 435 (N435, *Candida antarctica* lipase immobilized on acrylic resin) was purchased from Novozymes Biotechnology Co., Ltd. Vinyl hexanoate, vinyl caprylate, vinyl decanoate, vinyl laurate and vinyl myristate were purchased from TCI Development Co. Ltd. Diesel oil was purchased from China Sinopec gas station. All other chemicals were of commercially analytical grade and used without further purification.

2.2 Preparation and functionalization of PCM

The PCM was fabricated according to Klein's report with minor modification [22]. Typically, a suspension containing 0.9 wt% Na-alginate, 7.3 wt% silica, 0.15 wt% citrate and 14.7 wt% alumina was added drop wise to a 0.1 mol/L CaCl2 water/ethanol (80/20-v/v) solution using a shortened hypodermic needle tip (sterican Ø 0.7 mm) at room temperature. After cross-linking 18 h, the formed microbeads were washed 3 times and poured in to molds. The beads were dried in a mold at room temperature and subsequently sintered at 1000 °C for 2 h, and then PCM was obtained.

For preparation of glutaraldehyde-activated APTES-functionalized PCM (Glu-APTES-PCM), 5 g of PCM was added to a solution consisting of 40 mL APTES and 100 mL toluene. After being reflux at 120 °C for 4 h, suspension was filtrated and washed three times with acetone. After drying under vacuum, the material was added to aqueous glutaraldehyde solution (10 wt%) and the mixture was shaken at room temperature for 2.5 h. The excess glutaraldehyde was removed by washing repeatedly with deionized water. Finally, the sample was dried under vacuum and then the Glu-APTES-PCM was obtained.

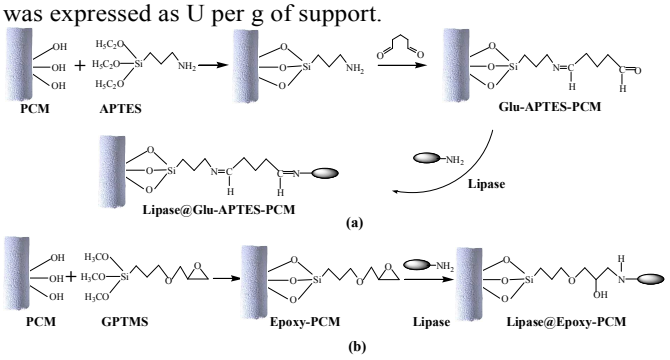
For preparation of epoxy-modified PCM (Epoxy-PCM), 5 g of PCM was added to a solution consisting of 40 mL GPTMS and 100 mL toluene. After being reflux at 120 °C for 4 h, suspension was filtrated and washed three times with acetone. The sample was dried under vacuum and then the Epoxy-PCM was obtained.

2.3 Immobilization of lipase on Glu-APTES-PCM and Epoxy-PCM

Glu-APTES-PCM and Epoxy-PCM were used as carriers for performing covalent immobilization of lipase. The immobilization process and the interactions between lipase and carrier were shown in Scheme 1. Typically, 3 g of Glu-APTES-PCM or Epoxy-PCM was mixed with 10 mL of lipase from *Candida sp.* 99-125 solution (100 mM sodium phosphate buffer, pH 7.0). The mixture was incubated at room temperature under shaking condition (120 r/min) for different times. Then, the samples were taken out at each time point. After that, the immobilized lipase was washed with deionized water three times. The sample was dried under vacuum and then the immobilized lipases Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM were obtained. The lipase concentration and immobilizing time were optimized.

The activity of immobilized lipase was measured with the hydrolysis of 4-nitrophenyl palmitate. Typically, 100μL of 4-nitrophenyl palmitate ethanol solution (13.2 mmol/L) was added into the mixture consisting of 5 mL phosphate buffer solution (pH 7.0) and 100 mg immobilized lipase, and reacted 1 min at room temperature. After the reaction, the

mixture was filtered and the absorbance of filtrate was measured at 405 nm. One unit of enzyme activity was defined as the mount of enzyme which liberates 1 μmol of p-nitrophenol per minute under the assay conditions. The specific activity of native lipase was expressed as U per mg of protein and the specific activity of immobilized lipase was expressed as U per g of support.



Scheme 1 (a) Lipase immobilized on glutaraldehyde-activated APTES-functionalized porous ceramic monoliths; (b) Lipase immobilized on epoxy-functionalized porous ceramic monoliths.

