Effects of Solvents on the Glycerolysis Performance of the SBA-15 Supported Lipases

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Abstract: In this study, *Candida antarctica* lipase B (CALB), *Rhizomucor miehei* lipase (RML) and Lecitase® Ultra (LU) were immobilized onto the mesoporous silica SBA-15. The glycerolysis performance of the obtained supported lipases (lipase@SBA-15) in solvent systems was carefully investigated. LU@SBA-15 exhibited good glycerolysis performance in solvent-free system, with diacylglycerols (DAG) content and triacylglycerols (TAG) conversion at 52.4 and 98.6% respectively obtained after 12 h reaction at 60°C. CALB@SBA-15 showed good glycerolysis activity in *tert*-pentanol and *tert*-butanol systems, with TAG conversion over 90% obtained. In addition, the present CALB@SBA-15 exhibited selectivity for monoacylglycerols (MAG) production, with glycerol to TAG molar ratio increased to 3:1, MAG content over 80% and TAG conversion over 99% could be obtained from both *tert*-pentanol and *tert*-butanol systems. However, RML@SBA-15 showed low glycerolysis activity neither in solvent nor in solvent-free systems. The present results favor the practical enzymatic design for MAG and DAG production.

Key words: solvent, lipase, glycerolysis, immobilization, SBA-15

1 Introduction

Glycerolysis reaction is of importance in lipid modification field, it is the primary reaction route for monoacylglycerols (MAG) and diacylglycerols (DAG) production¹⁾. MAG are food grade emulsifiers, widely used in food and cosmetic industries; DAG, especially 1,3-DAG, are recognized as functional cooking oil, due to their ability to reduce postprandial serum triacylglycerols (TAG) levels and prevent obesity, without loss of taste and processing functions^{2,3)}. Compared with chemical glycerolysis, enzymatic glycerolysis was more attractive, since its mild reaction conditions and no or less byproducts formation⁴⁾. Yet the substrates of glycerolysis reaction, namely TAG and glycerol, are immiscible at low temperatures. Therefore, to enhance the low-temperature glycerolysis reaction, solvents with amphiphilic properties, are usually introduced into the system to increase the miscibility between glycerol and TAG⁵⁾.

Previous studies indicated that solvents of *tert*-pentanol and *tert*-butanol were effective to improve the production of MAG and DAG from low-temperature glycerolysis⁶⁻¹³⁾. In addition, combination of *tert*-butanol or *tert*-pentanol with other solvents, like isopropanol or hexane, was also sufficient to improve the low-temperature glycerolysis reaction¹⁴⁻¹⁷⁾. In addition to the organic solvents, ionic

liquids¹⁸⁾, surfactants (like Tween and AOT)¹⁹⁻²¹⁾, or supercritical carbon dioxide (SC-CO₂)²²⁾, were also introduced to enhance the glycerolysis reaction. Despite the extensive studies on the reaction medium for glycerolysis enhancement, however, less attentions have been paid to the enzymatic glycerolysis activity. Aside from the miscibility, enzymatic activity in glycerolysis reaction is another issue, which has not been received attention presently. In our previous study, we found that the enzymatic activity of the SBA-15 supported Lecitase® Ultra (LU) in glycerolysis reaction was impaired by *tert*-pentanol, and considerable content of DAG was obtained from solvent-free system²³⁾. Similar results were also observed from the SBA-15 supported Lipase from *Thermomyces lanuginosus* (TLL)⁴⁾. Therefore, study on the enzymatic glycerolysis activity in solvent systems would shed light on the foundation of enzymatic design for practical application.

SBA-15 was one of the mesoporous silicate, it was introduced in 1998; its average pore diameter was approximately 8 nm, making it especially suitable for lipases immobilization. In addition, SBA-15 was tunable, it had sufficient

Abbreviations: CALB, *Candida antarctica* lipase B; RML, *Rhizomucor miehei* lipase; LU, Lecitase® Ultra; TLL, Lipase from *Thermomyces lanuginosus*; DAG, diacylglycerols; TAG, triacylglycerols; MAG, monoacylglycerols.

