



Immobilization of *Candida antarctica* lipase B onto SBA-15 and their application in glycerolysis for diacylglycerols synthesis



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ABSTRACT

In this study, *Candida antarctica* lipase B (CALB) was immobilized on SBA-15 with three pore diameters. CALB loading was found increased with CALB concentration increasing from 20.3 to 80.12 µg/ml. Higher CALB loading was observed from SBA-15 with pore diameters at 8.1 nm (SBA-15(8.1)), yet highest hydrolytic activity was found at SBA-15(12.5). Thermal stability of the immobilized CALB (SBA-15-CALB) samples was greatly influenced by their water content, after 4 h storage at 70 °C, 8.93 and 67.4% of the initial activity was observed from SBA-15-CALB samples with water content at 9.23 and 3.22% respectively. The SBA-15-CALB samples were then used in glycerolysis of corn oil, and 53.6 wt% of diacylglycerols was obtained after optimization. The operational stability was tested, and after 5 consecutive applications, 92.5 and 80.3% of the initial glycerolysis activity was remained respectively from SBA-15(6.6)-CALB and SBA-15(12.5)-CALB.

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1. Introduction

Immobilization of lipase facilitates the separation of products and the recovery of lipase for reuse. In addition, immobilization also confers advantageous features including enhanced activity, improved stability and increased selectivity (Chen, Miller, Miller, Maikner, & Gross, 2007; Forde et al., 2010). Generally, lipase can be immobilized through crosslinking, covalent attachment, or physical adsorption (Idris & Bukhari, 2012). Of which, physisorption may have higher commercial potential due to its simplicity, low-cost as well as retaining high lipase activity (Gao, Wang, Wang, Luo, & Dai, 2009; He, Song, Ma, Yang, & Guo, 2006). Nevertheless, the stability of the physically adsorbed lipase tends to be poor, because adsorption is generally a relative weak interaction and the lipase will desorb from support during reaction and washing (Gao, Wang, Diao, Luo, & Dai, 2010; Zheng et al., 2012). Covalent attachment can remedy the leaching disadvantage but it may significantly reduce the activity of lipase.

Carriers play an important part in the lipase immobilization. Leaching of lipase can be diminished without being covalently bonded to the wall, when ordered mesoporous materials (OMM) were used as the supports (Serra, Mayoral, Sakamoto, Blanco, &

Díaz, 2008). OMM are potential candidate carriers, due to their advantageous features, including high surface areas, good connectivity, big tortuosity as well as pore size tunability. SBA-15 is the most representative example, its average pore diameter is usually around 8 nm, which makes it an ideal candidate for the immobilization of lipase.

CALB is a highly versatile enzyme with approximate dimensions of 3 × 4 × 5 nm and molecular weight of 32 kDa, it can catalyze a variety of reactions including kinetic resolutions, aminolysis, esterification, transesterification and hydrolysis (Idris & Bukhari, 2012). In addition, CALB is also organic solvents resistant, reasonable thermal stable, stereospecific and enantiospecific (Forde et al., 2010). CALB in immobilized forms offer some operational advantages over their soluble counterparts in practical applications. Despite the already existence of commercially available Novozym 435, studies regarding CALB immobilization are continued at a rapid rate. The properties of the immobilized lipase were influenced greatly by the immobilization technique, immobilization conditions, nature of solvent and variety of reactor. Therefore, the properties of lipase for a particular application can be theoretically tailored through immobilization (Idris & Bukhari, 2012).

Immobilization of CALB on SBA-15 has been studied by some authors. Of which, Abdallah et al. used SBA-15 and a porous spherical silicate material (PPS) to immobilize CALB, they found that CALB exhibited higher activity and stability on SBA-15 than on PPS, due to the different physical properties of the SBA-15 and

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PPS (Abdallah et al., 2014). Laszlo et al. investigated the effects of matrix morphology and surface polarity on the CALB immobilization, and SBA-15 was found to enable to protect the immobilized CALB from denaturation and the all absorbed CALB was active (Laszlo, Jackson, & Blanco, 2011). Forde et al. employed three different types of mesoporous silicate to immobilize CALB, results illustrated that SBA-15 was able to create a stable environment for lipase and prevent damage by external shear forces. Approximately 30-fold stability improvement of the chemical modified CALB was observed after immobilization on SBA-15 (Forde et al., 2010). All these advances make SBA-15 potential candidate for CALB immobilization. However, systematic study on the immobilization of CALB onto SBA-15 has not been seen. In addition, applications of the SBA-15 supported CALB (SBA-15-CALB) as biocatalysts were still limited, catalytic performance of the SBA-15-CALB was largely evaluated by activity test through simple reactions, like hydrolysis of tributyrin or triacetin, some other important reactions have been rarely tested.

With its high time-space cost efficiency, glycerolysis of triacylglycerols (TAG) is quite important in lipid modification field (Huang, Gao, & Zhong, 2015). It is the primary reaction route for the production of monoacylglycerols (MAG) and diacylglycerols (DAG). MAG and DAG are widely used in food, pharmaceutical and cosmetic industries (Krüger et al., 2010). MAG are the most important food-grade emulsifiers, accounting for about 75% of the worldwide production of emulsifiers in food industry (Guo & Xu, 2005). DAG, especially 1,3-DAG, have been claimed to be capable of preventing body fat accumulation. DAG-enriched oil has been recognized as a functional cooking oil, which has gained tremendous interest as a functional food to replace the conventional TAG oil for obesity management (Phuah et al., 2015).

