Production and immobilization of lipase PCL and its application in synthesis of α-linolenic acid-rich diacylglycerol

Liu, Nan; Liu, Daoming; Wang, Weifei; Hollmann, Frank; Xu, Long; Ma, Yunjian; Yang, Bo; Bai, Weidong; Sun, Xiaotao ; Wang, Yonghua

DOI
10.1111/jfbc.12574

Publication date
2018

Document Version
Final published version

Published in
Journal of Food Biochemistry

Received: 3 January 2018 | Revised: 2 March 2018 | Accepted: 27 March 2018

DOI: 10.1111/jfbc.12574

FULL ARTICLE

WILEY Journal of Food Biochemistry

Production and immobilization of lipase PCL and its application in synthesis of α-linolenic acid-rich diacylglycerol

Nan Liu[1] | Daoming Li[1] | Weifei Wang[2] | Frank Hollmann[3] | Long Xu[1] | Yunjian Ma[1] | Bo Yang[4] | Weidong Bai[5] | Xiaotao Sun[6] | Yonghua Wang[1]

[1]School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China

[2]Sericultural and Agri-food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510610, China

[3]Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, Delft 2629HZ, The Netherlands

[4]School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China

[5]College of Food Science and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China

[6]Beijing Key Laboratory of Flavor Chemistry, Beijing Technology and Business University, Beijing 100048, China

Correspondence
Yonghua Wang, School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China.
Email: yonghw@scut.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 31501412, 31601398; Science and Technology Planning Project of Guangdong Province, Grant/Award Number: 2016B090920082; Open Project Program of Beijing Key Laboratory of Flavor Chemistry, Beijing Technology and Business University (BTBU), Beijing 100048, China, Grant/Award Number: SPFW-2017-YB01; National Science Fund for Distinguished Young Scholars, Grant/Award Number: 31725022

Abstract

α-Linolenic acid-rich diacylglycerol has been demonstrated with promising health promotion functions. This study examined the production, immobilization of lipase PCL, and its application in the synthesis of diacylglycerol by esterification of α-linolenic acid with glycerol. The resin ECR8806 was selected as an effective support for the immobilization of lipase PCL. Fourier transform infrared and Laser scanning confocal microscope analysis proved that the lipase was successfully immobilized on the resin. Compared with the free PCL, the immobilized one exhibited higher temperature tolerance. Under optimized reaction conditions, a DAG content of 54.49% were obtained. After further purified by molecular distillation, the purity of DAG was up to 99.28%. During esterification, the immobilized PCL was quite stable and retained more than 91.60% of its initial activity after 10 cycles. These new findings on the immobilized PCL will make it to be a prospective enzyme in oils and fats industry.

Practical applications

Immobilized PCL, a monoacylglycerol and diacylglycerol lipase, was first employed to synthesis of a high purity α-linolenic acid-rich diacylglycerol. The improved biocatalytic performance and reusability of the lipase make it to be a promising catalyst in industrial application.

KEYWORDS

diacylglycerol, esterification, immobilization, reusability, α-Linolenic acid

1 | INTRODUCTION

With increasing demands in functional food and health products also the demand for polyunsaturated (ω-3) fatty acid products is constantly rising. In food products especially monoacylglycerols and diacylglycerols (MAG and DAG, respectively) are frequently used due to their facile delivery to the human body and the simple processability of these compounds in the dietary products. Some very recent studies showed that α-linolenic acid-rich diacylglycerol has promising health promotion functions especially in reduction of visceral fat area (VFA), BMI and serum triacylglycerol (TAG) in men and women with overweight (Ando et al., 2016; Saito, Fukuhara, Osaki, Nakamura, & Katsuragi, 2016; Saito, Mori, Osaki, & Katsuragi, 2017; Yamanaka et al., 2016). Research showed that DAG activated enzymes involved in β-oxidation in the liver and small intestine (Murase et al., 2001; Murase, Aoki, Wakisaka, Hase, & Tokimitsu, 2002). In addition, α-linolenic acid, an abundant ω-3 fatty acid in linseed and perilla oil, was easier to be oxidized than palmitic, stearic, oleic or linoleic acid in rodent and human bodies

(Bessesen, Vensor, & Jackman, 2000; DeLany, Windhauser, Champagne, & Bray, 2000; Leyton, Drury, & Crawford, 1987). Therefore, α-linolenic acid-rich diacylglycerol is potentially useful to improve fat metabolism and utilization. In these respects, DAGs of α-linolenic acid have attracted very significant attention, motivating us to investigate their synthesis in more detail.

DAG can be produced via esterification, glycerolysis, and partial hydrolysis reactions. As biocatalyst for these reactions, the MAG and DAG lipases have attracted considerable attention due to few TAG generation during synthesis of DAG, which will help to further purify DAG. In this study, the lipase PCL from *Penicillieum camembertii* was chosen, which has a strong specificity toward MAG and DAG but not TAG. Lipase PCL has been intensively investigated by us and others for various applications such as synthesis of conjugated linoleic acid glycerides (Hernández-Martín, Hill, & Otero, 2009; Watanabe et al., 2002, 2004, 2005) or as catalyst for esterification and epoxidation reactions (Tang et al., 2016). Currently, no studies have specifically reported on the synthesis of α-linolenic acid-rich diacylglycerol using the lipase PCL.