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functional groups, and its thermal & mechanical stability was fine. Besides, the surface area was large and the pore size distribution was narrow. SBA-15 was therefore an ideal candidate for lipase immobilization⁴⁾. In this study, SBA-15 supported lipases, including CALB, LU and RML, were used to catalyze glycerolysis. The glycerolysis performance of the immobilized lipases in solvent systems was studied. In addition, the structural changes of the immobilized lipases in the present of solvents were studied using fluorescence.

2 Experimental

2.1 Materials and reagents

Refined, bleached and deodorized soybean oil was purchased from a local supermarket. SBA-15 silicates with pore diameters at 8.1 nm were purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). Glycerol with a purity of more than 99.0% was from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Organic solvents for reaction medium with a purity of more than 99.0% were from Aladdin Reagent Co., Ltd (Shanghai, China). The standards of 1-monolein, 1,3-diolein and triolein (>99.0%) for HPLC analysis were from Sigma (St. Louis, MO, USA). The commercial immobilized lipases of Novozym 435, Lipozyme[®]RM and Lipozyme TL IM were obtained from Novozymes (Beijing, China). The commercial TLL solution and RML solution, with activity respectively more than 100,000 and 20,000 U/g, were both purchased from Sigma-Aldrich (Shanghai, China). The commercial extract CALB with activity at 4340.4 U/mL, and commercial LU solution with activity at 426.7 U/mL, were both obtained from Novozymes (Beijing, China). All other solvents and reagents were of analytical or chromatographic grade.

2.2 Immobilization of lipase onto the SBA-15

Immobilization of lipases onto the SBA-15 was according to our procedure²⁴⁾. Typically, the required amounts of the lipases solution were dissolved in 40 mL phosphate buffer (25 mmol L⁻¹). Then, 100 mg of SBA-15 was added into the solution and magnetically stirred at 25°C for 30 min. After that, the suspensions were filtered and washed with the phosphate buffer. The immobilized lipases were dried in a vacuum oven (pressure at -0.093 MPa) at 30°C for 6 h and named as lipase@SBA-15. For example, the SBA-15 supported CALB was named as CALB@SBA-15.

2.3 Enzymatic glycerolysis of soybean oil

In a typical reaction, the reaction mixtures consisted of 3.52 g soybean oil, required amount of glycerol and 10.6 g solvent. The reactants were incubated in an oil bath at 60°C and mixed by magnetic stirring (600 rpm). Then 0.2 g of the immobilized lipase was added to initiate the reaction.

The reaction was allowed to proceed for 12 h, and then 30 µL of the reaction mixture was withdrawn and added to 4 mL of mixtures of acetonitrile, hexane and isopropanol (acetonitrile:hexane:isopropanol 270:80:100, v:v:v). The mixture was then filtered through a microfilter (0.45 µm) to remove the catalysts. All samples were stored at -20°C before HPLC determination.

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

The lipid profile was determined with RP-HPLC. Procedures were performed according to our previous RP-HPLC method²⁵⁾. Quantification and identification of compounds were described in detail in one of our previous studies¹⁶⁾. Double determinations were performed.

2.5 Fluorescence studies

The fluorescent spectra of free and immobilized lipases were performed using a fluorescence spectrophotometer (Thermo Scientific Lumina, USA) equipped with 1.0 cm quartz cells. The excitation wavelength was fixed at 280 nm, and the emission wavelength range was 300-500 nm²⁶⁾. Both the excitation and emission slit widths were kept at 5 nm²⁷⁾. The final protein concentration in all the media was 25 µg/mL.

2.6 Statistical analysis

SPSS 13.0 statistical analysis software was used for data analysis by one-way ANOVA. The level of confidence required for significance was defined at $p < 0.05$ using Tukey's test.

