Immobilization of Lecitase® Ultra onto the Amino-functionalized SBA-15 and their Applications in Glycerolysis

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Abstract: In this study, Lecitase® Ultra (LU) was immobilized onto the parent and the amino-functionalized SBA-15. The immobilization conditions were studied and the activity of the parent SBA-15 supported LU (SBA-15-LU) was found to be at 2177.78 ± 101.84 U/g. After 3-aminopropyl and n-(2-aminoethyl)-3-aminopropyl groups functionalization, enzymatic activity was increased to 3555.56 ± 200.21 and 3444.44 ± 346.41 U/g respectively. The immobilized LU samples were then used to catalyze glycerolysis. The possibility for diacylglycerols (DAG) and monoacylglycerols (MAG) production was evaluated and it was found only suitable for DAG production. In addition, the glycerolysis activity of the immobilized LU was impaired by the tert-pentanol and solvent-free was found suitable. Similar DAG content over 50 wt% could be obtained from glycerolysis by the three immobilized LU samples. The reusability in glycerolysis was evaluated, and 9.79% of the initial glycerolysis activity was remained from the SBA-15-LU after 5 cycles of reuse. Encouragingly, after 3-aminopropyl and n-(2-aminoethyl)-3-aminopropyl groups functionalization, 62.93% and 83.91% of their initial activity was respectively remained after 5 cycles of reuse.

Key words: amino-functionalized-SBA-15, glycerolysis, immobilization, Lecitase® Ultra

1 Introduction

Phospholipase A1 (PLA1, E.C.3.1.1.32) represents a very diverse subgroup of phospholipase with 1-acyl hydrolytic activity. It is of particular interest in industry for 2-acyl-ly-sophospholipids production and edible oil degumming, by cleaving sn-1 acyl group of phospholipids 5. PLA1 exhibits broad substrate specificity and harbors some lipase activity as well 6. Similarly to lipases, PLA1 has been described to suffer interfacial activation 7. Lecitase® Ultra (LU) is a phospholipase manufactured and marketed by Novozymes, Denmark. It was patented and made commercially available in 2007. LU is a new enzyme with a molecular mass of 35 kDa, it has a single active site which displays both the lipase and phospholipase activities 8. The commercial preparation of this new enzyme is a protein-engineered carboxylic ester hydrolase from the fusion of lipase genes from Thermomyces lanuginosa (to obtain good stability) and phospholipase genes from Pusarum oxysporum (to get the phospholipase activity) 9.

Commercial applications of LU are mainly focused on the edile oil degumming and phospholipids modifications 8, 9. In recent years, LU has also been used to synthetize diacylglycerols (DAG) through esterification 10, partial hydrolysis 11, 12 or glycerolysis reactions 13, 14. Partial acylglycerols are of great importance in food industry. Monoacylglycerols (MAG) are important food-grade emulsifiers, accounting for approximately 75% of the worldwide production of food emulsifiers 15. DAG-enriched oil has been recognized as a functional cooking oil due to its ability to reduce post-prandial serum triacylglycerols levels and prevent obesity 16. Enzymatic glycerolysis of triacylglycerols is the primary reaction route for MAG and DAG production, due to its high time-space cost efficiency and minimal glycidol fatty acid ester generation at mild reaction conditions 17, 18.

Free PLA1 is sensitive to pH and temperature and

Abbreviations: BET, Brunauer-Emmett-Teller; BJH, Barrett-Joyner-Halenda; CALB, Candida antarctica lipase B; DAG, diacylglycerols; FT-IR, Fourier transform infrared; HRTEM, high-resolution transmission electron microscope; IE, immobilization efficiency; LU, Lecitase® Ultra; MAG, monoacylglycerols; PLA1, Phospholipase A1; RML, Rhizomucor miehei lipase; TAG, triacylglycerols; TFE-SEM, thermal field emission-scanning electron microscopy; TLL, Thermomyces lanuginosus lipase; XPS, X-ray photoelectron spectroscopy; XRD, X-ray diffraction

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cannot be easily reused\(^{17}\). Immobilization facilitates the separation of products and the re-utilization of enzymes, permits the continuous use in industry\(^{20}\). In addition, enzymatic properties including stability, activity, selectivity, specificity and resistance to inhibitors, would be altered after immobilization\(^{20}\). For example, the activity and stability of LU was greatly improved when it was immobilized on cyanogen bromide agarose and on octyl agarose via physical coating with polyethyleneimine as ionic polymers\(^{20}\).