Immobilization is an effective method to improve the performance and recovery of lipase for industrial application. A successful application was reported that monoglycerides can be efficiently synthesized by *Penicillium camembertii* lipase immobilized on epoxy SiO_2-PVA composite (Freitas, Paula, dos Santos, Zanin, & de Castro, 2010). However, immobilization of this lipase by physical absorption, a simple operation with low cost, and can keep high stability of the obtained enzymes (Fernandez-Lafuente, Armisén, Sabuquillo, Fernández-Lorente, & Guisán, 1998; Li et al., 2017), is rarely can be seen in the open literature.

In the present study, the crude lipase PCL was produced, and six types of resins were screened to immobilize PCL by physical absorption. Subsequently, the immobilized PCL was used for the synthesis of diacylglycerol by esterification of α-linolenic acid with glycerol. The effects of reaction conditions, including water content, enzyme loading, α-linolenic acid/glycerol ratio, and temperature were investigated. Molecular distillation was used to purify DAG from the products. Finally, the reusability of immobilized PCL was evaluated.

2 | MATERIALS AND METHODS

2.1 | Materials

The resin ECR1030, ECR8806 was kindly provided by Purolite Co., Ltd (Zhejiang, China). The resin (Amberlite XAD1180N) was obtained from Rohm and Haas (USA). The resin AB-8 was purchased from Chemical Plant of Nankai University (Tianjin, China), and DA201, D380 were from Zhengzhou Qinshi Technology Co., Ltd. (Henan, China). Bradford reagent was obtained from Shanghai BioScience & Technology Company (Shanghai, China). α-Linolenic acid was purchased from Aladin Reagent (Shanghai, China). *n*-Hexane, isopropanol and formic acid were of HPLC grade from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Acylglycerol standards of dioleoylglycerol (15% of 1,2-dioleoylglycerol and 85% of 1,3-dioleoylglycerol, CAS no. 25637-84-7), monooleoylglycerol (CAS no. 113 47885-U) were obtained from Sigma-Aldrich (China). All other chemicals were of analytical grade and used without further purification.

2.2 | Production of crude lipase PCL

Lipase PCL was produced according to the method of Tang et al. (2016). The obtained suspension was concentrated by a 10 kDa molecular mass membrane (Vivaflow 200, Sartorius, Germany). The hydrolytic activity of PCL was determined according to the method of Wang et al. (2012).

2.3 | Immobilization of lipase PCL by physical absorption

Before immobilization, the pretreatment processes of resins (not including of ECR1030 and ECR8806) were carried out according to the method of Wang et al. (2017). Immobilization of lipase PCL on each resin was carried out as follows: a given amount of resins was equilibrated with sodium phosphate buffer (0.02 M, pH 5.6). Subsequently, an equal volume of supernatant of PCL was added to conical flask and stirred by thermostatic air bath shaker at 30°C with a speed of 180 rpm for 8 hr. After that, the suspension was filtered out and the resins were washed with buffer until no protein was detected in the eluate. Finally, the immobilized PCL was dried in a vacuum desiccator at 30°C for 8 hr and stored at 4°C before use. Next, the immobilization conditions of lipase/support ratio (10, 15, 20, 25, 30, 35, and 40 mg/g resin) were varied for optimization.

2.4 | Determination of protein loading and activity of immobilized PCL

Protein loading of immobilized PCL was determined according to the Bradford method and a calibration curve using BSA as protein standard for protein quantification (Bradford, 1976). The esterification activities of immobilized PCL were determined according to Novozymes Standard Method EB-SM-1069.02 (Basso, Froment, Hesseler, & Serban, 2013). One unit of lipase (U) was defined as the amount (g) of immobilized PCL produced 1 μmol of propyl laurate per minute. Specific activity of immobilized PCL was expressed as units per mg protein (U/mg).

2.5 | Characterization of immobilized PCL by FT-IR and CLSM

Fourier transform infrared (FT-IR) analysis of immobilized PCL, free PC, L and ECR8806 resin was carried out referring to the method of Liu et al. (2012). The images of ECR8806 before and after immobilization of PCL was investigated using laser scanning confocal microscope (CLSM) according to the method of Li et al. (2017).

2.6 | Effects of pH and temperature on the activity of free and immobilized PCL

The effects of pH on the hydrolytic activity of free and immobilized PCL were measured at various pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0)

at 35°C. Effects of temperature on the activity of free and immobilized PCL were assessed at pH 6.0 and pH 6.5, respectively, at which the temperature was ranged from 30 to 50°C. The hydrolytic activities of free and immobilized PCL were determined according to the method of Wang et al. (2012). One unit of lipase is defined as the amount of enzyme that hydrolyzes DAG oil releasing 1 μmol fatty acid per minute under the assay conditions. The relative activity was obtained with the highest enzyme activity of 100%.