3 Results and Discussion

3.1 Glycerolysis performance of the RML@SBA-15 in organic solvents

Several common organic solvents were introduced into the glycerolysis reaction systems. TAG conversion at about 45 and 37% was observed respectively from acetonitrile and *n*-heptane systems, with RML@SBA-15 as catalyst (Table 1). In other solvent systems, RML@SBA-15 exhibited quite low glycerolysis activity, with TAG conversion lower than 10%. In addition, in the solvent-free system, the glycerolysis activity of the RML@SBA-15 was also limited, with TAG conversion at only 16.8%. The results indicated that the present RML@SBA-15 catalyzed glycerolysis reaction was not limited by the mass transfer, since in the amphiphilic solvent systems (*tert*-butanol and *tert*-pentanol), quite low glycerolysis activity was obtained. To check if the low glycerolysis activity was ascribed to the stripped water caused by solvents, water content at 5-25% (based on glycerol weight) was added into the *tert*-butanol system. However, as indicated in Table 2, the glycerolysis reaction was not enhanced at all. On the other hand, with

Table 1 Effects of organic solvents on the glycerolysis performance of the RML@SBA-15^{a,b}.

Solvent	MAG%	DAG%	TAG%
Solvent-free	2.66 ± 0.82	14.14 ± 3.53	83.20 ± 4.34
<i>n</i> -Hexane	0.88 ± 0.07	4.89 ± 0.52	94.23 ± 0.45 ^a
<i>n</i> -Heptane	9.27 ± 4.88	27.49 ± 12.69	63.24 ± 17.57 ^b
<i>tert</i> -Butanol	0.82 ± 0.05	2.27 ± 1.19	96.91 ± 1.14 ^a
<i>tert</i> -Pentanol	0.50 ± 0.33	4.12 ± 2.03	95.38 ± 2.36 ^a
Acetone	0.24 ± 0.34	1.01 ± 0.46	99.75 ± 0.80 ^a
2-Butanone	0.06 ± 0.02	1.69 ± 0.16	98.25 ± 0.14 ^a
3-Pentanone	0.00 ± 0.00	0.73 ± 0.01	99.27 ± 0.01 ^a
Acetonitrile	29.14 ± 2.82	16.03 ± 2.53	54.82 ± 5.35 ^b
Chloroform	0.12 ± 0.00	2.12 ± 0.45	97.76 ± 0.45 ^a
Toluene	0.18 ± 0.26	1.38 ± 0.27	98.44 ± 0.53 ^a

^a Immobilization conditions: RML solution were dissolved in 25 mM phosphate buffer (pH 5.0), up to a total volume of 40 mL, with RML concentration at 38.19 µg/mL; then contacted with 100 mg of SBA-15 at 25°C for 30 min.

^b Glycerolysis reaction conditions: soybean oil 3.52 g, glycerol 0.184 g, RML@SBA-15 0.2 g, solvent 10.6 g in solvent reaction systems; reaction temperature 60°C and time 12 h with magnetic stirring at 200 rpm in solvent-free system and 600 rpm in organic solvent reaction system. RML, *Rhizomucor miehei* lipase; RML@SBA-15, SBA-15 supported RML; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

^{a, b} $p < 0.05$ compared with the solvent-free group of the same column, with different superscript differ (^a higher than the solvent-free group, and ^b lower than the solvent-free group).

Table 2 Effects of water content on the glycerolysis performance of the RML@SBA-15^{a,b}.

Water content%	MAG%	DAG%	TAG%
5	0.10 ± 0.14	0.88 ± 0.06	99.02 ± 0.08
10	0	0.83 ± 0.06	99.17 ± 0.06
15	0.13 ± 0.06	1.68 ± 0.33	98.19 ± 0.39
20	0.14 ± 0.10	1.54 ± 0.16	98.31 ± 0.26
25	0.21 ± 0.02	1.75 ± 0.11	98.04 ± 0.12

^a Glycerolysis reaction conditions: soybean oil 3.52 g, glycerol 0.184 g, RML@SBA-15 0.2 g, *tert*-Butanol 10.6 g; reaction temperature 60°C and time 12 h with magnetic stirring at 600 rpm. MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

^b Water content based on glycerol weight was added into the reaction system.

Lipozyme[®]RM as catalyst, the glycerolysis was improved significantly (Table 3). Also, the Lipozyme[®]RM showed selectivity towards DAG generation in solvent-free system (DAG/MAG ratio at 4.14); however, the selectivity decreased in *tert*-butanol and *tert*-pentanol systems, especially in *tert*-butanol system (DAG/MAG ratio at 1.92). This

results suggested that solvent affected the selectivity of the commercial Lipozyme[®]RM. In addition, organic groups modified SBA-15 supported RML exhibited good performance in the solvent-free glycerolysis reaction¹⁾. Therefore, the supports affect the performance of RML and the glycerolysis activity was impaired by solvents.