Immobilization of LU has been studied by some authors. Sheelu et al.\(^{21}\) had immobilized LU onto the gelatin hydrogel for rice bran oil degumming. The obtained immobilized LU displayed improved stability, it could be used efficiently in rice bran oil degumming in a spinning basket bioreactor, without activity loss after 6 recycles. Studies from Yu et al.\(^{22}\) indicated that, the immobilized LU exhibited a broader pH-activity and better stability profile, and more than 80% of its initial activity could be retained after 10 cycles reused in soybean oil degumming. Liu et al.\(^{12, 23}\) had studied the immobilization of LU onto the macroporous resin. They found that higher activity was obtained with macroporous resin DA-201 as support. The DA-201 displayed improved stability and it exhibited approximately 70% of relative esterification efficiency after 6 successive cycles reuse in esterification of glycerol and oleic acid\(^{23}\). In addition, the DA-201 supported LU was also used to produce DAG through glycerolysis, and DAG content up to 53.7% was obtained in solvent-free system under optimum conditions. Moreover, the DA-201 supported LU exhibited considerable reusability in glycerolysis, and 60% of its initial activity was retained after 28 consecutive batches reuse.

Mesoporous silica materials are promising supports for enzyme immobilization. Of which, SBA-15 is of particular interest, due to its large surface area, controlled pore size, and sufficient surface silanol groups which could be used for surface modification\(^{20, 25-28}\). In this study, LU was immobilized onto the parent and the \(n\)--(2-aminoothyl)--3-aminopropyl and 3-aminopropyl groups modified SBA-15 for MAG and DAG production. The enzyme immobilization conditions were optimized and the obtained immobilized LU was then used to catalyze glycerolysis. The possibility for MAG and DAG production was carefully evaluated, reaction conditions were studied and the reusability was investigated.

2 Experimental

2.1 Materials and reagents

Lecitase\(^{5}\) Ultra solution was obtained from Novozymes (Beijing, China). The hydrolysis activity of the LU solution was measured to be at 426.67 ± 28.28 U/mL (hydrolysis of tributyrin), and the protein concentration was 10.04 µg/ mg, determined by the Bradford assay. The standards of 1-monoolein, 1,3-diolein and triolein (>99.0%) for HPLC analysis were purchased from Sigma-Aldrich (Shanghai China). SBA-15 with diameters at 8.1 nm was from Nanjing XPnano Materials Tech Co., Ltd. (Nanjing, China). Refined, bleached and deodorized soybean oil was purchased from a local supermarket. Glycerol with a purity of more than 99.0% was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai China). The silane coupling agent of (3-aminopropyl)triethoxysilane (>98%) was from Sigma-Aldrich (Shanghai China), and N-[3-(trimethoxysilyl)propyl]ethylenediamine (>95%) was from Aladdin Reagents Co., Ltd. (Shanghai China). All other solvents and reagents were analytical or chromatographic grade.

2.2 Surface modification of SBA-15

Briefly, 2 g of dry SBA-15 was placed into a 150-mL three-necked flask, and 60 mL of toluene was slowly added to disperse the SBA-15, the mixture was kept refluxing under nitrogen atmosphere. Then 10 mmol of a silane coupling agent was added dropwise into the dispersion, and the mixture was stirred at 500 rpm for 8 h at 95°C. After that, the mixture was centrifuged (10 min, 3000 rpm) and the supernatant was discarded, the modified SBA-15 was washed with ethanol (50 mL×3) and diethyl ether (50 mL×3), and then dried under vacuum (pressure at 0.093 MPa) at 80°C for 6 h. The \(n\)--(2-aminoothyl)--3-aminopropyl and 3-aminopropyl groups functionalized SBA-15 were named as NH\(_2\)CH\(_2\)CH\(_2\)NHCH\(_2\)CH\(_2\)SBA-15 (Scheme 1a) and NH\(_2\)CH\(_2\)CH\(_2\)SBA-15 (Scheme 1b) respectively.