2.7 | Comparison of the catalytic ability of immobilized PCL during esterification of α-linolenic acid or ethyl linolenate with glycerol

The capability of α-linolenic acid and ethyl linolenate with glycerol by immobilized PCL in the synthesis of α-linolenic acid-rich diacylglycerol was compared under the following conditions: substrate molar ratio of α-linolenic acid or ethyl linolenate to glycerol of 1:4, temperature of 35°C, and enzyme loading of 5% (wt/wt, with respect to the total substrates). Samples were withdrawn at periodic intervals and prepared for HPLC analyses as described in section 2.11. The conversion rate at different reaction times was defined as the percentage of the reacted substrate (fatty acid or fatty acid ethyl ester) to the initial substrate.

2.8 | Synthesis of α-linolenic acid-rich diacylglycerol by immobilized PCL-catalyzed esterification in a solvent-free system

For the synthesis of α-linolenic acid-rich diacylglycerol, the reaction mixtures consisted of substrates (1:1, 1:2, 1:3, 1:4, 1:5, and 1:6) of molar ratio of α-linolenic acid to glycerol with 0, 1, 3, 5, 7, and 9% of different amount of water (wt/wt, with respect to the total substrates) in a conical flask. The reactions catalyzed by different amounts of immobilized PCL (2.5, 5, 7.5, 10, and 12.5%, wt/wt, with respect to the total substrates) were incubated at various temperature (30, 35, 40, 45, and 50°C) and agitated on magnetic stirrer at 200 rpm for 24 hr. Samples were withdrawn periodically to monitor the production of glycerides and their fatty acid profiles by HPLC and GC analysis, respectively. The conversion rate (namely, esterification degree in the reactions) at different reaction times was defined as the percentage of the reacted substrate (fatty acid) to the initial substrate.

2.9 | Purification of α-linolenic acid-rich diacylglycerol by molecular distillation

To obtain α-linolenic acid-rich diacylglycerol with high purity, the esterification reaction mixture obtained under the optimized conditions was further purified using molecular distillation (MD-S80 short path falling film distiller). The conditions of the purification made as follows: a feeding temperature of 60°C, an evaporating temperature of 170°C, a condenser temperature of 40°C, a pressure of 10 Pa, a feed flow rate of 1.5 mL/min, and a scraper speed of 250 rpm. The feed was divided into two fractions after molecular distillation: a large amount of DAG was acquired in the residues (final products) and MAG with FA from the reaction mixture were collected in the distillates. Samples were withdrawn and analyzed using HPLC and GC, respectively.

2.10 | Reusability of immobilized PCL

The reusability of immobilized PCL was evaluated by recovering and transferring the enzyme to a fresh substrate mixture. The reactions were carried out under the conditions of substrate molar ratio of 1:2 (α-linolenic acid to glycerol), and enzyme loading of 5% (wt/wt, with respect to the total substrates) at a temperature of 35°C for 24 hr. Then, the immobilized PCL was filtered out and washed with *n*-hexane for three times. Finally, the recovered enzyme was dried and added for the next cycle under the same reaction conditions. The activity of the first reaction was set as 100% and the activity in subsequent reactions was calculated accordingly.

2.11 | Analysis of the composition of the reaction mixture by HPLC and FA composition analysis by GC

The composition of the reaction mixture was analyzed by Normal-phase HPLC (NP-HPLC, refractive index detector) equipped with a Phenomenex Luna column (4.6 mm i.d. × 250 mm, 5 μm particle size, Phenomenex Corporation). The detailed analysis was carried out according to the method described by Wang et al. (2012). The mobile phase was a mixture of *n*-hexane, isopropanol with formic acid (21:1:0.003, vol/vol/vol) and its flow rate was 1 mL/min. Peaks in HPLC were identified by comparison of their retention times with those known standards. Waters 2695 integration software was used to calculate peak-areas percentages.

Before the analysis by GC, separation of different compositions in the reaction mixture was carried out using thin-layer chromatography method according to Li et al. (2016). Then, the scraped DAG, MAG, and the unreacted substrate (FA) were separately methylated to FAME according to the method described by Wang et al. (2010). Finally, the analysis of the FA composition was performed using GC (Agilent 7890A) equipped with a capillary column CP-Sil 88 (60 m × 0.25 mm × 0.2 μm) according to the method of Qin, Huang, Lan, Wang, and Yang (2014).

2.12 | Statistical analysis

All experiments were performed in triplicate and the data were presented as mean with the standard deviation. ANOVA ($p < .05$) was used to analyze the differences between the measured values.

3 | RESULTS AND DISCUSSION

3.1 | Production of crude lipase PCL

In this work, lipase PCL were overexpressed in recombinant *P. pastoris*. The cell suspension was centrifuged after 72 hr culture, then concentrated by a 10 kDa molecular mass membrane. The SDS-PAGE electrophoresis (Figure 1) showed the band of crude lipase PCL. The molecular weight was estimated to be about 35 kDa according to the

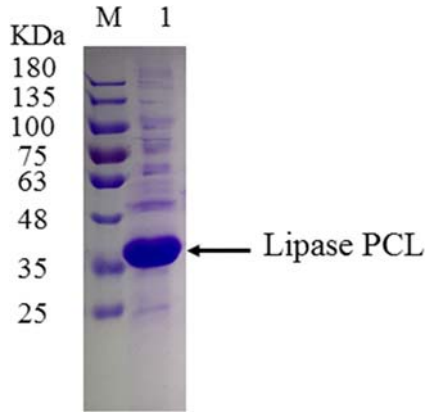

FIGURE 1 SDS-PAGE analysis for crude lipase PCL. Lane M, molecular mass standards indicated in kDa; lane 1, concentrated culture medium of lipase PCL

standard protein markers. The hydrolytic activity of lipase PCL was 315 U/mL.