The concentration of lipase solution was determined by the Bradford protein assay using bovine serum albumin as the standard. The loading amount of lipase on supports was calculated by difference in the protein mass of the solution before and after immobilized. The immobilization efficiency was calculated by the following equation:

$$\text{Efficiency (\%)} = (\text{observed activity})/(\text{immobilized activity}) \times 100\%$$

2.4 The thermal stability test

For assaying the thermal stability in water, the native lipase powder, Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM were incubated in water for different times at 50 °C and 60 °C. At the end of incubation times, the samples were taken out and the remaining hydrolytic activity of native or immobilized lipase was measured with the hydrolysis of 4-nitrophenyl palmitate.

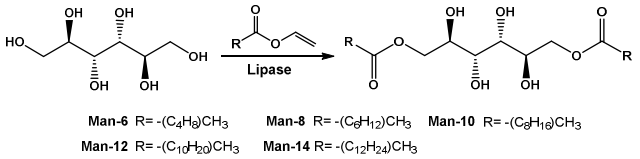
For assaying the thermal stability in acetone, the native lipase powder, Lipase@ Glu-APTES-PCM and Lipase@Epoxy-PCM were incubated in acetone for different times at 45 °C and 55 °C, respectively. At the end of incubation times, the samples were taken out and the remaining esterification activities were measured.

For assaying the thermal stability in isooctane, the native lipase powder, Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM were incubated in isooctane at 70 °C. After incubation for different times, the samples were taken out and the remaining esterification activity was measured.

The esterification activity was measured as follows: 0.8 g of immobilized lipase or 30 mg of native lipase was added into the mixture consisting of 0.42 g of oleic acid, 0.35 mL of ethanol and 5 mL of cyclohexane. Then, the mixture was incubated at 40 °C for 1 h under shaking condition (120 r/min). 0.5 mL of samples were withdrawn and mixed with 10 mL of ethanol/acetone (1:1, v/v). The ester conversions were determined by measuring the remaining acid value by titration with NaOH (50 mmol/L) in the presence of phenolphthalein.

2.5 Synthesis of oil gelling agent by using native lipase, Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM

The preparation process of the sugar-derived oil gelling agents including mannitol 1,6-dihexanoate (Man-6), mannitol 1,6-dicaprylate (Man-8), mannitol 1,6-didecanoate (Man-10), mannitol 1,6-dilaurate (Man-12), and mannitol 1,6-dimyristate (Man-14) were as follows(Scheme 2). Typically, N435, native lipase, Lipase@Glu-APTES-PCM or Lipase@Epoxy-PCM with equal activity (200 U) were added to a mixture of 3 mmol mannitol, 9 mmol fatty acid vinyl ester (vinyl hexanoate, vinyl caprylate, vinyl decanoate, vinyl laurate or vinyl myristate) and 40 mL acetone. The mixtures were shaken in an incubator shaker at 45 °C (120 r/min). The reaction was monitored by thin layer chromatography (TLC) visualized by staining with ethanolic phosphomolybdic acid. After each reaction the lipase was separated by filtration from the mixture and washed 3 times with acetone, and then reused.



Scheme 2 The synthetic route of oil gelling agent

The separated solvent from the mixture was removed in a rotary evaporator. The obtained crude products were purified by silica gel flash chromatography using chloroform: methanol (27:1) as an eluent. Flash column chromatography was performed on silica gel (200-300 mesh). The yield of oil gelling agent was calculated based on the obtained amount of oil gelling agent and the theoretical quantity of oil gelling agent, which can be calculated from "Equation (1)".

$$Y = m/M \times 100\% \quad (1)$$

Where, m (g) is the obtained amount of oil gelling agent, M (g) is the theoretical quantity of oil gelling agent. The theoretical quantity of oil gelling agent is calculated from "Equation (2)".

$$M = m1/M1 \times M2 \quad (2)$$

Where, m1 (g) is the amount of mannitol used, M1 (g/mol) is the molecular weight of mannitol, M2 (g/mol) is the molecular weight of the oil gelling agent.

2.6 Gelation test of the oil gelling agent

Gelation test of the sugar-derived oil gelling agents: Man-6, Man-8, Man-10, Man-12 and Man-14 were used to gel various organic solvents for testing their gelation ability. The concentration of Man-6 used for gelation of toluene, petrol, diesel, olive oil and soybean oil was 2.0%, 5.5%,

5.0%, 4.0% and 6.0%, respectively. While for Man-8, Man-10, Man-12 and Man-14, the concentration of 1.5%, 3.0%, 2.5%, 1.5% and 4.0% were used. The oil gelling agents were added to the organic solvents and heated until the solid was completely dissolved. The resulting solution was allowed to stand at room temperature, and then gelation was visually observed.