2.3 Immobilization of LU onto the (functionalized) SBA-15

Immobilization of LU was conducted according to our previous procedure with minor modifications\(^{8}\). Required amounts of the commercial LU solution was dissolved in 40 mL phosphate buffer (25 mM). The protein concentration of the LU solution was determined by the Bradford assay. Then 100 g of the (parent or organic group modified) SBA-15 was added, the obtained solution was magnetically stirred (200 rpm) at 25°C for 30 min. After that, the suspensions were filtered and washed with the phosphate buffer. The immobilized LU samples were dried in a vacuum oven (pressure at \(-0.093\) MPa) at 30°C for 6 h and named as (NH\(_2\)CH\(_2\)CH\(_2\)NHCH\(_2\)CH\(_2\)- or NH\(_2\)CH\(_2\)CH\(_2\))-SBA-15-LU (Scheme 1c or 1d, SBA-15-LU). The immobilization conditions, including time (15 to 75 min), pH of the incubating phosphate buffer (4 to 7), and LU concentration (160.64 to 281.12 µg/mL), were carefully studied. The immobilization efficiency (IE) was calculated based on the initial enzyme activity \(E_0\) and the final enzyme activity \(E_f\).

Enzymatic activity was assayed by hydrolysing of tributyrin\(^{8}\). Typically, 1 mL of tributyrin was added into 50 mL of phosphate buffer (25 mM, pH 7.0), the mixture was vigorously stirred at 40°C. Then 1 mL of the free LU solution
or 10 mg of the immobilized LU was added to catalyze the hydrolysis reaction, and the mixture was continuously titrated with 0.1M NaOH solution for 15 min to maintain a constant pH. Blank experiments were performed and one unit (U) of lipase was defined as the amount of lipase required to release 1 µmol of titratable free butyric acid per minute under assay conditions. All the experiments were conducted in triplicate.

2.4 Characterization

Small-angle powder X-ray diffraction (XRD) was carried on Bruker (D4) advance diffractometer, using Ni-filtered Cu Kα radiation at 40 kV and 40 mA in the 2θ range of 0.5-8°, at scan speed of 0.2°/min. Fourier transform infrared (FT-IR) spectra were obtained on a Nicolet 6700 FT-IR spectrophotometer (Thermo Scientific) in the 4000-400 cm⁻¹ wave number range using the standard KBr disk method. The surface chemistry and chemical state of the parent SBA-15, organic group functionalized SBA-15 and the following immobilized LU were analyzed by X-ray photoelectron spectroscopy (XPS; K-ALPHA®, Thermo Fisher Scientific), using monochromatic Al-Kα radiation of energy 1486.68 eV & 12 kV and operating in the constant analyzer energy mode. The pressure in the analysis chamber was around 3×10⁻⁸ mbar. The morphology was analyzed by the thermal field emission-scanning electron microscopy (TFE-SEM) on a GeminiSEM500 and the high-resolution transmission electron microscope (HRTEM) on a JEM-2100F microscope. The low-temperature N₂ adsorption-desorption experiments were carried out using Quantachrome (Autosorb-1Q) instruments. Before the measurement, samples were degassed in a vacuum at 80°C for 12 h. The pore diameter was determined and pore volume were calculated using the Barrett-Joyner-Halenda (BJH) method, and the surface area was calculated using the Brunauer-Emmett-Teller (BET) method.

2.5 Enzymatic glycerolysis of soybean oil

Enzymatic glycerolysis of soybean oil was conducted in a 100-mL round-bottom flask. Reaction mixture consisted of 3.52 g soybean oil and desired amount of glycerol, and 10.6 g tert-pentanol in solvent reaction system. Temperature was kept constant by oil bath and reaction mixture was magnetically stirred at 200 rpm. In tert-pentanol solvent system, the reaction mixture was kept refluxing. After that, the immobilized LU was added and the reaction was initiated. At predetermined intervals, 30 µL of the reaction mixture was withdrawn and incubated at 95°C for 10 min to inactivate the enzyme, then added into 4 mL mixtures of acetonitrile, hexane and isopropanol (acetonitrile: hexane: isopropanol 270: 80: 100, v: v: v). The immobilized LU remained in the mixture was removed by filtering through a microfilter (0.45 µm). Samples were stored at −18°C before HPLC determination.

