Organic Modifications of SBA-15 Improves the Enzymatic Properties of its Supported TLL

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Abstract: In this study, lipase from Thermomyces lanuginosus (TLL) was immobilized onto the parent and organic groups modified SBA-15, and the enzymatic properties of the obtained immobilized TLL samples were investigated. 1) Activity of SBA-15-TLL at 2862.78 ± 293.24 U/g was obtained. 2) Most of the organic groups modification favored a great improvement in activity, and higher activity over 12000 U/g was observed for N-phenylaminomethyl and phenyl group modification. 3) Most of the supported TLL showed better thermostability in air while poor in phosphate buffer, with over 80% versus less than 20% of their initial activity retained after 4 h incubation at 70°C. 4) The n-dodecyl, phenyl and N-phenylaminomethyl group functionalization decreased the sensitivity of immobilized TLL in extreme pH values. 5) The n-octyl and 2-(propoxymethyl)oxiran group modification confered the supported TLL good reusability, and over 60% of their initial activity was retained after five successive cycles of reuse.

Key words: lipase from Thermomyces lanuginosus, SBA-15, Immobilization, organic functionalization

1 Introduction

Enzymes are Nature’s sustainable catalysts. Compared with the traditional chemical processes, enzymatic processes are more environmentally friendly, more cost-effective and more sustainable. Lipases (triacylglycerols (TAG) ester hydrolases, E.C.3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. The active site of lipases is composed by a serine, an aspartate or glutamate, and a histidine. Because of their interfacial activation, lipases can manifest itself in two different conformations, an open (active) and a closed (inactive). The open form of lipases has been described as more stable than the closed form. Usually, the open form of lipases occurs with the movement of the lid in the presence of hydrophobic surfaces, increasing the enzyme activity. The physiologic function of lipases is to hydrolyze TAG into diacylglycerols (DAG), monoacylglycerols (MAG), fatty acids and glycerol. In addition to their natural function, lipases are also able to catalyze other reactions, including esterification, transesterification and transesterification reactions in nonaqueous media. Lipases are therefore widely used in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries. Limitations of the industrial use of lipases are mainly owing to their high cost. Immobilization of lipases onto the solid supports was one of the effective strategies to overcome this problem, since immobilization facilitated the separation of products and the recovery of lipases for reuse.

Lipases are very special enzymes, having a peculiar mechanism of action called interfacial activation. In homogeneous media, a large percentage of most lipase molecules have their active center covered by a polypeptide chain called lid, which may isolate it from reaction medium (closed form). In the presence of a hydrophobic surface, the enzyme becomes adsorbed on it, fixing a new structure (open form). In the presence of a hydrophobic surface, the enzyme becomes adsorbed on it, fixing a new structure (open form). This idea has been exploited to selectively immobilize many lipases in a variety of hydrophobic supports via their open forms. Moreover, the open form of a lipase molecule can stabilize the open form of other lipases, creating dimers with altered catalytic features. Therefore, the proper

Abbreviations: CALB, Candida antarctica lipase B; IE, immobilization efficiency; LU, Lecitase Ultra; NaH₂PO₄·2H₂O, sodium dihydrogen phosphate dihydrate; Na₂HPO₄·12H₂O, disodium hydrogen phosphate dodecahydrate; NaOH, sodium hydroxide; RML, lipase from Rhizomucor miehei; TLL, Lipase from Thermomyces lanuginosus.

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ties of the immobilized lipases were closely related to the immobilization protocols as well as the characteristics & structure of the carriers. Many kinds of carriers, such as alumina, diatomaceous earth, ion exchange resin, and celite, have already been developed and used for enzyme immobilization. Among these, mesoporous silicates, in particular SBA-15 silicates, are promising candidates for lipases immobilization, owing to their large surface areas, narrow pore size distribution, thermal and mechanical stability, as well as abundant surface silanol groups for surface modification. SBA-15 was introduced in 1998, its average pore diameter was usually around 8 nm and it was an ideal candidate for lipases immobilization.

Lipase from Thermomyces lanuginosus (TLL) is a basophilic and noticeably thermostable enzyme, commercially available in both soluble (Lipolase®) and immobilized (Lipozyme TL IM®) forms. TLL consists of a single chain protein containing 269 amino acids with a molecular weight of 31.7 kDa, isoelectric point of 4.4 and molecular volume of 35 Å × 45 Å × 50 Å. With its high catalytic activity, strict enantioselectivity and broad specificity, TLL has been found to catalyze a diversity of reactions towards a broad range of natural and unnatural substrates. TLL has been therefore widely used in many industrial areas from biodiesel production to fine chemicals, especially in oil and fat modification area.