Table 3 Effects of organic solvents on the glycerolysis by Lipozyme[®]RM^a.

Solvent	MAG%	DAG%	TAG%
Solvent-free	15.03 ± 1.12	62.16 ± 1.11	22.81 ± 0.01
<i>tert</i> -Butanol	24.75 ± 0.91	47.59 ± 1.48	27.66 ± 2.39
<i>tert</i> -Pentanol	18.03 ± 0.55	30.60 ± 0.10	51.38 ± 0.64

^a Glycerolysis reaction conditions: soybean oil 3.52 g, glycerol 0.184 g, Lipozyme[®]RM 0.2 g, solvent 10.6 g; reaction temperature 60°C and time 12 h with magnetic stirring at 600 rpm. MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

Table 4 Effects of organic solvents on the glycerolysis performance of the LU@SBA-15^{a,b}.

Solvent	MAG%	DAG%	TAG%
Solvent-free	37.47 ± 0.50	52.43 ± 1.64	1.40 ± 0.06
<i>n</i> -Hexane	1.58 ± 0.17	12.59 ± 0.45	85.83 ± 0.28*
<i>n</i> -Heptane	0.90 ± 0.59	12.49 ± 1.29	86.61 ± 0.69*
<i>tert</i> -Butanol	0.13 ± 0.03	1.22 ± 0.07	98.64 ± 0.04*
<i>tert</i> -pentanol	0.13 ± 0.02	1.21 ± 0.02	98.66 ± 0*
Acetone	0	1.07 ± 0.19	98.93 ± 0.19*
2-Butanone	—	—	—
3-Pentanone	0	0.95 ± 0.01	99.05 ± 0.01*
Acetonitrile	0.22 ± 0.31	0.94 ± 0.29	98.84 ± 0.59*
Chloroform	0	1.03 ± 0.06	98.97 ± 0.06*
Toluene	0.14 ± 0.05	1.00 ± 0.05	98.86 ± 0.10*

^a Immobilization conditions: LU solution were dissolved in 25 mM phosphate buffer (pH 6.0), up to a total volume of 40 mL, with LU concentration at 200.8 µg/mL; then contacted with 100 mg of SBA-15 at 25°C for 30 min.

^b Glycerolysis reaction conditions: soybean oil 3.52 g, glycerol 0.184 g, LU@SBA-15 0.2 g, solvent 10.6 g in solvent reaction systems; reaction temperature 60°C and time 12 h with magnetic stirring at 200 rpm in solvent-free system and 600 rpm in organic solvent reaction system. LU, Lecitase[®] Ultra; LU@SBA-15, SBA-15 supported LU; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

* $p < 0.05$ compared with the solvent-free group of the same column.

3.2 Glycerolysis performance of the LU@SBA-15 in organic solvents

With LU@SBA-15 as catalyst, good performance was observed from solvent-free glycerolysis. In solvent reaction systems, low glycerolysis activity was observed, with TAG conversion at about 13 and 14% from *n*-heptane and *n*-hexane system respectively, and TAG conversion lower than 5% from other solvent systems (Table 4). The results were agreed with our previous study, and even with organic group modified SBA-15 as support, the immobilized LU still exhibited low glycerolysis activity in *tert*-pentanol solvent system; in addition, the low glycerolysis activity was also

not due to the stripped water caused by solvent²³. Interestingly, SBA-15 supported TLL also showed good performance in solvent-free glycerolysis reaction, and the glycerolysis performance decreased in solvent systems⁴.

3.3 Glycerolysis performance of the CALB@SBA-15 in organic solvents

With CALB@SBA-15 as catalyst, good glycerolysis performance was obtained from *tert*-butanol and *tert*-pentanol systems, with TAG conversion over 90%; in addition, TAG conversion at about 42% was observed from 3-Pentanone system, and 33–35% from solvent-free, *n*-hexane and

Table 5 Effects of organic solvents on the glycerolysis performance of the CALB@SBA-15^{a,b}.