2.6 Determination of MAG, DAG and TAG by RP-HPLC

The lipid profile was determined with RP-HPLC-ELSD, procedures were performed in accordance with our previous method. Compounds were identified and quantified based on our previous study. Double determinations were performed.

2.7 Reusability of the immobilized LU in glycerolysis

The reusability of the immobilized LU was studied in five consecutive cycles. The model reaction mixtures consisted of 3.52 g soybean oil, 0.184 g glycerol and 0.25 g immobilized LU, reaction was progressed for 4 h at 30°C for a cycle. After that, the mixture was centrifuged and the separated immobilized LU was washed with 10 mL hexane and then used for the next run under the selected conditions. The relative activity of the lipase was defined as the ratio of DAG content obtained from each cycle to the DAG content obtained from the first cycle.
Relative activity (%) = \( \frac{\text{DAG content obtained from each cycle}}{\text{DAG content obtained from the first cycle}} \times 100 \)

2.8 Statistical analysis

All experiments were performed in triplicate and data were reported as mean values ± standard deviations. SPSS 14.0 statistical analysis software was applied for data analysis by one-way ANOVA. The level of confidence required for significance was defined at \( p < 0.05 \).

3 Results and Discussion

3.1 Characterization

Small-angle powder XRD is an efficient tool to characterize the mesoporous structure of SBA-15. Typical XRD signals of SBA-15 are the one intense peak attributed to reflections at (100) and two low-intensity peaks assigned to (110) and (200), which indicating a two-dimensional hexagonal pore regularity of a \( \text{P6}_3\text{mm} \) space group. In the present study, the parent SBA-15, the \( n\)-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 (Scheme 1a) and the thereafter LU immobilization (Scheme 1c), were characterized. As indicated in Fig. 1a, all retained the typical XRD signals, suggesting that the organic modification and the later LU immobilization did not destroy the mesoporous structure.

Shown in Fig. 1b are the FT-IR spectra of the SBA-15, Scheme 1a and Scheme 1c. Three typical Si-O-Si peaks attributed to the condensed silica network were observed, respectively centered at 1080 cm\(^{-1}\) (asymmetric stretching), 800 cm\(^{-1}\) (symmetric stretching) and 460 cm\(^{-1}\) (bending vibration). The broad envelope band located at approximately 3450 cm\(^{-1}\) could be assigned to the O-H stretching vibration caused by physisorbed water and surface hydroxyls, and IR band near 1630 cm\(^{-1}\) was attributed to the O-H deformation vibration. In addition, the adsorption band situated at about 970 cm\(^{-1}\) could be assigned to the bending vibration of framework Si-OH group in the SBA-15.

The intensity of the bands situated at 3450 and 970 cm\(^{-1}\) decreased after \( n\)-(2-aminoethyl)-3-aminopropyl group functionalization and the following LU immobilization, suggesting that some of the silanol group had been reacted with the \( n\)-(2-aminoethyl)-3-aminopropyl group through silanization. In addition, the weak peaks at 2930 and 685 cm\(^{-1}\) were ascribed to the stretching vibration of C-H and N-H, and the weak bands at 1567 and 1478 cm\(^{-1}\) represented the asymmetry and symmetry of primary amines (\( -\text{NH}_2 \)), indicating the successful introduction of the \( n\)-(2-aminoethyl)-3-aminopropyl group\(^{10}\). Moreover, prominent peaks at 1658 and 1538 cm\(^{-1}\) appeared after LU immobilization could be due to the vibration of the amines (\( -\text{NH}_2 \)) in the enzyme LU\(^{31}\).