Though Lipozyme TL IM® is already commercially available, immobilization of TLL is still the object of intense studies. One of the successful strategies to immobilize TLL is to adsorb TLL onto the hydrophobic supports via interfacial activation, and good results have been achieved from several hydrophobic supports: Accurel EP100, agarose beads activated with alkyl groups, and Sepabeads coated with octadecyl groups. Interestingly, granulated silica, the carrier of the commercial Lipozyme TL IM®, was hydrophobic. In our previous study, Candida antarctica lipase B (CALB) was immobilized on parent SBA-15 and the enzymatic activity was found to be 855 U/g; however, when lipase from Rhizomucor miehei (RML) was immobilized on the SBA-15, the enzymatic activity of the obtained immobilized RML was only 200 U/g. In addition, SBA-15 was organically functionalized by a series of silane coupling reagents and later used to immobilize CALB and RML, and the enzymatic properties of the obtained supported lipases were carefully studied. Results indicated that, besides the carriers, lipase itself also played a role in the properties. For example, highest enzymatic activity up to 13211.11 U/g was observed from the n-octyl group modified SBA-15 supported RML; while for the immobilized CALB samples, highest enzymatic activity up to 6100.00 U/g was obtained from the propyl methacrylate group modified SBA-15 supported CALB. Moreover, other enzymatic properties, such as thermostability and reusability, of the immobilized CALB and RML, also exhibited different behaviors with same support. It therefore deserves a continuation study on other lipases. In the present study, we intended to immobilize TLL onto the organically functionalized SBA-15, and to study the enzymatic properties of the obtained immobilized TLL samples.

Catalytic properties of the immobilized lipases were affected by the surface properties of the supports. Hydrophobic or proper/moderate hydrophobic surface would facilitate the activity improvement of the immobilized lipases. Therefore, in this study, SBA-15 was functionalized by organic groups through silanization. The organic modified SBA-15 samples were then used to immobilize TLL. The properties of the obtained immobilized TLL, including enzymatic activity, thermostability and reusability, as well as effects of pH on the enzymatic activity, were carefully studied.

### 2 Experimental

#### 2.1 Materials and reagents

SBA-15 with pore diameters of 8.1 nm was purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). Lipozyme TL IM was purchased from Novozymes (Beijing, China), the activity of TL IM was measured to be at 9666.67 U/g through hydrolysis of tributyrin. TLL solution with activity more than 100,000 U/g, tributyrin with a purity of over 97%, as well as the standards of 1-monoolein, 1,3-diolein and triolein (>99%) for HPLC analysis, were all from Sigma-Aldrich (Shanghai China). Organosilane compounds of (3-aminopropyl)triethoxysilane (>98%), 1-[3-(trimethoxysilyl)propyl]urea (>94%), N-octadecyltriethoxysilane (>85%), N-dodecyltrimethoxysilane (>98%) and 3-mercaptopropyltriethoxysilane (>98%) were respectively purchased from Sigma-Aldrich (Shanghai, China), Tokyo Chemical Industry Co. Ltd, Aike Reagent (Chengdu, China), Xiya Reagent (Shandong, China) and Shanghai Macklin Biochemical Co., Ltd (Shanghai, China); hexadecyltriethoxysilane, N-phenylaminomethyltriethoxysilane and N-butyltrimethoxysilane with purity of more than 95% were purchased from Gelest, Inc. (Shanghai, China); in addition, triethoxymethylsilane (>97%), 3-(trimethoxysilyl)propyl methacrylate (>97%), triethoxymethylsilane (>98%), N-[3-(trimethoxysilyl)propyl] ethylenediamine (>95%), 3-(triethoxysilyl)propyl isocyanate (>95%), 3-glycidyloxypropyltrimethoxysilane (>97%), triethoxyoctylsilane (>97%), and trimethoxyphenylsilane (>95%) were purchased from Aladdin Reagents Co., Ltd (Shanghai, China). Disodium hydrogen phosphate dodecahydrate (Na2HPO4·12H2O) and sodium dihydrogen phosphate dihydrate (NaH2PO4·2H2O) with a purity of more than 99% were from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All other chemicals and solvents & reagents were of analytical.