Solvent	MAG%	DAG%	TAG%
Solvent-free	9.42 ± 3.83	23.87 ± 2.13	66.71 ± 5.97
<i>n</i> -Hexane	20.86 ± 7.54	14.01 ± 10.75	65.13 ± 18.29
<i>n</i> -Heptane	7.32 ± 4.88	13.54 ± 11.06	79.14 ± 6.18
<i>tert</i> -Butanol	47.83 ± 0.13	44.81 ± 0.72	7.36 ± 0.85 ^b
<i>tert</i> -pentanol	49.50 ± 0.53	45.77 ± 0.44	4.73 ± 0.10 ^b
Acetone	7.18 ± 0.47	9.64 ± 0.42	83.18 ± 0.89 ^a
2-Butanone	13.81 ± 0.47	21.59 ± 3.24	64.59 ± 3.71
3-Pentanone	20.90 ± 1.13	21.32 ± 1.09	57.78 ± 0.04 ^b
Acetonitrile	3.04 ± 0.95	3.51 ± 0.57	93.44 ± 1.52 ^a
Chloroform	0.76 ± 0.01	1.82 ± 0.79	97.42 ± 0.80 ^a
Toluene	9.27 ± 2.80	6.75 ± 3.43	83.98 ± 6.23 ^a

^a Immobilization conditions: CALB solution were dissolved in 25 mM phosphate buffer (pH 7.0), up to a total volume of 40 mL, with CALB concentration at 60.90 µg/mL; then contacted with 100 mg of SBA-15 at 25°C for 30 min.

^b Glycerolysis reaction conditions: soybean oil 3.52 g, glycerol 0.184 g, CALB@SBA-15 0.2 g, solvent 10.6 g in solvent reaction systems; reaction temperature 60°C and time 12 h with magnetic stirring at 200 rpm in solvent-free system and 600 rpm in organic solvent reaction system. CALB, *Candida antarctica* lipase B; CALB@SBA-15, SBA-15 supported CALB; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

^{a, b} $p < 0.05$ compared with the solvent-free group of the same column, with different superscripts differ (^a higher than the solvent-free group, and ^b lower than the solvent-free group).

Table 6 Effects of organic solvents on the glycerolysis by Novozym 435^a.

Solvent	MAG%	DAG%	TAG%
Solvent-free	16.71 ± 1.63	65.22 ± 1.27	18.06 ± 0.36
<i>tert</i> -Butanol	53.23 ± 0.12	45.05 ± 0.17	1.72 ± 0.05
<i>tert</i> -Pentanol	31.73 ± 3.46	58.51 ± 3.35	9.76 ± 0.11

^a Glycerolysis reaction conditions: soybean oil 3.52 g, glycerol 0.184 g, Novozym 435 0.2 g, solvent 10.6 g; reaction temperature 60°C and time 12 h with magnetic stirring at 600 rpm. MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

2-Butanone systems; 16-21% from toluene, acetone and *n*-heptane systems (Table 5). Interestingly, with Novozym 435 as catalyst, TAG conversion over 90% was also observed from *tert*-butanol and *tert*-pentanol systems (Table 6). In addition, the commercial Novozym 435 exhibited better performance in solvent-free system, and it showed selective toward DAG formation. The present CALB@SBA-15 showed selective for MAG generation in both *tert*-butanol and *tert*-pentanol systems, while the Novozym 435 exhibited selective for MAG and DAG production respec-

tively from *tert*-butanol and *tert*-pentanol system. The results also indicated that, in addition to the glycerolysis activity, the supports affected the selectivity as well. The differences in selectivities in glycerolysis reaction from the commercial Novozym 435 and the present CALB@SBA-15 was mainly due to the differences in the supports. The support of the Novozym 435 was the macroporous acrylic resin, which was hydrophobic; while the SBA-15 was hydrophilic. Our previous study had suggested that, proper hydrophobicity favored the DAG selectivity of the support-

Table 7 Glycerolysis performance of the CALB@SBA-15 in *tert*-alcohol systems with different substrate molar ratio^a.