In this study, CALB was immobilized onto SBA-15 with three different pore diameters, 6.6, 8.1 and 12.5 nm (labelled as SBA-15 (6.6, 8.1 and 12.5) before and SBA-15(6.6, 8.1 and 12.5)-CALB after CALB immobilization respectively). Effects of the immobilization conditions were studied, the thermal stability of the obtained immobilized CALB was evaluated. The immobilized CALB was then used to catalyze the glycerolysis reaction for DAG production. Reaction conditions were optimized and the reusability of the immobilized CALB was evaluated.

2. Materials and methods

2.1. Materials and reagents

Refined, bleached and deodorized corn oil was purchased from a local supermarket. SBA-15 with three different pore diameters (6.6, 8.1 and 12.5 nm) were purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). Novozym 435 (acrylic resin immobilized CALB) and CALB was kindly provided by Novozymes (Beijing, China). Glycerol with a purity of >99.0% was from Sinopharm Chemical Reagent Co., Ltd (Shanghai China). Tributyrin (>97%) for activity analysis and the standards of 1-monolein, 1,3-diolein and triolein (>99.0%) for HPLC analysis were from Sigma (St. Louis, MO, USA). All other solvents and reagents were of analytical or chromatographic grade.

2.2. Immobilization of CALB onto SBA-15

The commercial extract CALB supplied by Novozymes (Beijing, China) had a protein concentration of 5.086 µg/mg, determined by the Bradford assay (Arice, Soydogan, & Bayramoglu, 2010). Required amounts of this extract were dissolved in 25 mM phosphate buffer, up to a total volume of 40 ml. Samples were withdrawn at this stage for the initial lipase activity (E_0)

measurement. Then 100 mg of SBA-15 were added into the solution, and magnetically stirred at room temperature. After that, the suspensions were filtered and washed with the phosphate buffer. Supernatants were tested for the final lipase activity (E_f). Unless otherwise stated, the immobilized CALB samples were dried in a vacuum oven (pressure at -0.093 MPa) at 25 °C for 2 h before activity analysis. Water content of the obtained immobilized CALB was at 17.59%. Samples with water content at 9.23% and 3.22% were obtained through being dried in a vacuum oven (pressure at -0.093 MPa) at 25 °C for 4 h and 30 °C for 6 h respectively. Water content was determined by heating samples at 105 °C for 1 h, and the mass of water was the weight loss during the heating process.

2.2.1. Immobilization efficiency measurement

To determine the immobilization efficiency, samples were withdrawn as mentioned above, and hydrolytic activities were measured. A blank assay was also conducted to evaluate a possible lipase deactivation under the immobilization conditions. For this purpose, a solution of CALB was placed in a reactor under the same conditions of immobilization, but without the SBA-15 addition. The immobilization efficiency (IE) was calculated through the following equation (Liu, Fu, et al., 2012; Liu, Wang, et al., 2012):

$$IE (\%) = (E_0 V_0 - E_f V_f) / E_0 V_0 \times 100$$

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).

2.2.2. Assay of enzymatic activity

The activity of the free or immobilized CALB was determined according to Wu et al. (2012) with some modifications. 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 CALB solution or 10 mg of the immobilized CALB 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 same procedures but without lipase addition. 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 the described conditions. The activity of the commercial CALB solution was found to be 4340.4 U/ml. All the experiments were conducted in triplicate.

2.2.3. Thermal stability

Thermal stability of SBA-15(6.6)-CALB with different water content was examined by measuring the activity of samples taken at regular time intervals and comparing the results with those at the beginning of the experiment, the initial activity was defined as 100% (Yang et al., 2013). The immobilized CALB samples were stored at 70 °C without incubation in any medium for a period of time and then withdrawn for activity assay. For comparison, sample of SBA-15(6.6)-CALB with water content at 17.59% was also incubated in hexane and phosphate buffer (pH 7.0) solution at 70 °C and then withdrawn at time intervals for activity measurement. In addition, thermal stability of the CALB solution was also evaluated. In this case, the CALB solution was incubated in phosphate buffer (pH 7.0 and the CALB concentration at 30.5 µg/ml) at 70 °C for 0 h, 0.5 h, 1 h, 2 h, 3 h and 4 h respectively. After that, the activity of the CALB solution was determined under the same reaction conditions as described above.

2.3. Characterization

Small-angle powder XRD was carried on Bruker (D4) advance diffractometer, using Ni-filtered Cu K α radiation at 40 kV and 40 mA in the 2θ range of 0.5–8°, at scan speed of 0.2°/min. The

low-temperature N₂ adsorption-desorption experiments were carried out using a Gold APP V-Sorb 2800P apparatus. Before the measurement, SBA-15 were degassed in a vacuum at 240 °C for 4 h, and SBA-15 with lipase were degassed in a vacuum at 80 °C for 12 h. The pore diameter distribution 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. The surface chemistry and chemical state of SBA-15 before and after CALB immobilization were analyzed by X-ray photoelectron spectroscopy (XPS; Thermo ESCALAB 250 instrument, Thermo Scientific), using monochromatic Al-K α radiation of energy 1486.6 eV and operating in the constant analyzer energy mode. The pressure in the analysis chamber was 9.8×10^{-8} Pa.