Gelation test of Man-8 in the presence of water: Man-8 (0.05 g) completely dissolved in ethanol was added to the mixture consisting of water (2 mL), organic phase (0.6 mL) and a magnetic stirring rotor (0.7 g) at room temperature. After 5 min, the gels were visually observed.

Recovery of diesel gel from diesel-water mixture: Man-8 (0.4 g) completely dissolved in ethanol (10 mL) was added to the mixture consisting of water (30 mL) and diesel (10 mL) at room temperature. After 1 h, gelation was visually observed and the gel can be separated with net. The diesel can be recovered from the gel by vacuum distillation.

All experiments were performed in triplicate, and the means ± standard deviation values were calculated and reported.

2.7 Characterizations of the PCM and oil gelling agents

The pore size, porosity and surface area of PCM were measured via mercury-intrusion porosimetry (PoreMaster 60GT, Quantachrome America), assuming the contact angle and surface tension of mercury to be 140o and 480 mNm-1, respectively. The Fourier transform infrared (FT-IR) spectra of PCM, Epoxy-PCM, APTES-PCM and Glu-APTES-PCM were collected using a Bruker vector 22 FT-IR spectrometer with a spectral resolution of 0.4 cm−1 (Bruker Corporation, Germany) by KBr pellet method. The sugar-derived oil gelling agents were dissolved in dimethyl sulphoxide (DMSO) solvent and analyzed by NMR spectroscopy. 1H and 13C-NMR spectra were recorded on a Bruker Avance III 400 NMR spectrometer (proton: 400.13 MHz, 295.8 °C; carbon: 100.62 MHz, 298.5 °C).

3. Results and discussion

3.1 Characterization of PCM, Glu-APTES-PCM and Epoxy-PCM

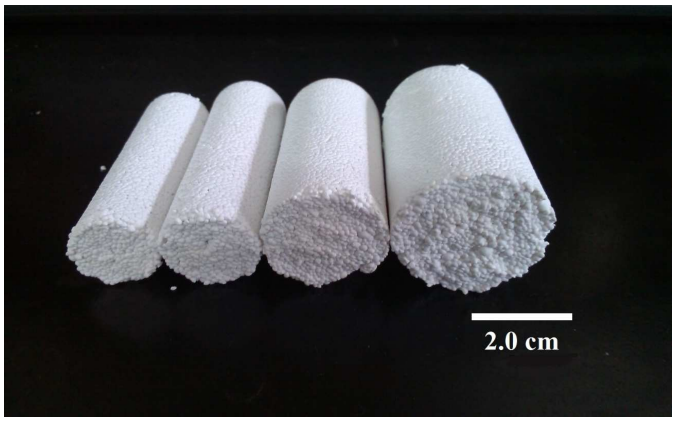


Fig. 1 Examples of the obtained PCM

The examples of the obtained PCM were shown in Fig. 1. FTIR spectroscopy was used to verify the presence of the functional groups on PCM involved in the modification processes. As shown in Fig. 2, bands at 2841, 2934, 2944 and 2975 cm^{-1} all corresponded to the C-H absorption and vibration from CH_3 and CH_2 [23]. These results indicated that organosilane had reacted with PCM, which was also reported in other literature [23].

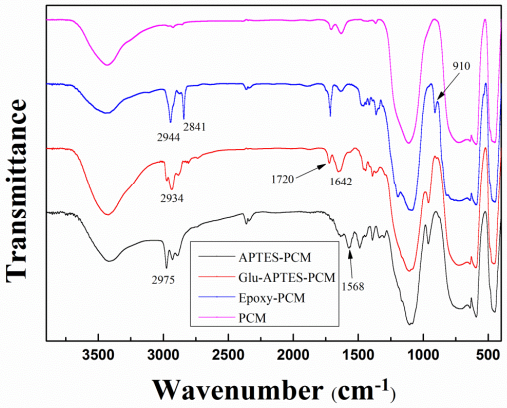


Fig. 2 FT-IR spectra of PCM, APTES-PCM, Glu-APTES-PCM and Epoxy-PCM

The FT-IR spectrum of APTES-PCM showed an absorption band at 1568 cm^{-1}, which can be attributed to N-H stretching vibration indicating the presence of APTES on the surface of PCM [23]. In the FT-IR spectrum of Glu-APTES-PCM, the peak at 1720 cm^{-1} corresponding to the C=O groups and the peak at 1642 cm^{-1} corresponding to the C=N groups were observed[23, 24]. This confirmed the successful preparation of Glu-APTES-PCM. In the FT-IR spectrum of Epoxy-PCM, the peak at 910 cm^{-1} corresponding to the epoxy groups was observed, which confirmed the presence of epoxy group on the Epoxy-PCM [25]. The pore size, porosity and surface area of PCM, Glu-APTES-PCM and Epoxy-PCM were shown in Table 1.