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2.2 Organic modification of SBA-15

SBA-15 was modified by organic functional groups through silanization. Typically, 2 g of dry SBA-15 was dispersed in 60 mL of dry methylbenzene in a 150 mL round-bottom flask, and then 10 mmol of silane coupling agent was added dropwise into the dispersion, which was kept refluxing under nitrogen atmosphere for 8 h at 95°C. After that, the silica gel was filtrated by centrifuge (10 min at 1,500 rpm) and washed with ethanol (50 mL × 3) and diethyl ether (50 mL × 3). Once finished, the modified SBA-15 was dried under vacuum (pressure at 0.093 MPa) at 80°C for 6 h. After the procedure above, the SBA-15 was functionalized by different silane coupling agents and named as R-SBA-15 (R was the functional group). The organic functionalized SBA-15 was labelled as R-SBA-15. For example, methyl group modified SBA-15 supported TLL was labelled as Scheme 1a.

2.3 Immobilization of TLL onto the (R-)SBA-15

The protein concentration of the commercial TLL solution was 18.3 µg/mg, determined by the Bradford assay. Immobilization of TLL onto the (R-)SBA-15 was conducted according to our previously reported method. Required amounts of the TLL solution were dissolved in 25 mM phosphate buffer, up to a total volume of 40 mL. Then 100 mg of (R-)SBA-15 were added into the solution, and magnetically stirred at 25°C. After that, the suspensions were filtered and washed with the phosphate buffer. The initial lipase activity ($E_0$) and the final lipase activity ($E_f$) was determined for the immobilization efficiency (IE) measurement. The IE was calculated through the following equation:

$$IE(\%) = \frac{(E_0V_0 - E_fV_f)}{E_0V_0} \times 100$$

Where $E_0$ is the initial lipase activity (U/mL), $V_0$ is the initial volume of enzyme solution (mL), $E_f$ is the lipase activity in the filtrate (U/mL), and $V_f$ is the filtrate volume (mL).

The immobilized TLL samples were dried in a vacuum oven (pressure at ~ 0.093 MPa) at 30°C for 6 h before activity analysis. In order to avoid the activity loss during storage, all the immobilized lipases were prepared freshly before use. Enzymatic activity was assayed through hydrolysis of tributyrin. Briefly, the tributyrin mixture consisted of 1 mL of tributyrin and 50 mL of phosphate buffer (25 mM, pH 7.0) was vigorously stirred at 40°C. Then 1 mL of the free TLL solution or 10 mg of the immobilized TLL was added, and the mixture was continuously titrated with 0.1 M NaOH solution for 15 min to maintain a constant pH. Blank experiments were performed through the same procedures, but without the addition of lipase. 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. In order to obtain the highest enzymatic activity for each (R-)SBA-15-TLL, at least three TLL concentrations were tested for every supports (including the R-SBA-15 samples and the parent SBA-15), and all the

Scheme 1 Chemical structures of the organic functionalized SBA-15 supported TLL samples referred in the text. TLL, Lipase from *Thermomyces lanuginosus*. R-SBA-15-TLL, TLL immobilized on the organic functionalized SBA-15, where the R is the modified organic group.
performance was evaluated using the each highest activity.

Immobilization time (from 15 to 60 min) and TLL concentrations (from 8.34 to 100.08 µg/mL) were evaluated with parent SBA-15 as support. Enzymatic activity of the R-SBA-15-TLL versus pH of the buffer solution used during the immobilization process was studied with pH values of the phosphate buffer solution ranging from 4.0 to 8.0.

2.4 Thermal stability of the immobilized TLL

Thermal stability of the immobilized TLL samples was examined according to our previous procedure. Samples were incubated in three different media, air, n-hexane and phosphate buffer (pH 7.0) respectively, and each was stored at 70°C for 0 h, 1 h, 2 h, 3 h and 4 h. After incubation, samples were recovered by filtration for activity determination (according to the above-mentioned reaction conditions), and the initial activity was defined as 100%.

2.5 Reusability of the immobilized TLL

Reusability of the immobilized TLL was tested by repeating batch experiments (five times) using the same conditions as the above-described enzymatic activity assay procedure. The immobilized TLL was recovered by filtration, and then used in the next reaction under otherwise identical conditions. The relative activity of lipase was defined as the ratio of enzymatic activity obtained from each cycle to the enzymatic activity obtained from the first cycle (Equation 1).