2.4. Enzymatic glycerolysis of corn oil

Unless otherwise stated, the reaction mixtures consisted of 4.4 g corn oil, required amount of glycerol and 17 ml of *tert*-pentanol were incubated in an oil bath, with magnetic stirring at 500 rpm. The reaction was initiated by the addition of lipase. Reaction conditions, namely reaction temperature, immobilized CALB concentration, substrates molar ratio and time progress, were evaluated. At pre-determined intervals, 30 μ L of the reaction mixture was withdrawn and added to 4 ml of mixtures of hexane and isopropanol (hexane:isopropanol 4:5, v:v). The mixture was then filtered through a microfilter (0.45 μ m) to remove the catalysts. All samples were stored at –20 °C before analysis.

2.5. Determination of MAG, DAG and TAG by RP-HPLC

The lipid profile was determined according to our previous RP-HPLC method (Huang et al., 2015). Quantification and identification of compounds were described in detail in another our previous study (Zhong et al., 2009). Double determinations were performed.

2.6. Reusability of the immobilized CALB

The reusability of the immobilized CALB was studied under the model reaction with 4.4 g corn oil, 0.23 g glycerol, 17 ml *tert*-pentanol and 0.23 g immobilized CALB. The reaction was progressed for 12 h at 50 °C for each cycle. At the end of the reaction, the reaction mixture was centrifuged and the separated catalyst was washed with 10 ml *tert*-pentanol, and then used for the next batch under otherwise identical conditions. The relative activity of lipase was defined as the ratio of TAG conversion obtained from each cycle to the TAG conversion obtained from the first cycle.

$$\text{Relative activity (\%)} = \frac{\text{conversion of TAG obtained from each cycle}}{\text{conversion of TAG obtained from the first cycle}} \times 100$$

2.7. Statistical analysis

An analysis of variance (ANOVA) was performed using the SPSS 13.0 statistical analysis system. The level of confidence required for significance was defined at $P < 0.05$ with Tukey's test.

3. Results and discussion

3.1. Characterization of the supports

CALB was immobilized on SBA-15 with three different pore diameters. Fig. S1a showed the X-ray diffractograms of the SBA-15 with pore diameters at 8.1 nm before (SBA-15(8.1)) and after CALB immobilization (SBA-15(8.1)-CALB). Both have an intense

peak attributed to reflections at (1 0 0) and two low-intensity peaks assigned to (1 1 0) and (2 0 0), corresponding to a two-dimensional hexagonal pore regularity of a *P6mm* space group (Zhao et al., 1998). The XRD patterns indicated that immobilization of CALB did not destroy the ordered mesostructure of the siliceous SBA-15. However, the immobilization caused remarkably decrease in the XRD reflection intensity, which was agreed with previous studies about the immobilization of porcine pancreas lipase onto SBA-15 (Zou et al., 2010). In addition, CALB immobilization caused a marginal shift of the order diffraction peak to the higher angle, indicating that CALB had entered into the mesopore channels of the SBA-15 (Hu et al., 2012).

Table S1 summarized the textural properties of SBA-15 before and after CALB immobilization. The surface area decreased dramatically after CALB immobilization, indicating that the lipase molecules stayed in the channels rather than merely on the external surface of the mesoporous materials. Pore volume and average pore diameter did not change much because there was a distribution of pores, and a little reduce in the average pore diameter may indicate that a large number of channels were filled with lipases and had a slight decrease in diameter (Gao et al., 2010).

XPS spectras of SBA-15 before and after CALB immobilization were presented in Fig. S2, and the XPS elemental concentrations summarized in Table S2. As demonstrated, after CALB immobilization, the C_{1s} and N_{1s} (Binding Energy at 285 and 400 eV respectively) peaks increased, as well as the C_{1s} and N_{1s} elemental concentrations raised. This phenomenon was attributed to the presence of amino acid groups (Khoobi et al., 2014), which confirmed the successful immobilization of CALB.

3.2. Immobilization of CALB onto SBA-15

SBA-15 with three different pore diameters was used as supports for CALB immobilization. Immobilization conditions including time, pH and CALB concentrations, were evaluated carefully. Results were summarized in Tables 1 and 2. In addition, thermal stability of the immobilized CALB was examined.

3.2.1. Effects of pH

Higher IE was observed at pH 6.0 and 7.0 than at pH 8.0, and no much difference was found between pH 6.0 and 7.0 (entry 1–6 and 13–15 in Table 1). Higher IE value implied higher lipase loading.

Table 1
Immobilization of CALB onto SBA-15 with pore diameters at 8.1 nm.^a

Entry	pH	Time (min)	CALB concentration (μ g/ml)	IE (%) ^b	Activity (U/g) ^c
1	6.0	15	40.6	86.08 \pm 0.89	300.00 \pm 33.33
2	6.0	30	40.6	87.84 \pm 0.34	288.89 \pm 38.49
3	6.0	60	40.6	89.71 \pm 0.29	222.22 \pm 38.49
4	7.0	15	40.6	82.36 \pm 0.89	300.00 \pm 33.33
5	7.0	30	40.6	85.85 \pm 0.34	444.44 \pm 38.49
6	7.0	60	40.6	89.15 \pm 0.34	344.44 \pm 19.25
7	7.0	90	40.6	89.39 \pm 0.18	388.89 \pm 19.25
8	7.0	120	40.6	93.07 \pm 0.00	237.78 \pm 26.94
9	7.0	30	20.3	92.17 \pm 1.04	122.22 \pm 19.25
10	7.0	30	40.6	85.85 \pm 0.34	444.44 \pm 38.49
11	7.0	30	60.9	74.24 \pm 0.69	855.56 \pm 69.39
12	7.0	30	80.12	69.35 \pm 0.65	388.89 \pm 19.25
13	8.0	15	40.6	63.90 \pm 0.75	211.11 \pm 19.25
14	8.0	30	40.6	66.08 \pm 0.33	244.44 \pm 38.49
15	8.0	60	40.6	68.00 \pm 0.34	255.56 \pm 19.25

^a Immobilization conditions: Required amounts of the CALB solution were dissolved in 25 mM phosphate buffer, up to a total volume of 40 ml, contacted with 100 mg of SBA-15 at room temperatures. Note: Standard deviation values were calculated from triplicate experiments.