Table 1 Properties of PCM, Glu-APTES-PCM and Epoxy-PCM

	Total Surface Area (m^2/g)	Pore Size (μm)
PCM	47.01	0.01-0.20
Glu-APTES-PCM	44.93	0.01-0.15
Epoxy-PCM	35.75	0.02-0.20

The pore size distribution was shown in Supplementary Information Fig.S1. The pores inside were formed by the calcium alginate gel fiber after calcined [20]. As can be seen in Table 1 and Fig.S1, the pore size and surface area of the modified PCM were decreased in comparison to PCM. This may be attributed to the occupation of the functional groups in the pore channels [26]. According to the previous report, the pore sizes necessary for efficient enzyme immobilization were reported to be about 40-200 nm, which depended on the size of the enzyme [27]. Thus, Glu-APTES-PCM and Epoxy-PCM have the suitable pores which can immobilize

lipase effectively. In addition, based on Klein's results, the special properties of PCM such as high permeability and porous structures are favorable to the immobilization of enzyme and the transport of substrates and products [20].

3.2 Optimization of the lipase immobilization conditions

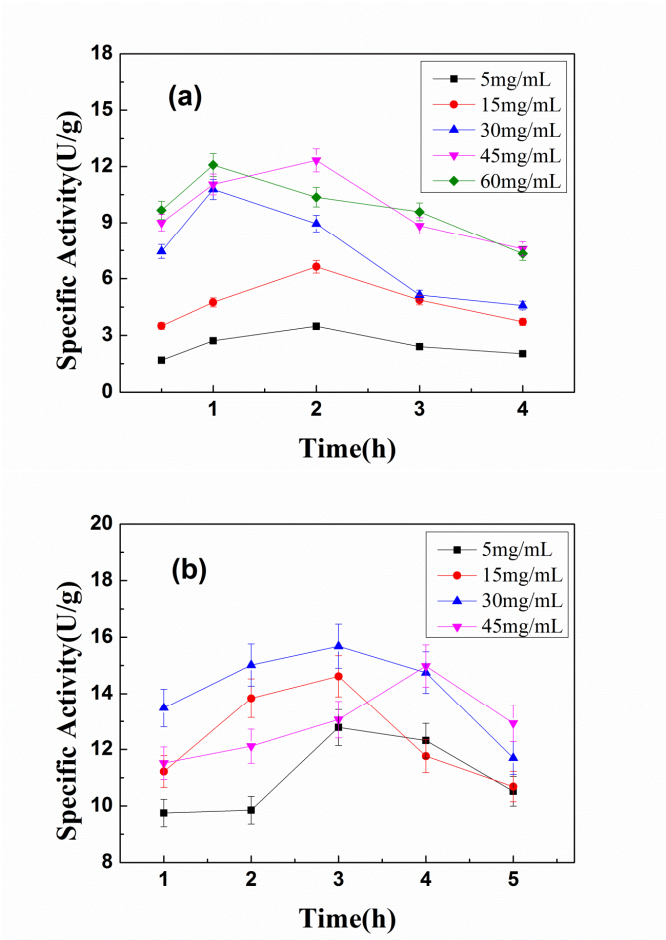


Fig. 3 Effect of lipase concentration and immobilizing time on the activity of Lipase@ Glu-APTES-PCM (a) and Lipase@Epoxy-PCM (b)

The effect of lipase concentration and immobilizing time on the specific activity of Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM was investigated and shown in Fig. 3. With the increase of immobilizing time, the activities of the immobilized lipases increased first and then decreased under a certain concentration of lipase solution. This can be explained as follows: with the immobilizing time increase, more lipase would be immobilized on the carriers and the specific activities of the immobilized lipases would increase; after that, the specific activities decreased with the immobilizing time was prolonged further, which may be a consequence of protein-protein interactions taking place at high lipase loading [28]. In addition, the specific activity of the immobilized lipase increased with increasing the initial lipase concentration and reached a maximum value at 45 mg/mL for Lipase@ Glu-APTES-PCM and 30 mg/mL for Lipase@Epoxy-PCM. The corresponding lipase loadings on Glu-APTES-PCM and Epoxy-PCM were 5.7 mg/g and 6.4 mg/g, respectively. After that, the specific activity decreased when further increased the initial lipase concentration. This can be mainly due to the steric hindrance effect of the enzyme molecules at high loading densities that induced by the high initial lipase concentration [29, 30].