XPS spectra of the parent SBA-15 and the \( n\)-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 before and after LU immobilization are presented in Fig. S1, and XPS elemental concentrations are summarized in Table 1. Both the C\(_1s\) and N\(_1s\) peaks increased gradually after the \( n\)-(2-aminoethyl)-3-aminopropyl group functionalization and the later LU immobilization. Correspondingly, the C\(_1s\) and N\(_1s\) elemental concentrations raised increasingly. It was reasonable since both the LU molecules and the \( n\)-(2-aminoethyl)-3-aminopropyl group contain C and N elements. It therefore suggested the successful functionalization and LU immobilization.

Figure 2 shows the SEM and TEM images of the parent SBA-15 and the \( n\)-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 before and thereafter LU immobilization. All the TEM images (Figs. 2a, 2b and 2c) exhibited wavy or short, rod-shaped morphology. The uniform morphology suggested that the textural properties of the mesoporous SBA-15 preserved after the organic modification and the following LU immobilization. The TEM images (Figs. 2d, 2e and 2f) showed the arrays of long-range regular mesopore channels. The straight arrays of mesoporous channels could be clearly observed after the function-

![Fig. 1](image-url) Low-angle XRD patterns (a) and FT-IR spectra of SBA-15 and the \( n\)-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 before and after LU immobilization. \( n\)-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15; \( n\)-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 immobilized onto LU; SBA-15, LU, Lecitase Ultra.
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### Table 1 XPS elemental concentrations of the parent SBA-15 and the \textit{n-}(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 before and after LU immobilization.

|                | C_{1s} (%) | N_{1s} (%) | O_{1s} (%) | Si_{2p} (%) |
|----------------|------------|------------|------------|-------------|
| SBA-15         | 2.24       | 0.22       | 66.54      | 31.00       |
| NH\textsubscript{2}CH\textsubscript{2}NH(CH\textsubscript{3})\textsubscript{3}-SBA-15\textsuperscript{a} | 17.57      | 3.90       | 51.67      | 26.86       |
| NH\textsubscript{2}CH\textsubscript{2}NH(CH\textsubscript{3})\textsubscript{3}-SBA-15-LU\textsuperscript{b} | 24.71      | 6.79       | 47.01      | 20.24       |

\textsuperscript{a} \textit{n-}(2-aminoethyl)-3-aminopropyl group functionalized SBA-15, see Scheme 1a.

\textsuperscript{b} \textit{n-}(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 supported LU, see Scheme 1c; LU, Lecitase\textsuperscript{®} Ultra.

3.2 Immobilization of LU onto the (functionalized) SBA-15

Immobilization of LU onto the (functionalized) SBA-15 was studied. Immobilization conditions including time, pH of the incubating phosphate buffer and LU concentration, were firstly studied with parent SBA-15 as support. Then, immobilization of LU onto the 3-aminopropyl and \textit{n-}(2-aminoethyl)-3-aminopropyl groups functionalized SBA-15 was investigated.

Effects of the immobilization buffer pH on the activity of the SBA-15-LU was illustrated in Fig. 3a. As indicated, higher activity up to 2177.78 \pm 101.84 U/g was observed from buffer solution pH at 6.0, however, higher IE was found at pH 4 and 5 (Fig. 3b). Therefore, pH of the buffer solution at 6.0 was selected for further study. Interestingly, pH 7.0 was found suitable for LU immobilization, when LU was immobilized on the macroporous resins DA-201, and the activity at 1652 to 1682 U/g was obtained\textsuperscript{12, 22}.

Activity of the SBA-15-LU was increased with LU content was observed after further LU immobilization. The result was agreed with a previous study, the pore diameter did not change because there was a distribution of pores\textsuperscript{22}.

Fig. 2 SEM images of the parent SBA-15 (a) and the \textit{n-}(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 before (b) and after LU immobilization (c); TEM images of the parent SBA-15 (d) and the \textit{n-}(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 before (e) and after LU immobilization (f). LU, Lecitase\textsuperscript{®} Ultra.
concentration increasing from 160.64 to 200.8 µg/mL, and no further increment in activity was observed when LU concentration was increased from 200.8 to 281.12 µg/mL (Fig. 4a). Higher IE was however obtained from LU concentration at 160.64 µg/mL, and it decreased with LU concentration further increment (Fig. 4b). Combined with Figs. 4a and 4b, it can be estimated that, the LU loading was increased with LU concentration increasing from 200.8 to 281.12 µg/mL. However, no increment in activity was observed and it could be explained from the following aspects. On one hand, with more LU entering into the pore of SBA-15, some might get aggregated and did not distributed as a one-layer at the inner surface of the channels, leading to no contribution to enzymatic activity increment since the bottom layer LU cannot contact with substrates. On the other hand, high load of LU may also result in LU immobilization in wrong orientation, which obstructed the active site and in turn leading to low enzymatic activity.