Solvent	Ratio ^b	MAG%	DAG%	TAG%
<i>tert</i> -Butanol	1:1	63.05 ± 1.10	35.19 ± 0.53	1.76 ± 0.57
<i>tert</i> -Pentanol	1:1	65.40 ± 0.13	33.16 ± 0.10	1.44 ± 0.03
<i>tert</i> -Butanol	2:1	69.39 ± 0.77	28.56 ± 0.62	2.05 ± 0.15
<i>tert</i> -Pentanol	2:1	74.34 ± 2.88	25.05 ± 2.75	0.61 ± 0.13
<i>tert</i> -Butanol	3:1	82.91 ± 1.20	16.80 ± 1.17	0.28 ± 0.02
<i>tert</i> -Pentanol	3:1	81.52 ± 1.20	18.22 ± 0.22	0.27 ± 0.11

^a Glycerolysis reaction conditions: soybean oil 3.52 g, CALB@SBA-15 0.2 g, solvent 10.6 g; reaction temperature 60°C and time 12 h with magnetic stirring at 600 rpm. CALB, *Candida antarctica* lipase B; CALB@SBA-15, SBA-15 supported CALB; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

^b Molar ratio of glycerol to oil.

ed lipase²⁸).

Considering that the present CALB@SBA-15 was selective for MAG production in *tert*-butanol and *tert*-pentanol systems, glycerol amount was increased to prepare MAG. As expected, MAG content over 80% and TAG conversion over 99% was obtained from both *tert*-butanol and *tert*-pentanol systems, with glycerol to TAG molar ratio at 3:1 (Table 7). It indicated that the present CALB@SBA-15 was potential for MAG production.

Compared with RML@SBA-15 (Table 1), LU@SBA-15 (Table 4) and CALB@SBA-15 (Table 5), it could be seen that, CALB@SBA-15 exhibited relatively better stability in solvents than the other two immobilized lipases. It exhibited good glycerolysis performance in *tert*-butanol and *tert*-pentanol systems, with TAG conversion over 90% obtained (Table 5), suggesting that their glycerolysis activity was not impaired by these two solvents, and that the introduction of these two solvents helped enhance the glycerolysis reaction (which could be seen by comparing with the solvent-free system). Actually, the reusability of the CALB@SBA-15 in *tert*-pentanol system was also studied in our previous study²⁴. And 92.5 and 80.3% of the initial glycerolysis activity was retained from the SBA-15 supported CALB samples. The glycerolysis activity of CALB@SBA-15 decreased in other solvents, could be mainly ascribed to that, the glycerolysis activity was impaired by these solvents. It could be ruled out the possibility of substrates miscibility issue, since TAG conversion from these solvents was lower than that from solvent-free systems, except for the 3-pentanone system. Therefore, the decreased glycerolysis activity of CALB@SBA-15 from other solvents could be mainly due to that, the glycerolysis activity was impaired by these solvents, not the miscibility issue. Poor performance of LU@SBA-15 in glycerolysis reaction from solvent systems could be due to that its glycerolysis activity was impaired (Table 4). Its excellent performance in solvent-free system had ruled out the possibility of substrates

miscibility issue. As for the RML@SBA-15 (Table 1), its overall glycerolysis performance was poor; better performance from acetonitrile and *n*-heptane systems than that from solvent-free system was observed, however, its glycerolysis activity was also limited, with TAG conversion lower than 50% obtained. In other solvents, its glycerolysis performance was quite poor, with TAG conversion ≤ 5% observed, indicating that its glycerolysis activity was impaired by these solvents.

CALB was known for being thermostable, besides the present study, this concept was also supported by some other reports. For example, in the enzymatic esterification reaction, no significant decrease in the activity of Novozym 435 was obtained after 11 cycles of reuse, with reaction temperature at even 100°C²⁹. In addition, the Novozym 435 was also rather stable in the enzymatic production of DAG from high-acid soybean oil system, and no loss activity was observed after 10 consecutive uses, each lasting 12 h at 60°C³⁰.