^b Immobilization efficiency.

^c Activity of the immobilized CALB.

Table 2
Immobilization of CALB onto SBA-15 with pore diameters at 6.6 nm and 12.5 nm.^a

Entry	Supports	CALB concentration (μg/ml)	IE (%) ^b	Activity (U/g) ^c
1	SBA-15 (6.6) ^d	20.3	80.87 ± 0.26	200.04 ± 38.49
2		40.6	67.65 ± 0.55	322.22 ± 19.25
3		60.9	65.69 ± 0.27	944.44 ± 19.25
4	SBA-15 (12.5) ^e	80.12	61.81 ± 0.63	822.22 ± 38.49
5		20.3	52.41 ± 1.38	377.82 ± 38.49
6		40.6	53.13 ± 0.64	433.33 ± 19.25
7		60.9	58.15 ± 0.71	1188.89 ± 69.39
8		80.12	51.51 ± 0.86	755.56 ± 38.49

^a Immobilization conditions: required amounts of the CALB solution were dissolved in 25 mM phosphate buffer, up to a total volume of 40 ml, pH 7.0, contacted with 100 mg of SBA-15 at room temperatures for 0.5 h. Note: Standard deviation values were calculated from triplicate experiments.

^b Immobilization efficiency.

^c Activity of the immobilized CALB.

^d SBA-15 with pore diameters at 6.6 nm.

^e SBA-15 with pore diameters at 12.5 nm.

Such results were plausible, since pH around the isoelectric point of CALB (6.0), the net charge of the surface of CALB was small which minimising electrostatic repulsions, leading to higher loadings (Abdallah et al., 2014). Lower activity from immobilization pH at 8.0 was the result of two parallel effects. One was the aforementioned lower lipase loading at pH 8.0. The other was that, the lipase conformation which was vital for enzymatic activity, changed with pH, pH 8.0 seemed less suitable for CALB in right conformation, leading to lower activity. Higher activity was observed from immobilization pH at 6.0 and 7.0 (Miletić, Abetz, Ebert, & Loos, 2010).

3.2.2. Effects of immobilization time

Immobilization time from 15 to 120 min was evaluated (entry 4–8, in Table 1). Lipase loadings were increased with time increasing from 15 to 60 min, however, no increment was observed with time increasing from 60 to 90 min. Possibly there was an aggregation of lipase molecules at the orifice of SBA-15 at this stage, resulting in a blockage of the pores and a rejection of other CALB molecules into the channels (Gao et al., 2010). After that, some lipase molecules began to depart and let the pores expose again, and lipase can gradually enter into the channels, which was observed from time 90 to 120 min. Higher loading at 120 min also indicated there was still space for lipase entry.

3.2.3. Effects of CALB concentrations

The IE decreased with CALB concentration increasing from 20.3 to 80.12 μg/ml (entry 9–12 in Table 1), nevertheless, it can be estimated that lipase loading still increased but with a declined immobilization rate. It was reasonable since when more CALB molecules were concentrated at the orifice of SBA-15, the orifice tended to be blocked and the diffusion of lipase through into the inner channels became difficult, causing a declined immobilization rate.

The activity of the supported CALB increased firstly and then declined as the lipase loading went up (entry 9–12 in Table 1; entry 1–8 in Table 2). It can be presumed that with more lipase accessing 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, causing a decrease in enzymatic activity because of the non-contact with substrates of the bottom layer lipases (Gao et al., 2010). On the other hand, high loads of lipase may also be subject to diffusion limitations and steric hindrance (Silva, Macedo, Rodrigues, Giordano, & Gonçalves, 2012). Since with more lipases into the pore of the supports, substrates may become difficult to diffuse into the channels; in addition, lipase may be

immobilized with the wrong orientation in the case of high loads, which obstructed the active site, leading to low enzymatic activity (Silva et al., 2012).

3.2.4. Effects of pore diameters

Of the studied SBA-15 samples, lipase loading increased with the CALB concentration increasing from 20.3 to 80.12 μg/ml (Fig. S4a). However, the highest loading was not found in the SBA-15 with the largest pore diameter (SBA-15(12.5)), but in SBA-15(8.1). Larger pores usually favored more lipase entry into the channels and therefore a higher loading of lipase. Nevertheless, lipase loading was also affected by textural properties of the supports, such as surface area, morphology and pore size distribution. The present results may be due to the broader pore size distribution (Fig. S1b) and the lower surface area of SBA-15(12.5) (Table S1), which may reduce the enzyme loading (Abdallah et al., 2014). Consistently, the highest loading of *Candida rugosa* lipase was either not found in the SBA-15 with the largest pore diameter, whose pore size distribution was broader (Gao et al., 2010). Fig. S4b presented the relation between CALB loading and the activity of the immobilized CALB samples. Interestingly, SBA-15 with three pore diameters all exhibited similar trends, in which the activity increased and then decreased with CALB loading went up. The highest enzymatic activity was observed in the SBA-15 (12.5), possibly due to that larger pore size favored the diffusion of substrates, which helped to improve the activity.