After organic functionalization, the enzymatic activity of the immobilized LU increased significantly (Table 2). In fact, quite some other organic groups functionalization was also studied (data not shown), and the highest activity up to 4777.78 ± 115.47 U/g was observed from propyl methacrylate group modified SBA-15 supported LU, and in turn the Table 2 listed two immobilized LU samples. However, better performance, in terms of glycerolysis activity and reusability in glycerolysis reaction, was observed from 3-aminopropyl and n-(2-aminooethyl)-3-aminopropyl groups seemed to make more LU (the increased LU) in right orientation, which contributed to enzymatic activity improvement.

Immobilization time at 15 and 30 min, higher activity was obtained; further prolonged time to 75 min, activity was decreased (Fig. 5a). However, the LU loading was in fact similar, since no difference in IE was observed within the studied time range (Fig. 5b). The decreased activity from longer immobilization time may be due to that, the immobilized LU in wrong orientation or LU got aggregated with prolonged immobilization, which obstructed the active site and in turn leading to low enzymatic activity.

Immobilization time at 15 and 30 min, higher activity was obtained; further prolonged time to 75 min, activity was decreased (Fig. 5a). However, the LU loading was in fact similar, since no difference in IE was observed within the studied time range (Fig. 5b). The decreased activity from longer immobilization time may be due to that, the immobilized LU in wrong orientation or LU got aggregated with prolonged immobilization, which obstructed the active site and in turn leading to low enzymatic activity.
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| Entry (functionalized) SBA-15 | Activity (U/g) | IE (%) |
|-------------------------------|----------------|--------|
| 1 SBA-15-LU | 2177.78 ± 101.84 | 73.83 ± 6.34 |
| 2 NH₂CH₂CH₂-SBA-15-LU | 3555.56 ± 200.21 | 91.66 ± 2.70 |
| 3 NH₂CH₂CH₂NH(CH₂)₃-SBA-15-LU | 3444.44 ± 346.41 | 87.33 ± 2.24 |

LU solution was dissolved in 40 mL phosphate buffer (25 mM, pH 6), with concentration at 200.8 µg/mL; then contacted with 100 mg of (modified) SBA-15 at 25°C for 30 min. LU, Lecitase Ultra. Enzymatic activity of the immobilized LU. Immobilization efficiency. SBA-15 supported LU. 3-aminopropyl group functionalized SBA-15 supported LU, see Scheme 1d. n-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 supported LU, see Scheme 1c.

3.3 Glycerolysis of soybean oil by the immobilized LU

In general, LU concentration at 200.8 µg/mL, phosphate buffer pH at 6 and immobilization time 30 min was selected for LU immobilization. Under the conditions, activity up to 2177.78 ± 101.84 U/g was obtained from SBA-15-LU. After 3-aminopropyl and n-(2-aminoethyl)-3-aminopropyl groups modification, enzymatic activity was increased to 3555.56 ± 200.21 and 3444.44 ± 346.41 U/g respectively.

3.3.1 Glycerolysis of soybean oil for DAG and MAG production

The immobilized LU was applied to catalyze glycerolysis reaction. The possibility for DAG and MAG production was firstly evaluated; conditions for DAG production were then studied and the reusability of the immobilized LU was investigated.
in solvent-free system and no increment in glycerolysis activity was obtained after organic modification. The present immobilized LU could therefore be considered for DAG production. In our previous studies, considerable glycerolysis activity was also obtained from SBA-15-TLL (data not shown); however, low glycerolysis activity was observed from SBA-15-CALB and SBA-15-RML in solvent-free system. Therefore, aside from the supports, lipases themselves also play a part in glycerolysis performance.