3.4 Fluorescence analysis

Fluorescence spectroscopy is a facile technique to study the conformation structure of proteins, the conformation change can be followed by both changes in the maximal intensity of fluorescence ($I_{\max}$) and the shift of the maximal emission wavelength ($\lambda_{\max}$). Lipase comprises of three intrinsic fluorophores that can be quenched, tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe), with Trp being chief intrinsic fluorophore³¹. Trp fluorescence was often used to probe structural changes of the immobilized lipases compared with the free counterpart, since wavelength and intensity of Trp fluorescence indicates the surroundings and changes when proteins have a different conformation. Figure 1 shows the spectra of three free and immobilized lipases in phosphate buffer (25 mM and pH 7.0). As indicated, after immobilization, a significant decrease in the fluorescence intensity was observed from

RML and CALB; interestingly, a slight increase in the fluorescence intensity was obtained from LU. In addition, a red shift of the λ_{max} was also observed, with CALB the larger (from 322 to 341 nm), and then the RML (from 335 to 340 nm) and LU, indicating some microenvironmental change near the Trp residues³²⁾. The decreased fluorescence intensity and the red shift of λ_{max} observed in RML@SBA-15 and CALB@SBA-15 spectra could probably be explained by a classical unfolding process of proteins, which usually resulted in an activity loss (Tables 1 and 5)³³⁾. While little changes in the spectra of the LU@SBA-15 indicated less extensive irreversible unfolding for LU (Table 4)³⁴⁾. The results could partially explain the observed decrease in glycerolysis activity of the RML@SBA-15 and CALB@SBA-15 in solvent-free system (Tables 1 and 5), and the observed good glycerolysis performance of the LU@SBA-15 (Table 4).

The fluorescence emission spectra of immobilized lipases, in phosphate buffer and organic solvents (*tert*-pentanol and acetonitrile), were properly acquired and compared (Fig. 2). The λ_{max} of RML@SBA-15, LU@SBA-15 and CALB@SBA-15 in phosphate buffer was 340, 339 and 341 nm respectively; while exposed to *tert*-pentanol, the λ_{max} of the three immobilized lipases respectively moved to 327, 331 and 330 nm; and exposed to acetonitrile, moved to 333 nm, 328 and 332 nm, respectively (Fig. 2). The blue shift of the λ_{max} (from phosphate buffer to *tert*-pentanol

and acetonitrile) indicated some microenvironmental change near the Trp residues, and the blue shift may be due to the exposure of Trp residues to a more hydrophobic environment³⁵⁾.

In addition, the three immobilized lipases exhibited higher fluorescence intensity when exposed to *tert*-pentanol and acetonitrile than that when exposed to phosphate buffer. Moreover, the fluorescence intensity of RML@SBA-15 and LU@SBA-15 exposed to *tert*-pentanol and acetonitrile significantly increased, compared to that of the free counterparts exposed to phosphate buffer (Figs. 2A and 2B). The blue shift of the λ_{max} and the increase in fluorescence intensity may indicate the protein changed to an active conformation with high activity³³⁾. However, quite poor glycerolysis performance was observed in *tert*-pentanol and acetonitrile solvent systems with RML@SBA-15 and LU@SBA-15 as catalysts (Tables 1 and 4). Interestingly, study from lipases incubated in ionic liquids suggested that, higher fluorescence intensity occurred when lipase was exposed to a hydrophobic environment, favoring the open conformation of the lipase and consequently an increase in activity³⁶⁾. In addition, the CALB@SBA-15 exhibited even higher fluorescence intensity when exposed to acetonitrile than that when exposed to *tert*-pentanol, yet it exhibited good glycerolysis performance in *tert*-pentanol, and quite poor glycerolysis performance was obtained from acetonitrile system (Table 5).

Fig. 1 The fluorescent spectra of free lipase and immobilized lipases in phosphate buffer. RML, *Rhizomucor miehei* lipase; LU, Lecitase® Ultra; CALB, *Candida antarctica* lipase B; lipase@SBA-15, SBA-15 supported lipase.

Fig. 2 Fluorescence spectra of the lipases in phosphate buffer and organic solvents (*tert*-pentanol and acetonitrile) obtained initially. Abbreviations please see Fig. 1.

Fig. 3 Fluorescence spectra of the immobilized lipases incubated in phosphate buffer and organic solvents (*tert*-pentanol and acetonitrile). All experiments were carried out after incubation at 60°C for 4 h. (A) RML@SBA-15, (B) LU@SBA-15 and (C) CALB@SBA-15. Abbreviations please see Fig. 1.