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\text{Relative activity (\%) = } \frac{\text{enzymatic activity obtained from each cycle}}{\text{enzymatic activity obtained from the first cycle}} \times 100
\]

2.6 Statistical analysis

An analysis of variance (ANOVA) was performed using the SPSS 13.0 statistical analysis system. Significance of differences was defined at \( p < 0.05 \) with Tukey’s test. The results were expressed as mean ± SD.

3 Results and discussion

3.1 Immobilization of TLL onto the parent SBA-15

TLL was immobilized onto the SBA-15, immobilization conditions including time and TLL concentration were firstly studied and results were listed in Table 1. When immobilization time was 15 min, the activity of the obtained

| Entry | Time (min) | TLL concentration (µg/mL) | IE (%) \( ^b \) | Activity (U/g) \( ^c \) |
|-------|------------|---------------------------|---------------|---------------------|
| 1     | 15         | 8.34                      | 91.68 ± 0.00  | 848.89 ± 48.93      |
| 2     | 15         | 16.68                     | 91.49 ± 1.79  | 1220.00 ± 81.92     |
| 3     | 15         | 33.36                     | 92.39 ± 0.32  | 2120.74 ± 232.34    |
| 4     | 15         | 66.72                     | 82.11 ± 1.63  | 2862.78 ± 293.24    |
| 5     | 15         | 100.08                    | 67.69 ± 1.22  | 2133.33 ± 485.67    |
| 6     | 30         | 8.34                      | 97.01 ± 1.51  | 1051.11 ± 64.98     |
| 7     | 30         | 16.68                     | 90.35 ± 2.22  | 1333.34 ± 127.32    |
| 8     | 30         | 33.36                     | 91.03 ± 1.44  | 2348.89 ± 199.30    |
| 9     | 30         | 66.72                     | 85.41 ± 1.47  | 2615.88 ± 271.78    |
| 10    | 30         | 100.08                    | 69.92 ± 4.72  | 2316.67 ± 308.61    |
| 11    | 60         | 8.34                      | 95.94 ± 0.00  | 1037.50 ± 43.49     |
| 12    | 60         | 16.68                     | 88.74 ± 1.87  | 1579.36 ± 155.72    |
| 13    | 60         | 33.36                     | 93.60 ± 0.43  | 2265.56 ± 408.48    |
| 14    | 60         | 66.72                     | 87.66 ± 0.95  | 2622.22 ± 346.20    |
| 15    | 60         | 100.08                    | 77.06 ± 1.39  | 3177.78 ± 346.20    |

\( ^a \) Immobilization conditions: Required amounts of the TLL solution were dissolved in 25 mM phosphate buffer (pH 5.0), up to a total volume of 40 mL, contacted with 100 mg of SBA-15 at 25°C. TLL, Lipase from Thermomyces lanuginosus. Note: Standard deviation values were calculated from triplicate experiments.

\( ^b \) Immobilization efficiency.

\( ^c \) Activity of the immobilized TLL.
SBA-15-TLL was increased with TLL concentration increasing from 8.34 to 66.72 µg/mL (entry 1-4), and no further increment in activity was observed with TLL concentration increasing to 100.08 µg/mL (entry 4-5). While the immobilization time was 30 or 60 min, the activity of the SBA-15-TLL was increased with TLL concentration increasing from 8.34 to 33.36 µg/mL (entry 6-8 and 11-13). This phenomenon was also observed in our previous study. The enzymatic activity of the immobilized lipase was not always increased with lipase loading increment, and it could be explained from the following aspects. On one hand, with more lipase 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 enzymatic activity decrease because of the non-contact with substrates of the bottom layer lipases. On the other hand, high load of lipase may also result in the immobilized lipases in wrong orientation, which obstructed the active site and in turn leading to low enzymatic activity. Furthermore, diffusion limitations and steric hindrance may occur in the case of high load of lipase, since substrates may become difficult to diffuse into the channels when more lipases entered into the pore of the supports.