Glycerol amount was increased to aim for MAG production, with glycerol/TAG molar ratio at 5/1, and results were presented in Table S2. However, the increased glycerol did not convert to MAG or DAG, but less TAG conversion was observed, with TAG conversion at about 40 wt% obtained from SBA-15-LU (entry 1), and about 10 wt% from the organic functionalized SBA-15 supported LU (entry 2 and 3). Reason for this was that, glycerol and oil were immiscible, and with more glycerol addition into the reaction system, the enzyme will be trapped by the glycerol, resulting in the enzyme being difficult to contact with oil, which slowed the glycerolysis reaction and led to poor TAG conversion. To improve the miscibility of soybean oil and glycerol, 

| Entry | (functionalized) SBA-15-LU | MAG (%) | DAG (%) | TAG conv (%) | DAG/MAG |
|-------|--------------------------|---------|---------|--------------|---------|
| 1     | SBA-15-LU\(^b\)          | 37.47 ± 0.50 | 52.43 ± 1.64 | 89.89 ± 1.14 | 1.40 ± 0.06 |
| 2     | NH\(_2\)CH\(_2\)CH\(_2\)-SBA-15-LU\(^c\) | 32.56 ± 0.69 | 56.33 ± 1.53 | 88.90 ± 2.21 | 1.73 ± 0.01 |
| 3     | NH\(_2\)CH\(_2\)CH\(_2\)NH(CH\(_3\))\(_2\)-SBA-15-LU\(^d\) | 20.82 ± 6.90 | 56.21 ± 0.93 | 77.03 ± 5.96 | 2.86 ± 0.99 |

\(^a\) Reaction conditions: Soybean oil 3.52 g, glycerol 0.184 g, the immobilized LU 0.2 g, reaction temperature 60°C and time 24 h with magnetic stirring at 200 rpm. LU, Lecitase\(^b\) Ultra; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols; conv, conversion. Note: Standard deviation values were calculated from double experiments.

\(^b\) SBA-15 supported LU.

\(^c\) 3-aminopropyl group functionalized SBA-15 supported LU, see Scheme 1d.

\(^d\) \(n\)-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 supported LU, see Scheme 1c.

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To further check if the glycerolysis activity was affected by the solvent, tert-pentanol was added into the less glycerol reaction system (glycerol/TAG molar ratio at 1/2 for DAG production with SBA-15-LU as catalyst). Interestingly, the glycerolysis activity decreased greatly (compared with the solvent-free system in entry 1, Table 3), with TAG conversion only at approximately 17 wt% (entry 1 in Table S3). Even with water addition, no improvement but a decrease in TAG conversion was observed (entry 2 to 6, Table S3). Therefore, the glycerolysis activity of the immobilized LU was impaired by the tert-pentanol. Consistently, with macroporous resin DA-201 supported LU as catalyst, better performance for DAG production was also obtained from solvent-free system. Interestingly, poor performance from solvent reaction system was ascribed by the authors to the water molecules being stripped from the enzyme by hydrophilic solvents. In the present study, however, poor performance in solvent system was due to that, the glycerolysis activity of the immobilized LU was greatly impaired in by the tert-pentanol.

Therefore, the present immobilized LU was not suitable for MAG production through glycerolysis reaction. Because in solvent-free system, glycerol and oil were immiscible, and with more glycerol addition into the reaction system, the enzyme will be trapped by the glycerol, resulting in difficult contact of enzyme with oil and the in turn poor TAG conversion. While in solvent (tert-pentanol) system, the glycerolysis activity was impaired by the solvent. Therefore, the present immobilized LU was used for DAG production in solvent-free system in the following study, reaction conditions were evaluated and reusability was studied.