Fig. S5 presented the relation between pore diameter and CALB loading, pore diameter and activity, surface areas and CALB loading, as well as surface areas and activity. It seemed that both pore diameter and surface areas at median range gave higher CALB loading and lower activity for most cases. However, when pore size, surface areas, CALB loading and activity were compared, little correlation was observed.

3.2.5. Thermal stability of the immobilized CALB

Thermal stability of the immobilized CALB samples was greatly influenced by their water content. Quite poor thermal stability was observed in the samples with water content at 9.23% and especially 17.59%; while water content decreasing to 3.22%, thermal stability was improved significantly (Fig. 1a). Poor thermal stability was also observed when samples incubating in hexane or phosphate buffer (pH 7.0) solution (Fig. 1b). Results indicated that though SBA-15 could provide a good environment for lipase, water content of the immobilized lipase should be low, or thermal stability of the supported lipase (at least for CALB) would be poor. Therefore, it is reasonable to observe the good thermal stability of the commercial Novozym 435, whose water content was usually at 1–2%. To study further, Novozym 435 samples were exposed to the humid air for a time with water content up to 18.50%, then the thermal stability was tested. Consistently, only 22.58 ± 5.59% of the initial activity was remained after 4 h incubation in hexane at 70 °C (Fig. S3).

3.3. Enzymatic glycerolysis of corn oil

The immobilized CALB was then used to catalyze glycerolysis of corn oil for DAG production. The performance of the present three immobilized CALB (immobilized on SBA-15 with three different pore diameters) was compared; reaction conditions including temperature, catalysts concentration, substrates molar ratio, reaction time, were studied; and the reusability of the immobilized CALB was evaluated.

3.3.1. Glycerolysis of corn oil catalyzed by the immobilized CALB

In solvent-free systems, both DAG content and TAG conversion was low (<30 wt%). Interestingly, approximately 60 wt% of DAG was obtained from Novozym 435 mediated glycerolysis in

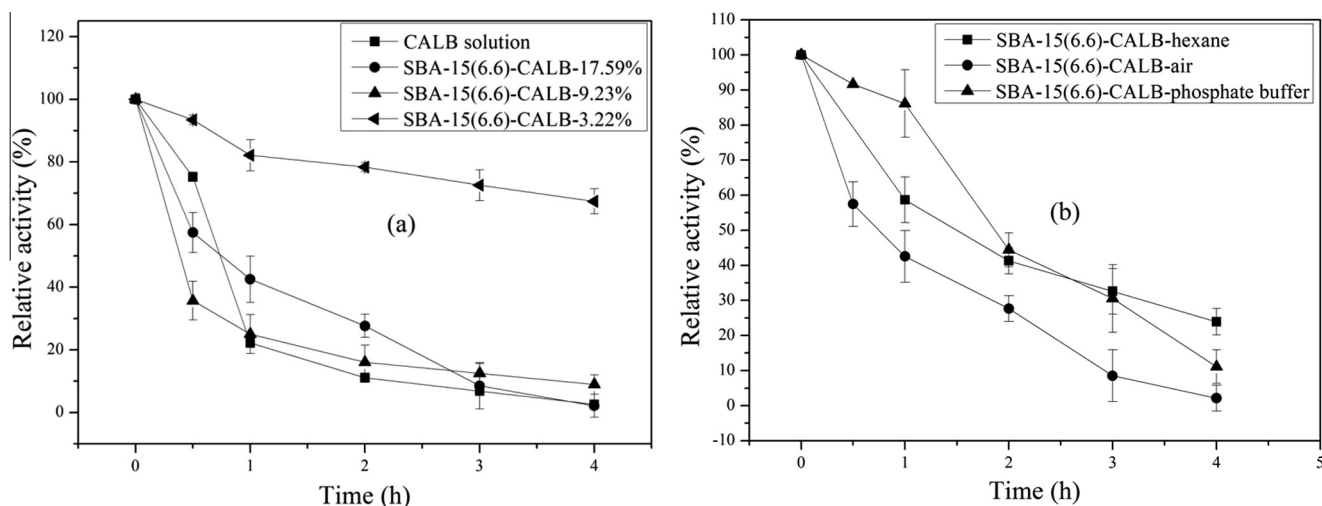


Fig. 1. (a) Thermal stability of CALB solution and SBA-15(6.6)-CALB with different water content. Conditions: CALB solution was incubated in phosphate buffer (pH 7.0) with CALB at 30.5 µg/ml and temperature 70 °C; SBA-15(6.6)-CALB samples were stored at 70 °C without incubation in any medium. (b) Effects of the incubation medium on the thermal stability of SBA-15(6.6)-CALB with water content at 17.59%. Conditions: samples were stored at 70 °C 1) without incubation in any medium (SBA-15(6.6)-CALB-air), 2) incubating in hexane (SBA-15(6.6)-CALB-hexane), 3) incubating in pH 7.0 phosphate buffer (SBA-15(6.6)-CALB-phosphate buffer). The initial activity was defined as 100%. CALB, *Candida antarctica* lipase B; SBA-15(6.6)-CALB, pore diameters of SBA-15 at 6.6 nm before CALB immobilization, and -CALB means CALB immobilized on the SBA-15; SBA-15(6.6)-CALB-(numbers%), numbers in the bracket mean the water content of the immobilized CALB.