Considering that hydrolytic activity not fully equated with glycerolysis activity¹⁾, hydrolytic activity (hydrolysis of tributyrin) of the present immobilized lipases in the studied media was also investigated. However, no hydrolytic activity improvement but a decrease in the *tert*-pentanol and acetonitrile media compared with that in the phosphate buffer was observed for all the three immobilized lipases (Tables S1 to S3). Therefore, despite the blue shift of the $\lambda_{\max}$ and the increase in fluorescence intensity, no improvement in activity was obtained from the present study.

After incubation at 60°C for 4 h (Fig. 3), no much difference was observed in the fluorescence spectra of the immobilized lipases, compared with their respective initial spectra. Additionally, Fig. S1 to Fig. S3 depict the evolution of both $I_{\max}$ and $\lambda_{\max}$ fluorescence parameters of the three immobilized lipases with incubation time in all the evaluated media at 60°C. As can be seen in Fig. S1A, a continuous decrease in $I_{\max}$ over time was observed when CALB@SBA-15 was incubated in acetonitrile; the $I_{\max}$ also decreased after 8 h incubation in *tert*-pentanol. On the contrary, a slight increase in $I_{\max}$ was observed when incubated in phosphate buffer. This may indicate a higher exposure of Trp to *tert*-pentanol and acetonitrile when the CALB@SBA-15 was incubated at different times, promoting a higher quenching effect of Trp fluorescence³⁷⁾. However, while the CALB@SBA-15 was incubated in three solvents at 60°C for 8 h, the position of the $\lambda_{\max}$ of was almost fixed (Fig. S1B), which indicated that there was no significant change in the secondary structure of CALB³⁸⁾.

A slight increase in $I_{\max}$ over time was observed when RML@SBA-15 incubated in phosphate buffer, and no much difference was observed when it incubated in *tert*-pentanol and acetonitrile (Fig. S2A). As for the $\lambda_{\max}$, the RML@SBA-15 exhibited similar behavior to the CALB@SBA-15, with also almost the fixed $\lambda_{\max}$ (Fig. S2B). In addition, a decrease in $I_{\max}$ was observed when LU@SBA-15 incubated in *tert*-pentanol for 8 h, and a continuous increase in $I_{\max}$ over time was observed when incubated in phosphate buffer (Fig. S3A). The $\lambda_{\max}$ of the LU@SBA-15 kept almost constant after 8 h incubation in acetonitrile and *tert*-pentanol, yet a slight decrease in $\lambda_{\max}$ (from 339 to 337 nm) was observed in phosphate buffer after 8 h incubation (Fig. S3B).

The present results from the evolution profiles of both $I_{\max}$ and $\lambda_{\max}$ fluorescence parameters may indicate a stable thermostability, to check this, thermostability of the immobilized lipases in the studied media was evaluated. Results indicated the thermostability was however normal; in addition, a relative higher stability was observed from phosphate buffer than that from *tert*-pentanol and acetonitrile (Tables S1 to S3).

4 Conclusions

Both support and lipase itself affect the glycerolysis performance of the immobilized lipase. LU@SBA-15 exhibited good glycerolysis performance in solvent-free system, while CALB@SBA-15 showed good glycerolysis activity in *tert*-pentanol and *tert*-butanol systems. LU@SBA-15 and CALB@SBA-15 are respectively suitable for DAG and MAG production. While low glycerolysis activity was observed from RML@SBA-15, neither in solvent nor in solvent-free system. Interestingly, when LU@SBA-15 and RML@SBA-15 incubation in phosphate buffer was shifted to *tert*-pentanol and acetonitrile media, an increase in fluorescence intensity and an blue shift of the $\lambda_{\max}$ was observed; nevertheless, LU@SBA-15 and RML@SBA-15 exhibited low activity (both glycerolysis and hydrolytic) in *tert*-pentanol and acetonitrile systems. In addition, despite the evolution profiles of both $I_{\max}$ and $\lambda_{\max}$ fluorescence parameters kept roughly stable, the thermostability of the immobilized lipases was normal.

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Conflict of Interest

The authors have declared no conflict of interest.

Supporting Information

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