3.2 | Immobilization of lipase PCL by physical absorption

To identify a suitable carrier material, a range of different supports were screened with respect to the activity of immobilized PCL (Table 1). While all resins evaluated could immobilize the enzyme in an active form, the performance of Purolite ECR8806 (octadecyl methacylate) was exceptional with an esterification activity of 459.98 U/g, and a specific activity of 23.40 U/mg. The higher activity of lipase immobilized on octadecyl resin is consistent with very recent studies (Steinhagen et al., 2016; Akanbi & Barrow, 2017). Therefore, lipase PCL immobilized on Purolite ECR8806 was used for all further experiments.

Next, the immobilization conditions of lipase PCL were investigated in some more details. The results of various ratios of enzyme to resin are shown in Figure 2. Up to a formal protein loading of 30 mg/g resin, efficient immobilization of lipase PCL was observed. The esterification activity increased significantly. Subsequently, it slowly decreased, which may be attributed to the formation of various layers

TABLE 1 Comparison of activities of lipase PCL immobilized by different resins

Resin	Protein loading (mg/g)	Esterification activity (U/g)	Specific activity (U/mg)
AB-8	17.44 ± 0.23	207.38 ± 3.01	11.89 ± 0.18
D380	5.52 ± 0.14	50.93 ± 0.88	9.23 ± 0.21
DA201	17.43 ± 0.11	152.50 ± 1.73	8.75 ± 0.34
XAD1180N	22.28 ± 0.22	249.54 ± 3.21	11.20 ± 0.38
ECR1030	24.10 ± 0.32	213.77 ± 1.08	8.87 ± 0.21
ECR8806	19.66 ± 0.38	459.98 ± 5.12	23.40 ± 0.52

Conditions for immobilization: lipase/support, 25 mg/g; phosphate buffer (pH 5.6), 1:1 (vol/vol liquid lipase); temperature, 30°C; absorption time, 8 hr.

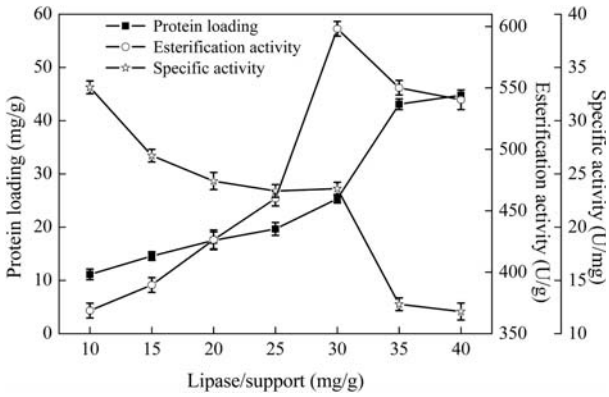

FIGURE 2 Effect of lipase/support ratio on the activity of immobilized PCL. Conditions for immobilization: lipase/support, 10, 15, 20, 25, 30, 35, 40 mg/g; phosphate buffer (pH 5.6), 1:1 (vol/vol liquid lipase); temperature, 30°C; absorption time, 8 hr

with the outer ones shielding the inner ones from substrate access and thereby rendering them inactive.

3.3 | Characterization of immobilized PCL by FT-IR and CLSM

The FT-IR characterization confirmed the successful absorption of the enzyme to the carrier material (Figure 3). The carbonyl functional groups of acrylates of 1,730 cm^{-1} and 1,738 cm^{-1} were observed in the spectra of ECR8806 and immobilized PCL, respectively. In addition, the amide group absorption band of free PCL appeared at 1,650 cm^{-1}, and the immobilized one appeared at 1,652 cm^{-1}, which indicated that the free PCL was successfully immobilized on the resin of ECR8806.

The successful immobilization was also confirmed by laser scanning confocal microscopy (Figure 4). After ECR8806 and immobilized PCL being stained with a fluorescent amine, it can be seen that the immobilized PCL displayed a clear fluorophore whereas ECR8806 did not. It is suggested that the free PCL is successfully absorbed on resin ECR8806. Because fluorescent amines cannot exhibit fluorescent until the proteins bound to them.