solvent-free systems (Kristensen, Xu, & Mu, 2005a, 2005b). Poor performance of the present SBA-15-CALB may be due to its hydrophilic carrier (SBA-15), which easily leading to the glycerol layer formation around the hydrophilic carrier, and in turn limiting contact between the lipase and the hydrophobic oil phase (Kristensen et al., 2005a). Lipase carrier was important for catalysis. Various carriers led to different performance for the immobilized CALB in glycerolysis reaction. For example, 42 wt% of DAG was obtained with Chirazyme L2 (lipase immobilized on acrylic resin from *Candida antarctica* lipase B) as catalyst through glycerolysis of fish oil, after 24 h reaction at 60 °C in solvent-free systems (Torres, Lin, & Hill, 2002). In addition, glycerolysis did not take place with Novozym CALB L (immobilized on macroporous anion-exchange resin) as catalyst in solvent-free systems. Stepwise addition of glycerol allowed the catalysis to proceed, however, only 19 wt% of DAG was obtained (Kristensen et al., 2005a).

DAG content and TAG conversion has been increased significantly (Fig. 2) after *tert*-pentanol introduction. The amphiphilic

tert-pentanol solvent was efficient in glycerolysis reaction, it can create a homogeneous reaction system of the immiscible reactants glycerol and oil (Damstrup et al., 2006), which helps to enhance the reaction.

Of the three studied cases, SBA-15(8.1)-CALB gave a little better performance than the other two SBA-15 supported CALB. Yet hydrolytic activity of SBA-15(8.1)-CALB was lower than that of other two SBA-15 supported CALB (Tables 1 and 2). It indicated that SBA-15(8.1)-CALB possessed higher glycerolysis activity despite its lower hydrolytic activity. This phenomenon was not strange since these two activities were different, higher hydrolytic activity not meant higher glycerolysis activity (Noureddini & Harmeier, 1998).

3.3.2. Effects of catalyst concentration, substrates molar ratio, reaction temperature, and time on the glycerolysis reaction

Reaction conditions were studied with SBA-15(6.6)-CALB as catalyst. Reaction temperature was an important parameter for

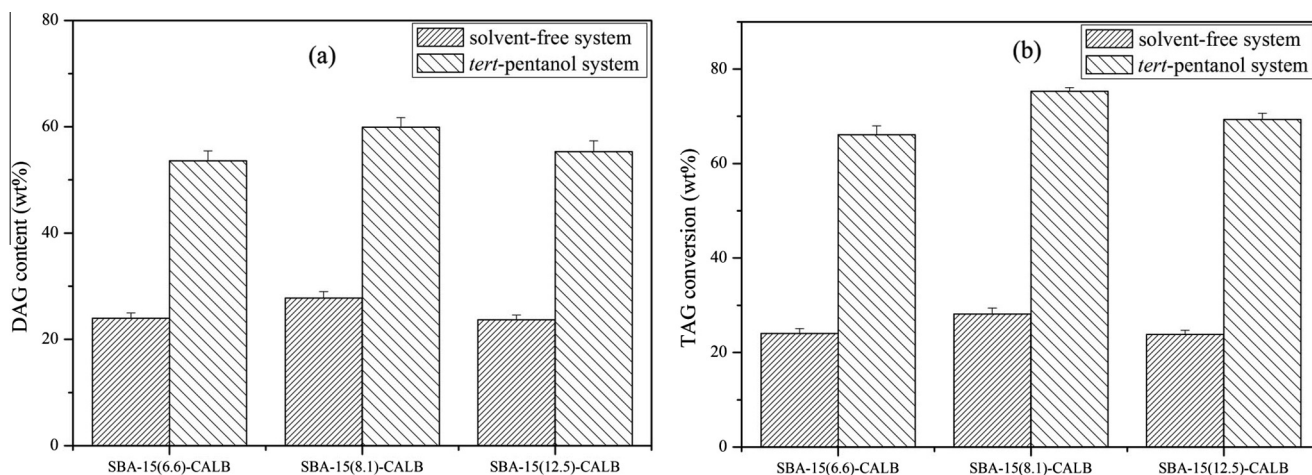


Fig. 2. Enzymatic glycerolysis of corn oil by SBA-15-CALB with three different pore diameters. Reaction conditions: corn oil 4.4 g, glycerol 0.23 g, catalysts 5 wt% of oil and glycerol, *tert*-pentanol (in solvent system) 17 ml, agitation speed 500 rpm, reaction temperature 50 °C and time 24 h. DAG, diacylglycerols; TAG, triacylglycerols; CALB, *Candida antarctica* lipase B; SBA-15(numbers)-CALB, numbers in the bracket mean the pore diameters of the SBA-15 before CALB immobilization, and -CALB means CALB immobilized on the SBA-15.

glycerolysis. Increasing temperature helps to improve the miscibility of substrates (TAG and glycerol), which facilitates the reaction enhancement. However, higher temperature would affect the enzymatic activity. As indicated in Fig. 3a, no much difference in DAG content was observed when temperature increasing from 40 to 60 °C. However, DAG content and TAG conversion declined significantly ($P < 0.05$) when temperature up to 65 and 70 °C, possibly due to the glycerolysis activity loss at high temperature. Because water content of the used immobilized CALB was at 17.59%. As indicated, the high water content led to poor thermal stability, therefore glycerolysis activity loss and in turn low TAG conversion was observed.

With the investigated ranges, enzyme concentration at 5 wt% gave the best performance in terms of DAG content and TAG conversion (Fig. 3b). Enzyme concentration affects the reaction rate, at certain ranges, more enzyme leads to higher reaction rate. However, excess enzyme would decrease the efficiency per mass unit of biocatalysts in enzymatic reactions. The slight decrease in DAG content with further increase of lipase over 5 wt% may be due to the lipase agglomeration and possible diffusion problems, similar trends have also been observed in other studies (Duan, Du, & Liu, 2010; Liu, Fu, et al., 2012; Liu, Wang, et al., 2012).