Based on the above results and discussion, the optimal conditions for preparing immobilize lipases in subsequent experiments were determined. For preparation of Lipase@Glu-APTES-PCM, the initial lipase concentration and immobilizing time were 45 mg/mL and 2 h, respectively. The corresponding lipase loading amount, specific activity, and the immobilization efficiency were 5.7 mg/g, 12.3 U/g, and 9.7%, respectively. For preparation of Lipase@Epoxy-PCM, the initial lipase concentration and immobilizing time were 30 mg/mL and 3 h, respectively. The corresponding lipase loading amount, specific activity, and the immobilization efficiency were 6.4 mg/g, 15.7 U/g, and 11.0%, respectively. Compared to our previous reports, the lipase loading amount, the specific activity, and immobilization efficiency were low, which may be due to the relative low surface area of the PCM supports [12, 31]. New approaches are required to improve the porous structure and the surface area of the PCM supports in the future studies.

3.3 Thermal stability of native and immobilized lipase

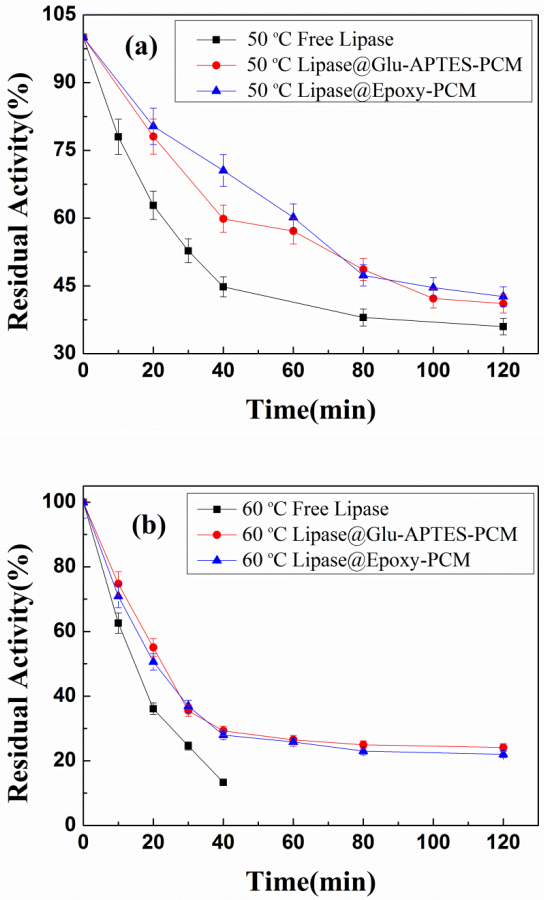


Fig. 4 The thermal stability of native lipase, Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM in water at 50 °C (a) and 60°C (b)

Generally, the lipase-catalyzed reactions are conducted in elevated temperatures. Thus, the thermal stabilities of native lipase, Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM in water, acetone and isooctane were investigated. As can be seen in Fig. 4 (a) and (b), all lipases denatured and showed

degradation in activity at 50 °C and 60 °C in water, however, the Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM were more stable than that of free one. Similar phenomena were also observed by Soni and coworkers when immobilizing *Thermomyces lanuginosus* lipase on ZnO nanoparticles [32].

As can be seen in Fig. 5 (a), at 45 °C, the native lipase and immobilized lipase were significantly activated when they were incubated in acetone with short time. The native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM showed the highest activity after 12h (130%), 24 h (133%) and 24 h (130%) of incubation, respectively. Similar phenomena were also observed by Wu and coworkers [33]. Their results indicated that the lipase from *Mucor javanicus* had a high stability and an increased activity (246%) after 2 h of incubation in acetone at 25 °C [33]. This may be attributed to the change of the lipase's active conformation induced by the extraction of the essential water in the lipase molecules to acetone, which caused the opening of the lid that covered the active site and then the super-activation of lipase can be observed [12]. As the incubation time increase, the catalytically active conformation of lipase was changed, which resulted in the decrease of the activity and/or the enzyme inactivation[34, 35]. After 240 h of incubation in acetone at 45 °C, the native lipase maintained 40% of initial activity, while the Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM can maintain 66.7% and 60% of the initial activities, respectively. As shown in Fig. 5 (b), the similar phenomena were also observed at 55 °C. The lipases activities also have an increase when incubated in acetone with short time. At 55 °C, the highest activities of native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM can achieve more than 120% of their initial activities. However, the native lipase was inactivated at a faster rate compared to the immobilized lipases. The improved stability of Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM can be attributed to the restricted conformational mobility of lipase molecules within the pore channels of the modified-PCM and the covalent binding between the lipase molecules and supports [36].