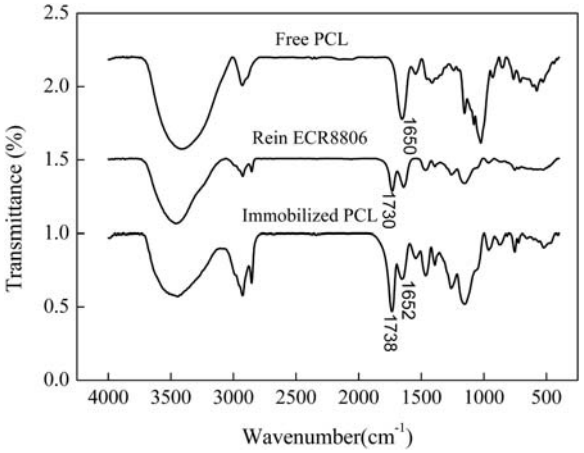

FIGURE 3 FT-IR spectra of free PCL, rein ECR8806 and immobilized PCL

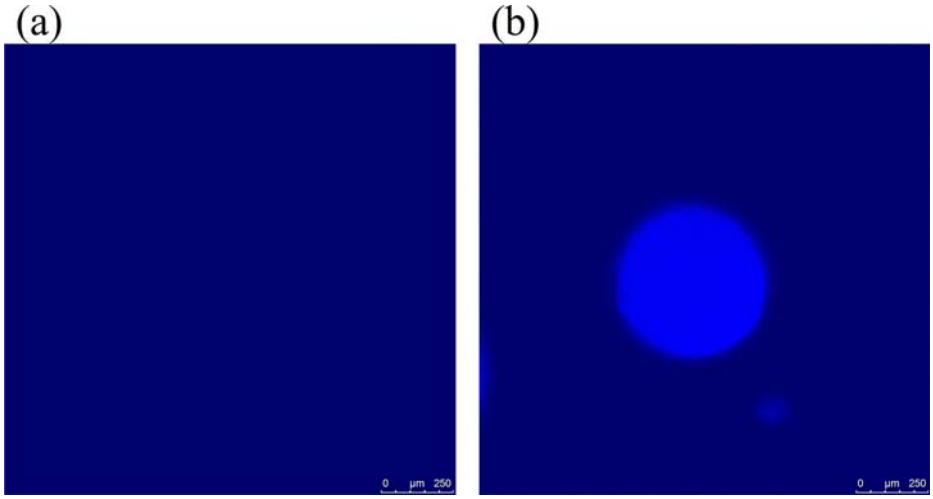

FIGURE 4 CLSM images of ECR8806 (a) and immobilized PCL (b)

3.4 | Effects of pH and temperature on the activity of free and immobilized PCL

The properties of the immobilized PCL were investigated under different conditions of pH and temperature. Compared to the free PCL, the pH profile (Figure 5a) of the immobilized PCL was somewhat shifted to more alkaline values (pH_{opt} being 6 and 6.5 for the free and the immobilized PCL, respectively). Thus, the tolerance of immobilized PCL in alkaline environment was improved to some extent, and it is observed that the activities of the enzyme were inactivated under the conditions of over acid/alkaline.

Next, the thermal robustness of the immobilized PCL in comparison with its free counterparts was studied (Figure 5b). Both forms of lipase PCL showed a steadily increasing hydrolytic activity until 40°C. Above this temperature, the free PCL rapidly decreased with only 20% of the maximal activity at 50°C. We attribute this result to thermal inactivation of the enzyme counteracting the expected increase in catalytic activity with temperature. Quite expectedly, immobilization very significantly increased the thermal stability of the enzyme leading to the observed lower decrease in catalytic activity at elevated temperatures.

3.5 | Comparison of the catalytic ability of immobilized PCL during esterification of α-linolenic acid or ethyl linolenate with glycerol

Having performed preliminary properties of the immobilized PCL, we advanced to evaluate it as catalyst for the desired esterification reaction. α-Linolenic acid and its ethyl ester as different acyl donors were compared (Figure 6).

Quite surprisingly, the reaction rates and conversions were by far better using the free fatty acid as acyl donor as compared to the use of the activated ethyl ester. One possible explanation of this observation may by that in case of the transesterification reaction ethanol accumulated in the reaction mixture (as compared to water in case of the esterification reaction). While water represents the natural solvent for lipase PCL, ethanol may negatively affect the structural integrity of lipase PCL by replacing water molecules on the surface of the enzyme.

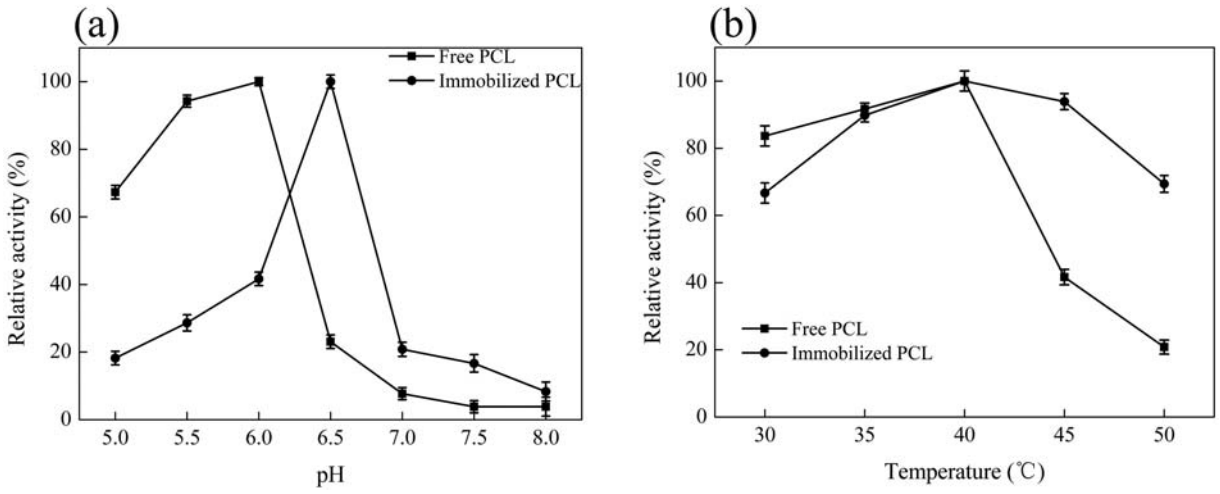

FIGURE 5 Effects of pH and temperature on the activity of free and immobilized PCL