Of the studied oil/glycerol molar ratios, higher content of DAG was expectedly observed at oil/glycerol 2:1, while higher TAG conversion was found at 2:3 (Fig. 3c). Oil/glycerol at 2:1 was the stoichiometric ratio in glycerolysis and higher content of DAG was usually obtained at this ratio (Guo, Kahveci, Özcelik, & Xu, 2009). More glycerol favored TAG conversion, on the other hand, it would also shift the reaction equilibrium towards the MAG formation side, which decreased the DAG content. Since the glycerolysis was a multi-step reversed reaction and DAG were the intermediate products (Zhong et al., 2014).

Glycerolysis time courses of corn oil were presented in Fig. 3d. DAG content up to 53.6 wt% based on acylglycerols was obtained after 12-h reaction. No much difference in DAG content was observed with time increasing from 12 to 72 h. Nevertheless, the reaction was still in progress up to 36 h to reach the equilibrium, with MAG content went up and TAG content decreased to a stable level. The time courses of glycerolysis indicated the reaction rate was lower, when compared with previous studies using Novozym 435 as catalyst. Lower reaction rate could be partly attributed to the smaller pore size of SBA-15 than that of macroporous acrylic resin (carrier of Novozym 435), which provided less room for reactants and products diffusion, leading to reaction slower.