As for the immobilization time, no increment in enzymatic activity was observed with time increasing, except for two cases. One was that immobilization time from 15 to 30 min with TLL concentration at 8.34 µg/mL (entry 1 and 6), and the other was time from 30 to 60 min with TLL concentration at 100.07 µg/mL (entry 10 and 15). No improvement in activity with prolonged immobilization time could possibly be due to the fact that, the lipase loading was not increased with time increasing at the same TLL concentrations (The lipase loading could be estimated based on the TLL concentration and IE data). In addition, it should be pointed out that, the activity also depends on the strategy, and other factors.

In addition, enzymatic activity of the SBA-15-TLL versus pH of the buffer solution used during the immobilization process was also studied, results were presented in Fig. S1. The pH of the immobilization buffer significantly affected the activity of the SBA-15-TLL. The highest enzymatic activity was observed when adsorption conducted near the TLL isoelectric point (pI 4.4). It could be attributed to two parallel aspects: one was that higher lipase loading was obtained from pH 5.0 and increasing pH the lipase loading lower (detail data not shown); the other was that, lipase configuration, which was vital for enzymatic activity, changed with pH, and pH 5.0 seemed suitable for TLL in its active configuration while immobilization. Consistently, other studies also indicated that, the highest activity was obtained while immobilization conducted near the isoelectric point of lipase.

Therefore, the optimum conditions for TLL immobilized onto the parent SBA-15 could be time 30 min, pH 5.0 and TLL protein concentration 66.72 µg/mL for 100 mg SBA-15 incubated in 40 mL phosphate buffer.

3.2 Immobilization of TLL onto the R-SBA-15

After organic functionalization, the enzymatic activity of the immobilized TLL samples increased significantly, except for the methyl group and 1-propanethiol group modified SBA-15 supported TLL samples (Scheme 1a and 1b, in entry 1 and 10), with activity respectively at 2500.00 ± 208.17 and 2950.00 ± 346.41 U/g (Table 2). Interestingly, the activity of CH3-SBA-15-CALB, CH3-SBA-15-RML and CH3-SBA-15-LU was 866.67, 533.33 and 2444.44 U/g, respectively. No or less increment in activity when compared with their parent SBA-15 counterparts (855.56 U/g for SBA-15-CALB, 200.00 U/g for SBA-15-RML and 2177.78 U/g for SBA-15-LU) (data for LU have not been published, LU, Lecitase Ultra). It indicated that methyl group modification did not led to much improvement in activity. Despite the activity was increased from 200.00 to 533.33 U/g for the immobilized RML after methyl group modification, it still quite lower when compared with other organic groups modified SBA-15 supported RML samples. The 1-propanethiol group modification of SBA-15 did not led to its supported RML improvement in activity either, which agreed with the present immobilized TLL. However, the 1-propanethiol group modification of SBA-15 led to its supported CALB improvement in enzymatic activity, from 855.56 to 1488.89 U/g.

Higher enzymatic activities were observed from N-phenylaminomethyl group and phenyl group modified SBA-15 supported TLL samples (Scheme 1c and 1d, in entry 16 and 15), with activity over 12000 U/g. Activities over 10000 U/g were also observed from n-octadecyl group and propyl methacrylate group modified SBA-15 supported TLL samples (Scheme 1e and 1f, in entry 6 and 14). Interestingly, highest enzymatic activity up to 13211.11 U/g was observed from n-dodecyl group modified SBA-15 supported RML (C12H25-SBA-15-RML), and 855.56 U/g for SBA-15-LU, respectively. No or less increment in activity when compared with their parent SBA-15 counterparts. As for the immobilized CALB samples, the highest activity was observed from propyl methacrylate group modified SBA-15 supported CALB (Scheme 1f), with activity at 6100 U/g.

The results indicated that, besides the carriers, lipase itself also played a role in the properties. Activity up to 2862.78 U/g for the parent SBA-15 supported TLL (SBA-15-TLL) was much higher than that of the SBA-15-CALB (855.56 U/g) and SBA-15-RML (200.00 U/g). Hydrophobic supports favor the activity increment, and organic modification of supports is therefore usually applied to improve the enzymatic activity. However, not the more hydrophobic supports, the better increment in activity. In addition, for different lipases, the most suitable "hydrophobic degree" was also different. Since the highest activity for the different immobilized lipases was obtained from different organic groups modification.
In order to obtain the highest enzymatic activity, at least three TLL concentrations were tested for every support.

Note: Standard deviation values were calculated from triplicate experiments.

Thermomyces lanuginosus.