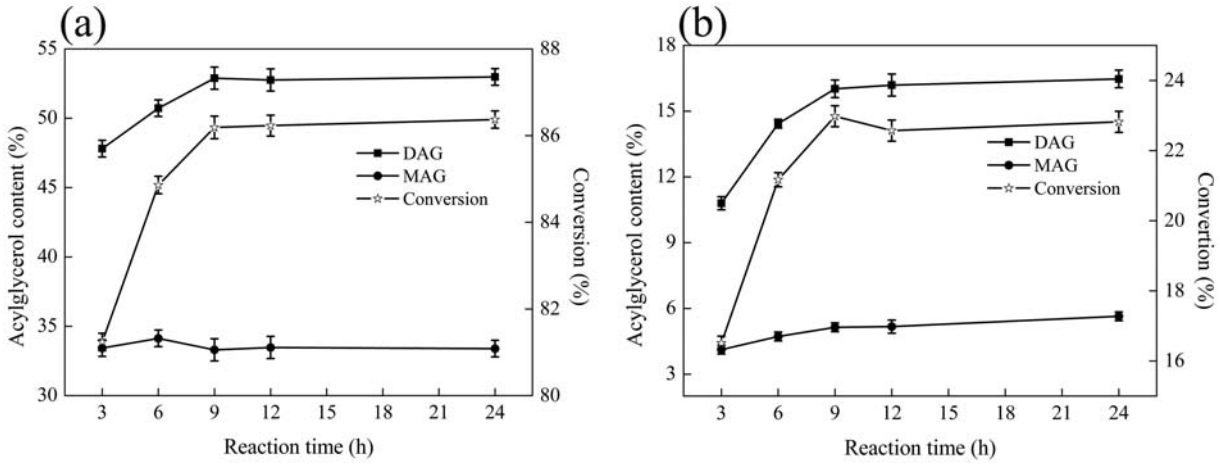

FIGURE 6 Comparison of the immobilized PCL-catalyzed synthesis of DAG and α-linolenic acid (a) or ethyl linolenate (b) as acyl donor. Reaction conditions: α-linolenic acid or ethyl linolenate/glycerol, 1:4 (mol/mol); immobilized PCL, 5% (wt/wt, with respect to the total substrates); temperature, 35°C

In addition, the reaction of the latter maybe a-two step for synthesis of DAG (firstly, ethyl linolenate transformed into α-linolenic acid, then the α-linolenic acid esterified with glycerol). Therefore, it is highly effective to synthesis of α-linolenic acid-rich DAG using α-linolenic acid as the substrate.

3.6 | Synthesis of α-linolenic acid-rich diacylglycerol by immobilized PCL-catalyzed esterification in a solvent-free system

3.6.1 | Effect of water content on the esterification reaction

The water content not only influences enzyme activity and stability but obviously influences the equilibrium of the esterification reaction. Interestingly, the effect of increasing the water content was less pronounced than expected (Figure 7a).

The immobilized PCL itself contains moisture content of 2%, keeping the water content in the reaction mixture to a minimum appeared to be the best case to attain high conversions. When the reaction reached equilibrium, the amount of water affected the esterification rate but did not significantly affect the ratio of DAG to MAG in the product. Further studies will be necessary to fully understand this observation.

3.6.2 | Effect of substrate molar ratio on the esterification reaction

The molar ratio of α-linolenic acid and glycerol also mildly influenced the equilibrium of the esterification reaction (Figure 7b). Increasing this value increased the conversion of α-linolenic acid from roughly 65% (1:1) to almost 90% (1:4). At higher glycerol loadings (1:6) the MAG content increased at the expense of DAG. This equilibrium shift in favor of the MAG was observed previously (Wang et al., 2014). In addition, the increased viscosity of the reaction mixture in the presence of high glycerol contents also effected the mass transfer efficiency of the reaction. Therefore, the molar ratio of α-linolenic acid to glycerol of 1:4 appeared optimal for the synthesis of DAG.

3.6.3 | Effect of enzyme loading on the esterification reaction

Figure 7c shows the effect of enzyme loading on the esterification reaction. Increasing the enzyme loading from 2.5% (wt/wt) to 5% (wt/wt), the content of DAG increased, whereas the content of MAG decreased. Meanwhile, the esterification degree increased slightly. Further increases of the enzyme loading, however, did not significantly influence the content of DAG. Moreover, the degree of esterification decreased when the amount of enzyme from 2.5% (wt/wt) to 5% (wt/wt) and did not vary with the continuous increase of enzyme loading. This is probably because that the enzyme reached saturation and a high enzyme loading would cause a decline of reaction efficiency (Liu et al., 2012). On account of this, enzyme loading of 5% (wt/wt) was selected as the optimal parameter for the subsequent experiments.