The thermal stability in non-polar solvent of isooctane was also studied and super-activation phenomena were also observed (see Supplementary Information Fig. S2). The highest activities of native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM in isooctane (70 °C) were 116.7%, 120.6% and 114%, respectively. The half-life of native lipase at 70 °C was about 11.9 h, while the half-life of Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM were 15.5 h and 19.5 h, respectively.

The improved stability of Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM can be attributed to the restricted conformational mobility of lipase molecules within the pore channels of the modified-PCM and the covalent binding between the lipase molecules and supports [36]. Similar results were also found in the previous report that immobilized lipase from *Thermomyces lanuginosus* on mesoporous silica yolk–shell spheres[37].

The thermal stability in non-polar solvent of isooctane was also studied and super-activation phenomena were also observed (see Supplementary Information Fig. S2). The highest activities of native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM in isooctane (70 °C) were 116.7%, 120.6% and 114%, respectively. The half-life of native lipase at 70 °C was about 11.9 h, while the half-life of Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM were 15.5 h and 19.5 h, respectively.

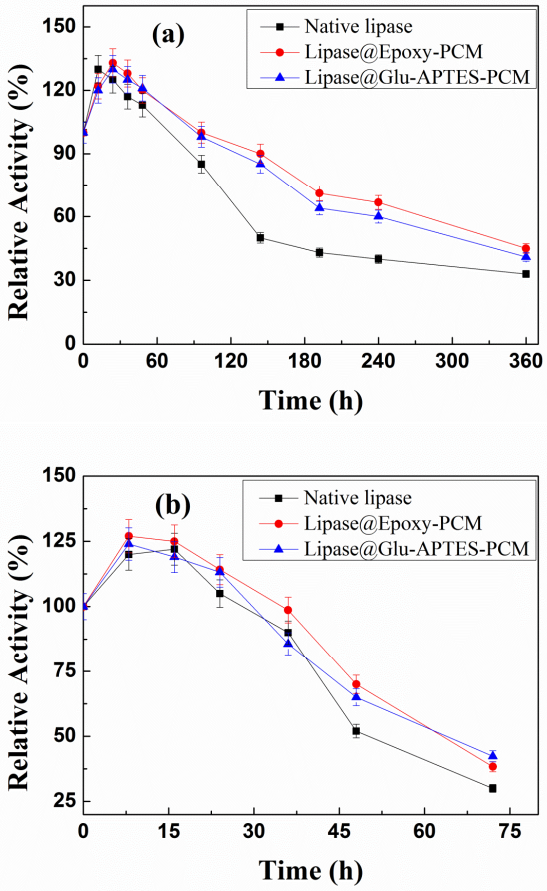


Fig. 5 Thermal stability of native lipase, Lipase@Epoxy-PCM and Lipase@ Glu-APTES-PCM in acetone at 45 °C (a) and 55 °C (b)

These improved thermal stabilities of immobilized lipase can be explained as follows: the lipase molecules were covalently combined on the supports through the reactions between supports and lipase molecules and it needs more energy to change the conformation for immobilized lipase[34, 38]. The greatly improved thermal stability made the immobilized lipases promising for industrial applications like ester synthesis, enantiomer resolution, and biodiesel production [14].

Based on the above tests and results, the catalytic characteristics of native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM were summarized in Table 2. As can be seen, although the specific activity was decreased, the half-life of the lipase was prolonged, which confirmed that PCM supports were highly efficient to stabilize lipase versus high temperature and organic solvents.

Table 2 Catalytic characteristics of native lipase, Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM

	Native lipase	Lipase@Glu-APTES-PCM	Lipase@Epoxy-PCM
specific activity (U/mg lipase)	22.7±1.1	2.2±0.1	2.5±0.1
loading protein (mg/g carrier)	--	5.7±0.3	6.4±0.3
half-time in water at 50 °C (min)	33.3±1.7	77.0±3.9	75.4±3.8
half-time in water at 60 °C (min)	14.5±0.7	22.8±1.1	19.8±1.0
half-time in acetone at 45 °C (h)	144.5±7.2	301.9±15.0	332.5±16.6
half-time in acetone at 55 °C (h)	50.4±2.5	63.8±3.2	62.9±3.1
half-time in isooctane at 70 °C (h)	11.9±0.6	19.5±1.0	15.5±0.7