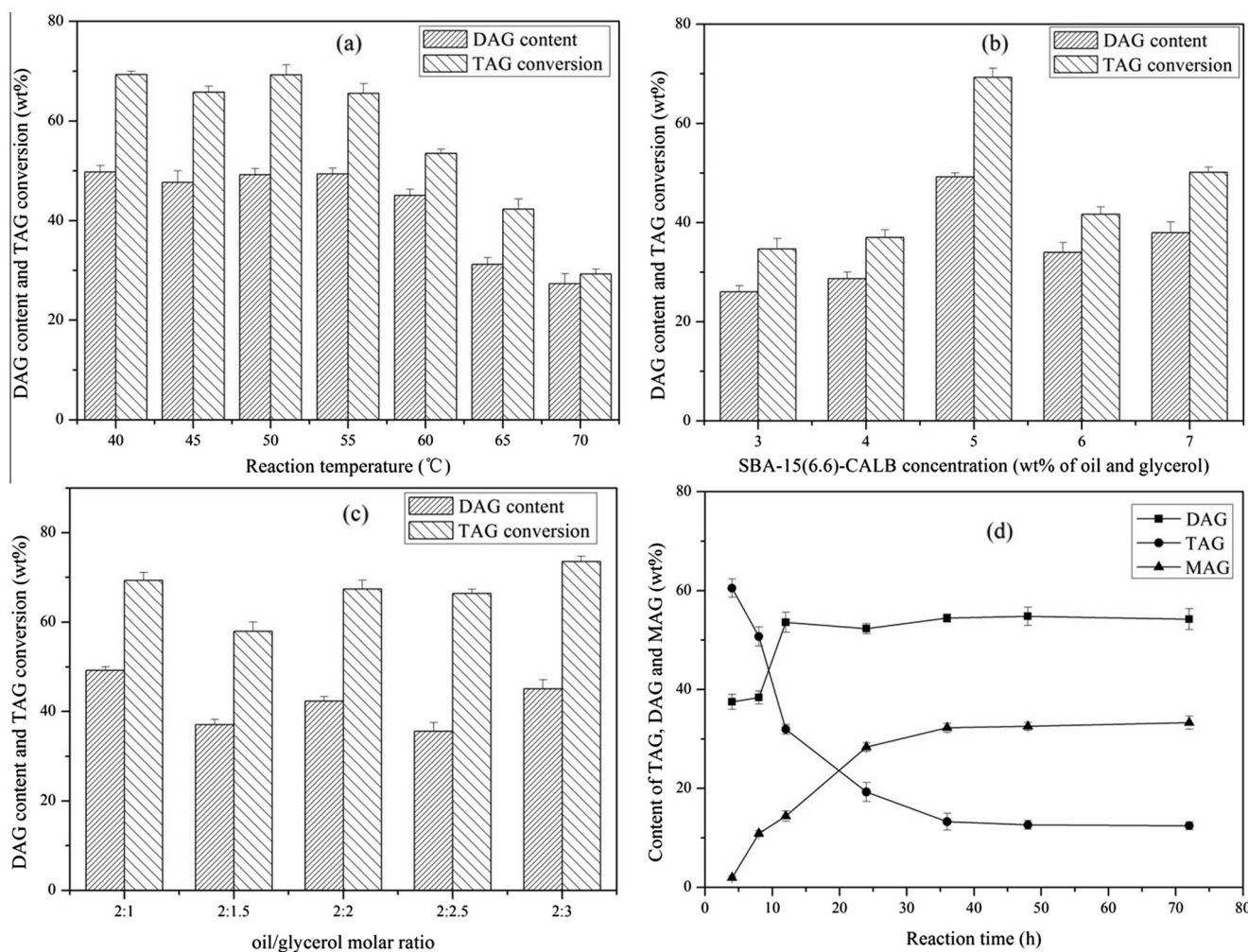


Fig. 3. Effects of temperature (a), enzyme concentration (b), substrates molar ratio (c) on the glycerolysis reaction; and time progress of the glycerolysis reaction (d). Reaction conditions: (a) corn oil 4.4 g, glycerol 0.23 g, catalysts SBA-15(6.6)-CALB 5 wt% of oil and glycerol, *tert*-pentanol 17 ml, agitation speed 500 rpm, reaction time 12 h; (b) corn oil 4.4 g, glycerol 0.23 g, *tert*-pentanol 17 ml, agitation speed 500 rpm, reaction temperature 50 °C and time 12 h; (c) corn oil 4.4 g, catalysts SBA-15(6.6)-CALB 5 wt% of oil and glycerol, *tert*-pentanol 17 ml, agitation speed 500 rpm, reaction temperature 50 °C and time 12 h; (d) corn oil 8.8 g, glycerol 0.46 g, catalysts SBA-15(6.6)-CALB 5 wt% of oil and glycerol, *tert*-pentanol 34 ml, agitation speed 500 rpm, reaction temperature 50 °C. DAG, diacylglycerols; TAG, triacylglycerols; CALB, *Candida antarctica* lipase B; SBA-15 (6.6)-CALB, pore diameters of SBA-15 at 6.6 nm before CALB immobilization, and -CALB means CALB immobilized on the SBA-15.

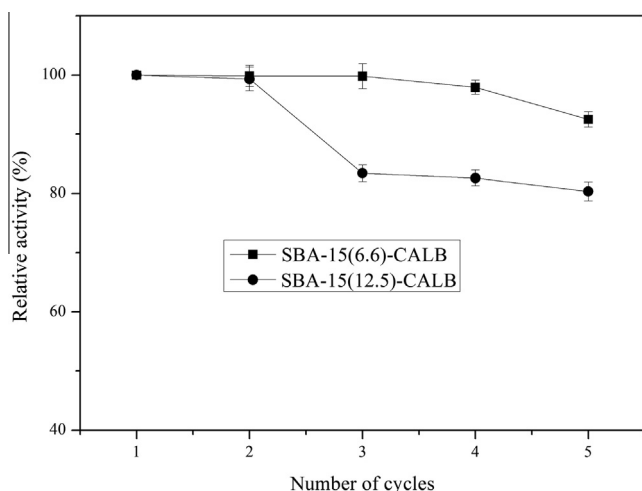


Fig. 4. Reusability of the immobilized CALB samples. Reaction conditions: corn oil 4.4 g, glycerol 0.23 g, catalysts amount 5 wt% of oil and glycerol, *tert*-pentanol 17 ml, reaction temperature 50 °C and time 12 h. CALB, *Candida antarctica* lipase B; SBA-15(6.6 or 12.5)-CALB, pore diameters of SBA-15 at 6.6 (or 12.5) nm before CALB immobilization, and -CALB means CALB immobilized on the SBA-15.

Overall, reasonable content of DAG was obtained from the present SBA-15-CALB mediated glycerolysis. However, it was not promising when compared with some previous studies. For example, 57 wt% of DAG was obtained from Novozym 435 mediated glycerolysis with *tert*-butanol as reaction medium (Voll et al., 2011). Different performance from SBA-15-CALB and Novozym 435 could be due to their different carriers. SBA-15 was hydrophilic while the carrier of Novozym 435 (macroporous acrylic resin) was hydrophobic, such difference may result in their different catalytic behaviors. This assumption was supported by the performance from Novozym 435 and Novozym CALB L (immobilized on macroporous anion-exchange resin, which was hydrophilic) mediated glycerolysis, in which lower content of DAG was observed from Novozym CALB L (imm.) than that from Novozym 435, even glycerolysis with glycerol absorbed on silica gel (Kristensen et al., 2005a). Remarkably, DAG content up to 70 wt% was obtained from Novozym 435 mediated glycerolysis with binary ionic liquids ([TOMA]₂[Tf₂N]/Amoeng 102) as reaction system (Kahveci, Guo, Özçelik, & Xu, 2010). The significant performance was attributed to used binary ionic liquids, in which Amoeng 102 resulted in high TAG conversion, and [TOMA]₂[Tf₂N] afforded selectivity for DAG production. On the other hand, 23.7 wt% of DAG was obtained from glycerolysis of olive oil in hexane system, with CALB immobilized on cellulose acetate-coated Fe₂O₃ as catalyst.

3.3.3. Reusability of the immobilized CALB

The reusability of the immobilized CALB is rather important in practical applications. In the current study, 92.5% of the initial glycerolysis activity of SBA-15(6.6)-CALB remained after 5 cycles, each lasting 12 h; while 80.3% was obtained for SBA-15(12.5)-CALB (Fig. 4). Such differences may be ascribed to the different pore diameters of SBA-15, which related to the lipase leaching. Supports with small pore diameters would offer more resistance to the exit of enzyme, while large pore diameters offer small resistance to the enzyme release (Serra et al., 2008).

4. Conclusions

CALB loading was increased with CALB concentration increasing from 20.3 to 80.12 µg/ml during immobilization. Higher CALB loading was observed from SBA-15(8.1), yet highest hydrolytic activity was found at SBA-15(12.5). Thermal stability of the

SBA-15-CALB samples was greatly influenced by their water content. DAG content at 53.6 wt% was obtained from the SBA-15-CALB mediated glycerolysis after optimization. 92.5 and 80.3% of the initial glycerolysis activity was remained respectively from SBA-15(6.6)-CALB and SBA-15(12.5)-CALB, after 5 consecutive applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.05.167>.

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