3.6.4 | Effect of temperature on the esterification reaction

Interestingly, the reaction temperature had only a minor effect on the lipase PCL-catalyzed esterification of α-linolenic acid with glycerol (Figure 7d). The esterification degree increased slowly with temperature and reached a maximum at 40°C. Subsequently, the esterification degree did not increase anymore when temperature was further increased from 40 to 50°C. However, the DAG content was increased slowly while MAG content decreased, which indicated that higher temperature may be benefit for the synthesis of DAG. Significantly, it can be seen that the immobilized PCL have a good thermal stability. Possibly this can be attributed to the restriction of the lipase flexible conformation, which due to multiple attachment point of enzyme on the support (Wang et al., 2014).

3.7 | Purification of α-linolenic acid-rich diacylglycerol by molecular distillation

Usually, molecular distillation is an effective method for separating acylglycerol mixtures. The esterification reaction mixtures mainly

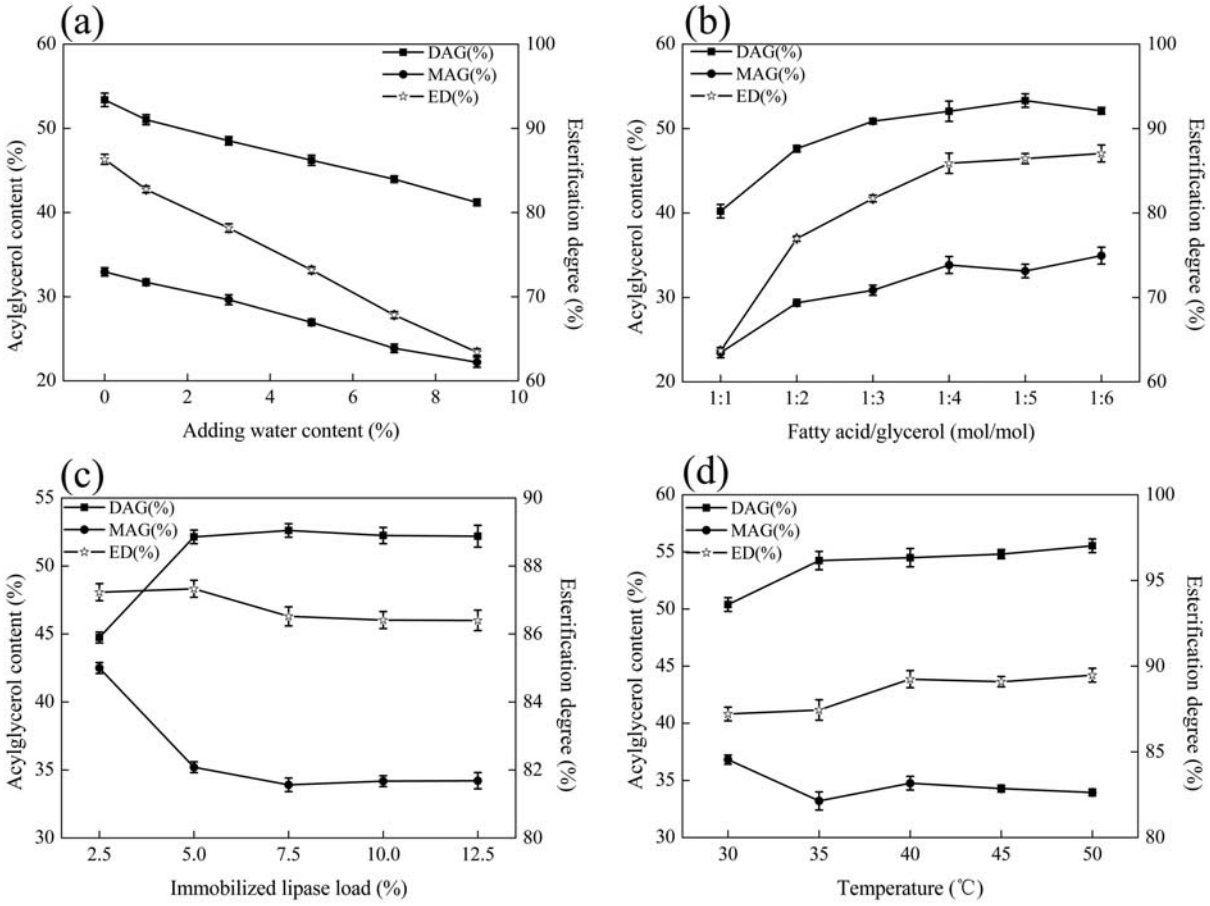

FIGURE 7 Synthesis of α-linolenic acid-rich diacylglycerol by immobilized PCL-catalyzed esterification in a solvent-free system. (a) Effect of water content on the esterification. Reaction conditions: α-linolenic acid/glycerol, 1:4 (mol/mol); immobilized PCL, 5% (wt/wt, with respect to the total substrates); water content, 0, 1, 3, 5, 7, and 9% (wt/wt, with respect to the total substrates); temperature, 35°C; time, 24 hr. (b) Effect of substrate molar ratio on the esterification. Reaction conditions: α-linolenic acid/glycerol, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6 (mol/mol); immobilized PCL, 5% (wt/wt, with respect to the total substrates); temperature, 35°C; time, 24 hr. (c) Effect of enzyme loading on the esterification. Reaction conditions: α-linolenic acid/glycerol, 1:4 (mol/mol); immobilized PCL, 2.5, 5, 7.5, 10, and 12.5% (wt/wt, with respect to the total substrates); temperature, 35°C; time, 24 hr. (d) Effect of temperature on the esterification. Reaction conditions: α-linolenic acid/glycerol, 1:4 (mol/mol); immobilized PCL, 5% (wt/wt, with respect to the total substrates); temperature, 30, 35, 40, 45, 50°C; time, 24 hr