3.4 Preparation of sugar-derived oil gelling agents

Conventional routes to such mannitol 1,6-diesters involve multiple steps with protection and deprotection methods. In the study, we choose a single step method by employing regiospecific enzyme catalysis that has been reported by John and coworkers [2]. Five kinds of mannitol 1,6-diesters including Man-6, Man-8, Man-10, Man-12 and Man-14 with different alkyl chain length were prepared respectively by using native lipase from *Candida sp.* 99-125 as catalyst. The obtained products were determined by 1H-NMR and 13C-NMR spectrum (see Supplementary Information Fig. S3-S7), and the NMR spectra fit the molecular structure of Man-6, Man-8, Man-10, Man-12 and Man-14, respectively, which was consistent with the previous reports [2, 39]. The yield of mannitol 1,6-diesters were also investigated and the results showed that more than 59% of yield can be obtained from all fatty acid vinyl esters (Fig. S8). The yields of Man-6 (82%) and Man-8 (81%) were comparable and higher than that of Man-10 (70%), Man-12 (61%) and Man-14 (59%), which indicated that the yields of mannitol 1,6-diesters were decreased with the increase of the carbon number of fatty acid vinyl ester. This can be explained by the steric effects of long chain fatty acid vinyl ester that blocked the access of mannitol to the lipase's active site and then led to reduced yields [40].

For the practical production of oil gelling agents using lipase from *Candida sp.* 99-125, it is critical to maintain the initial activity of the biocatalysts in consecutive batch reactions. Thus, the recycling stability of native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM was studied. For comparison, the catalytic property of N435 was also tested under the same operational conditions. Take Man-8 as an example, time courses of biotransformation of sugar to Man-8 that catalyzed by N435, native lipase, Lipase@Glu-APTES-PCM and Lipase@Epoxy-PCM were shown in Fig. 6.

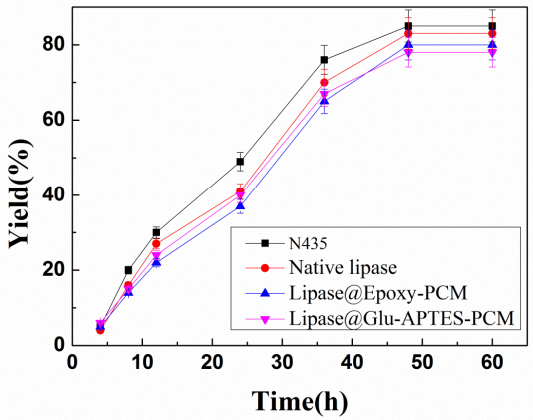


Fig. 6 Time course of N435, native lipase, Lipase@Glu-APTES-PCM, and Lipase@Epoxy-PCM in preparation of Man-8

Increase of the reaction time from 0 to 48 h can improve the Man-8 yield significantly for all the lipase preparations, and at 48 h, the yields of Man-8 catalyzed by N435, native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM reached 85%, 83%, 80% and 78%, respectively. Further increase of reaction time did not influence the Man-8 yield too much. Accordingly, in the recycling stability tests, 48 h of reaction time was used. As shown in Fig. 7, the recycling stabilities of N435, native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM were investigated. After 5 consecutive reuses, the yields of Man-8 that catalyzed by N435, native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM were 38%, 40%, 50% and 53%, respectively. The decrease of the yields can be explained by the decreased enzymatic activity that induced by lipase leakage from the supports and inherent lipase denaturation in acetone at high temperature [12]. Additionally, the native lipase from *Candida sp.* 99-125 has a comparable catalytic activity with N435 in the first and second cycle of reaction. Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM have higher catalytic activity than N435 in the reactions after the second reaction cycle, which indicated that the stability of Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM

was better than that of N435. The reduction in the activity of N435 may be a result of the lipase molecules desorbing from the acrylic beads because of the weak electrostatic interactions

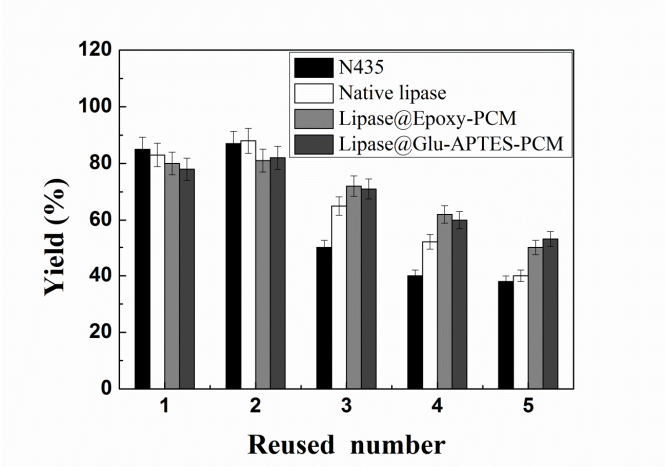


Fig. 7 Recycling stability of N435, native lipase, Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM in preparation of Man-8