3.3.2 Production of DAG through glycerolysis by the immobilized LU

The 3-aminopropyl group functionalized SBA-15 supported LU (Scheme 1d) was selected for DAG production. Reaction conditions including the immobilized LU amount, reaction temperature and time were carefully studied. As demonstrated in Fig. 6, DAG content was increased with the immobilized LU amount increasing from 4.0 to 5.4 wt% (based on soybean oil and glycerol), and no further increase was observed with the LU increasing from 5.4 to 9.4 wt% (Fig. 6a). Interestingly, temperature at 30°C was suit-
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able for the present immobilized LU in glycerolysis reaction (Fig. 6b). To our knowledge, temperature at 50 to 70°C range was suitable for most of the immobilized lipases in practical use, like Novozym 435, Lipozyme TL IM and RM IM. On the other hand, the commercial LU solution was found suitable to be at 40°C in glycerolysis for DAG production by Liu et al.11, in addition, they also immobilized the LU onto the macroporous resin DA-201, and 45°C was found suitable for the obtained supported LU in DAG production through glycerolysis12. Actually, we have also evaluated the glycerolysis performance of the SBA-15-LU and n-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 supported LU (Scheme 1c) at 30°C. And Scheme 1c exhibited similar performance to that of the Scheme 1d, however, lower glycerolysis activity was observed from SBA-15-LU (data not shown). It therefore indicated that, properties of the supports influenced the glycerolysis performance of the immobilized LU at relatively low temperatures, n-(2-aminoethyl)-3-aminopropyl and 3-aminopropyl groups modification of SBA-15 favored the supported LU suitable in practical use at 30°C.

Reaction progress at 30 and 60°C was studied and results were presented in Figs. 6c and 6d, respectively. With temperature at 30°C, glycerolysis reaction proceeded rapidly at the first 2 h, and higher DAG content was obtained after 4 h reaction. Interestingly, DAG content decreased slightly and MAG content increased accordingly with further prolongation of reaction time. As for temperature at 60°C, 45.82% of DAG was obtained after 0.5 h reaction, and it reached at 53.70% at 4 h. No improvement in DAG content was observed with reaction time further prolonging to 24 h.

3.3.3 Reusability of the immobilized LU in glycerolysis reaction

The reusability of the immobilized enzyme is of significant importance in practical applications. As indicated in Fig. 7, with parent SBA-15 as support, the immobilized LU exhibited poor performance in reusability, with only 9.79% of its initial glycerolysis activity remained after 5 cycles of reuse, each lasting 4 h. Encouragingly, the reusability was improved significantly after n-(2-aminoethyl)-3-aminopro-
pyl and 3-aminopropyl group functionalization, with the initial glycerolysis activity retained respectively at 83.91 and 62.93% after 5 cycles of reuse. The results indicated that the organic functionalization of SBA-15 favored its supported LU improvement in regard to operational stability and in turn the reusability.

4 Conclusions

In this study, LU was immobilized onto the parent SBA-15, as well as the n-(2-aminoethyl)-3-aminopropyl and 3-aminopropyl groups functionalized SBA-15 mesoporous silicates. Immobilization conditions were studied, and activities up to 2177.78, 3555.56 and 3444.44 U/g were respectively obtained. The immobilized LU samples obtained were then used to catalyze glycerolysis. The possibility for DAG and MAG production was evaluated and it was found only suitable for DAG production. In addition, the glycerolysis activity of the immobilized LU was impaired by the tert-pentanol, and solvent-free was suitable for glycerolysis. Considerable DAG content (over 50 wt%) could be obtained from the three immobilized LU samples, yet the parent SBA-15 supported LU exhibited poor reusability, and the reusability was improved significantly after n-(2-aminoethyl)-3-aminopropyl and 3-aminopropyl groups functionalization.

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Conflict of Interest
The authors have declared no conflict of interest.

Supporting Information
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Fig. 7 Reusability of the immobilized LU in glycerolysis.

Reaction conditions: soybean oil 3.52 g, glycerol 0.184 g, the immobilized LU 0.25 g, magnetic stirring 200 rpm, temperature 30°C and reaction time 4 h for each cycle. LU, Lecitase® Ultra; SBA-15-LU, SBA-15 supported LU; NH₂CH₂CH₂CH₂-SBA-15-LU, 3-aminopropyl group functionalized SBA-15 supported LU, see Scheme 1d; NH₂CH₂CH₂NH(CH₂)ₓ-SBA-15-LU, n-(2-aminoethyl)-3-aminopropyl group functionalized SBA-15 supported LU, see Scheme 1c.
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