Activity (U/g)
3.3 Thermal stability of the immobilized TLL samples

Thermal stability of the immobilized lipase is of paramount importance in practical applications, since most of the enzymatic reactions are conducted at around 60-70°C in different reaction media, and organic solvents are sometimes introduced as reaction media to enhance the reactions. Organic solvents affect the stability of lipases. Normally, hydrophobic solvents (Log P > 4) cause less inactivation than hydrophilic solvents, since the polar solvents would strip off essential water from enzyme surface, leading to an insufficiently hydrated enzyme molecule, which decreases enzymatic activity. Lipases usually exhibit higher stability in hydrophobic organic solvents, such as n-hexane. In addition, thermal stability of the immobilized lipases also depends on the lipases preparation strategy as well as the lipases themselves. With different preparation strategy, the thermostability of the obtained immobilized lipases may vary; in addition, the thermostability may also vary with different lipases, even with same preparation strategy.

Thermostability of the R-SBA-15-TLL samples in three different media was studied, results were presented in Fig. 1 to Fig. 4. In general, the immobilized TLL samples exhibited better thermostability in air and relatively poor performance in phosphate buffer. Most of the samples could retain over 80% of their initial activity after 4 h incubation in air at 70°C; while incubation in phosphate buffer, less than 20% of their initial activity was retained for most of the immobilized TLL samples. The R-SBA-15-TLL samples all exhibited better thermostability in air than that in phosphate buffer media. The 3-aminopropyl group modified SBA-15 supported TLL(Scheme 1g) exhibited better thermostability in n-hexane than that in air(Fig. 2c). For the n-octadecyl, 1-propanethiol, vinyl, phenyl and N-phenylaminomethyl group modified SBA-15 supported TLL samples(Scheme 1e, 1b, 1h, 1d, and 1c), no much difference in thermostability was observed from air and n-hexane(Figs. 2b, 3b, 3d, 4c and 4d).

Phenomenon that activity improved and higher than their initial activity after incubation was observed from Fig. 2d (incubation in n-hexane), Fig. 3a (incubation in air) and Fig. 3c (incubation in air). It is not strange and it could also be observed from the immobilized RML and CALB samples. It suggested that the immobilized TLL samples were not properly conditioned, and the incubation could favor it in better conditioning, which improved the activity. Enzymes, particularly if not properly conditioned, usually make better conditioning in the first reaction and demonstrate higher activity in the second and even third reactions.

In addition, thermostability of the SBA-15-TLL, the commercial TL IM and the TLL solution was also studied,
Fig. 2  Effects of the incubation medium on the thermal stability of the immobilized TLL samples. Conditions: samples were incubated in three different medium, air, n-hexane and phosphate buffer (pH 7.0) respectively, and each was stored at 70°C for 0h, 1h, 2h, 3h and 4h. The initial activity was defined as 100%. TLL, Lipase from Thermomyces lanuginosus; R-SBA-15-TLL, TLL immobilized on the organic functionalized SBA-15, and the R was the modified organic group.

Fig. 3  Effects of the incubation medium on the thermal stability of the immobilized TLL samples. Conditions: samples were incubated in three different medium, air, n-hexane and phosphate buffer (pH 7.0) respectively, and each was stored at 70°C for 0h, 1h, 2h, 3h and 4h. The initial activity was defined as 100%. TLL, Lipase from Thermomyces lanuginosus; R-SBA-15-TLL, TLL immobilized on the organic functionalized SBA-15, and the R was the modified organic group.
Organic Modification of SBA-15 for TLL Immobilization

Results were shown in Fig. 5. SBA-15-TLL exhibited better thermostability in air, and no much difference in thermostability was observed from n-hexane and phosphate buffer media. The commercial TL IM exhibited better thermostability in air and n-hexane media, and poor stability in phosphate buffer media. The TLL solution retained only 17.72 ± 4.40% of its initial activity after 4 h incubation at 70°C.