between the lipase and the support [41]. While the strong chemical linkage between the lipase molecules and functionalized PCM resulted in the increase of the recycling stability of Lipase@Epoxy-PCM and Lipase@Glu-APTES-PCM. Compared with the previous reports that used N435 as catalyst for selective synthesis of 1,6-diesters, the immobilized lipase from *Candida sp.* 99-125 used in this study exhibited comparable activity, synthesis yield and improved stability. Thus, the immobilized lipase presented in this study showed a promise for industrial production of sugar-derived oil gelling agents[2,42,43].

3.5 Characterization of sugar-derived oil gelling agent

Man-6, Man-8, Man-10, Man-12 and Man-14 were used to gel toluene, petrol, diesel, olive oil and soybean oil, respectively. As can be seen in Fig. S9, all of the gels were formed within 0.5 h at room temperature and they were strong enough to bear themselves weight in the inverted beaker. The formation of gels can be explained by that the oil gelling agents can form a three-dimensional structure through hydrogen bonding, π-π bonding, electrostatic and hydrophilic interactions, and the oils can be trapped in this structure [43-45]. The gelling abilities of the sugar-derived oil gelling agents were assayed quantitatively and the minimum gelation concentrations (MGC) were summarized in Table S1. As can be seen in Table S1, Man-6, Man-8, Man-10, Man-12 and Man-14 can gel numerous organic liquids with MGC ranging from 0.87 to 5.5% wt/v. In most of the cases, the MGC required to induce gelation was strongly dependent on the alkyl chain length of these oil gelling agents.

These results were similar with the previous report that showed the gelling ability can be modulated by altering the hydrophobic fatty acid chain of the oil gelling agents [2].

To show the gelation ability of the oil gelling agents further, we chose Man-8 as a model agent in the subsequent experiments because of its relative higher yield and lower MGC. As shown in Fig. 8 (a), Man-8 can gel the oil phase while the aqueous phase was left intact. After 1h, the formed gels were strong enough to hold not only their weights but also the weight of the water on top (Fig. 8 (b)), which was consistent with the previous report [2]. We further performed a gelation test of diesel (4 mL) in the presence of 50 mL of water by using Man-8 (0.15 g). The result indicated that the gel was strong enough

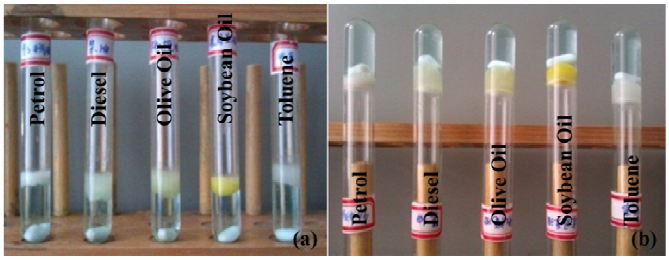


Fig. 8 Gelation of different hydrocarbons and oils in the presence of water by using Man-8

to bear its weight plus that of 50 mL water (see Video S1 in Supplementary Information). Fig. S10 showed the recovery of diesel gel from diesel-water mixture, which indicated that Man-8 can be used to separate oil from water effectively (see Video S2 in Supplementary Information). The recovery of diesel from the diesel gel can be realized by vacuum distillation and more than 90% of the initial diesel can be recovered. Overall, combining the ease of synthesis and excellent gelling ability, the oil gelling agents provide a promising approach for the cleaning and recovery of oil spills.

4. Conclusions

Lipase from *Candida sp.* 99-125 was immobilized on the Glu-APTES-PCM and Epoxy-PCM, and the thermal stabilities of immobilized lipases in water, acetone, and isooctane were improved. The mannitol-derived oil gelling agents can be successfully synthesized by using immobilized lipase from *Candida sp.* 99-125 as catalysts and the yield was more than 59%. Take Man-8 as an example, the oil gelling agent can selectively solidify various hydrocarbons and oils in the presence of water at room temperature. When the oil gelling agent was used to recover diesel from a biphasic mixture, more than 90% of the initial diesel can be recovered. Thus, this study provides not only a method for preparing robust lipase-based biocatalysts but also a promising approach for the industrial production of oil gelling agents.

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