3.4 Effects of medium pH on the activity of the immobilized TLL samples

Medium pH plays key role on the enzymes. Stabilization & destabilization of the enzymes was affected by pH. The
free enzyme may experience protein aggregation (mainly near to the isoelectric point), which may be caused by undesired enzyme-interactions where inactivation that can stabilize incorrect enzyme structures. In addition, dissociation state and behavior of the enzyme are also affected by the pH in the system. Effects of the medium pH on the enzymatic activity of the immobilized TLL samples was measured within the range of 4.0 to 8.0 at 40°C, results were presented in Fig. 6. As demonstrated, optimum pH of both the 3-aminopropyl group modified SBA-15 supported TLL (Scheme 1g) and the N1-propylethane-1,2-diamine group modified SBA-15 supported TLL (Scheme 1i) was found at 8.0 (Fig. 6b), with enzymatic activity from pH 8.0 slightly higher than that from pH 7.0. On the other hand, optimum pH at 6.0 was found for the 1-propylurea group modified SBA-15 supported TLL (Scheme 1j, Fig. 6c). For the n-dodecyl, phenyl and N-phenylaminomethyl group modified SBA-15 supported TLL samples (Scheme 1k, 1d and 1c), no much difference in enzymatic activity was observed at the studied pH ranges (Figs. 6b and 6d). It suggested that the n-dodecyl, phenyl and N-phenylaminomethyl group functionalization of SBA-15 had decreased the sensitivity of immobilized TLL in extreme pH values, and increased the stability in harsh conditions. For other immobilized TLL samples, optimum pH was found at 7.0 or 7.0-8.0.

Fig. 6 Effects of medium pH on the enzymatic activity of the immobilized TLL samples. Conditions: Required amounts of the TLL solution (the exact amount for each R-SBA-15 was the TLL concentration that exhibited highest activity, based on the results of Table 2) were dissolved in 25 mM phosphate buffer (pH from 4.0 to 8.0), up to a total volume of 40 mL, contacted with 100 mg of R-SBA-15 at 25°C for 30 min. TLL, Lipase from Thermomyces lanuginosus; R-SBA-15-TLL, TLL immobilized on the organic functionalized SBA-15, and the R was the modified organic group.

Effects of the medium pH on the enzymatic activity of the commercial TL IM was also studied, and optimum pH was found at 7.0-8.0 (Fig. S3).

3.5 Reusability of the immobilized TLL
Repeated use of the R-SBA-15-TLL samples was studied and results were shown in Fig. 7. As demonstrated, over 60% of their initial activity was retained after five successive cycles of reuse from the n-octyl and 2-(propoxymethyl) oxirane group modified SBA-15 supported TLL samples (Scheme 1l and 1m, Figs. 7a and 7d). In addition, 51.35 ± 0.22% of its initial activity was retained from N-phenylaminomethyl group modified SBA-15 supported TLL (Scheme 1c, Fig. 7d), and 20-50% of the initial activity was obtained from the rest of the samples. The reusability of the SBA-15-TLL and the commercial TL IM was also studied, 26.29 ± 1.13 and 27.54 ± 4.53% of the initial activity was respectively retained after five successive cycles of reuse (Fig. S4). The improvement in reusability after organic modification, may be due to the interaction between the organic groups and TLL, which facilitated the TLL more stable, when compared to the parent SBA-15 supported TLL. In addition, poor reusability of the parent SBA-15 supported TLL may probably be attributed to the protein diffusional limitations imposed by the considerably high amount of de-
posits of reaction product accumulated on the matrix surfaces, which in turn causing mass transfer restrictions to the protein molecules. Interestingly, catalytic activity above 50% after five cycles of reuse was retained, when TLL was immobilized onto the polyurethane foam (PU) by entrapment. The present immobilized TLL samples could be used for preparation of monoacylglycerols and diacylglycerols through glycerolysis.

4 Conclusions

TLL was immobilized onto the parent and organic groups modified SBA-15. Buffer solution at pH 5.0 was found suitable for TLL immobilization. Activity of SBA-15-TLL at 2862.78 ± 293.24 U/g was obtained. Most of the organic groups modification favored a great improvement in activity, and higher activity over 12000 U/g was observed for N-phenylaminomethyl and phenyl group modification. In general, the supported TLL samples exhibited better thermostability in air and relatively poor performance in phosphate buffer. Most of them could retain over 80% of their initial activity after 4 h incubation in air at 70°C; while incubation in phosphate buffer, less than 20% was observed. The n-dodecyl, phenyl and N-phenylaminomethyl group functionalization decreased the sensitivity of the immobilized TLL in extreme pH values. The n-octyl and 2-(propoxymethyl)oxirane group modification conferred the supported TLL good reusability, and over 60% of their initial activity was retained after five successive cycles of reuse.

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Conflict of interest

The authors have declared no conflict of interest.

Supporting Information

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