contain DAG and MAG obtained by immobilized PCL under the optimized conditions. And the final products in the residues consisted of 99.28% DAG (67.76% 1,3-DAG, 31.52% 1,2-DAG) and 0.72% MAG with a short path falling film distiller. Table 2 shows the FA composition of the substrate (FA) and the final products (DAG). The FA composition of substrate mainly consisted of 78.00% α-linolenic acid, 14.43% linoleic acid, and 6.42% oleic acid. There were no significant differences in the FA compositions between the substrates and the final products. It could be concluded that the FA composition was not affected during the processes of molecular distillation.

TABLE 2 FA composition of the substrate (FA) and α-linolenic acid-rich DAG in the final products after molecular distillation

FA composition	Substrate α-linolenic acid-rich FA (%)	Product α-linolenic acid-rich DAG (%)
C16:0	0.26 ± 0.01	0.49 ± 0.01
C16:1	0.19 ± 0.04	0.19 ± 0.03
C18:0	0.09 ± 0.10	0.30 ± 0.10
C18:1 n9	6.42 ± 0.08	6.62 ± 0.21
C18:2 n6	14.43 ± 0.17	15.22 ± 0.12
C18:3 n3	78.00 ± 0.35	76.57 ± 0.41
Others	0.61 ± 0.41	0.61 ± 0.21

3.8 | Reusability of immobilized PCL

The reusability of the immobilized PCL was evaluated by measuring the esterification efficiency of immobilized PCL-catalyzed esterification of α-linolenic acid and glycerol and the results are shown in Figure 8. The immobilized PCL retained 91.60% of its initial activity after the tenth cycle, which indicates that immobilized PCL has a good operational stability in the esterification of α-linolenic acid with glycerol. Additionally, the immobilization method used in the present work was very simple

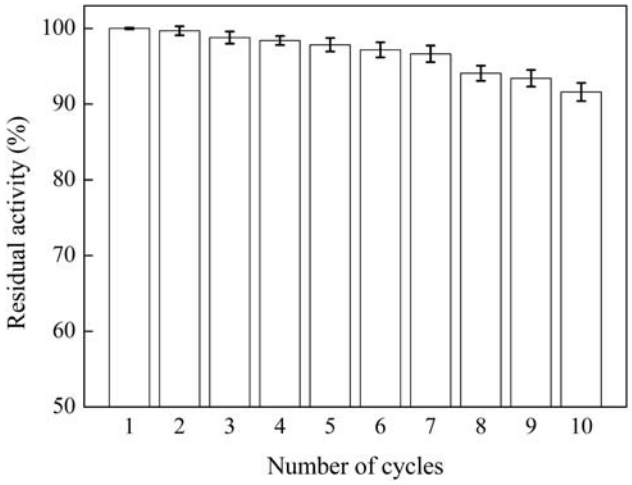

FIGURE 8 Operational stability of immobilized PCL during esterification of α-linolenic acid and glycerol. Reaction conditions: α-linolenic acid/glycerol, 1:2 (mol/mol); immobilized PCL, 5% (wt/wt, with respect to the total substrates); temperature, 35°C; time, 24 hr

and economic, so the immobilized PCL would have great potential in the industrial production of partial glycerides.

4 | CONCLUSIONS

The immobilized lipase prepared from PCL with Purolite ECR8806 as carrier was effective for the synthesis of α-linolenic acid-rich diacylglycerol. The immobilized PCL displayed a higher thermal stability than its free form. A high esterification degree (89.24%) with DAG content (54.49%) and MAG content (34.75%) was obtained under optimized conditions of immobilized PCL of 5% (wt/wt, with respect to total reactants), a fatty acid/glycerol molar ratio of 1:4, and at 40°C. The products were further purified by molecular distillation and the DAG purity reached to 99.28%. In addition, the immobilized PCL showed a good reusability during ten cycles of batch esterification of α-linolenic acid with glycerol. The results showed that immobilized PCL is a promising biocatalyst for the modification of oils and fats.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31501412, 31601398) and the Science and Technology Planning Project of Guangdong Province (2016B090920082), the Open Project Program of Beijing Key Laboratory of Flavor Chemistry, Beijing Technology and Business University (BTBU), Beijing 100048, China (SPFW-2017-YB01), and the National Science Fund for Distinguished Young Scholars (31725022).

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

ORCID

Yonghua Wang http://orcid.org/0000-0002-8790-4357

REFERENCES

Akanbi, T. O., & Barrow, C. J. (2017). Candida antarctica lipase A effectively concentrates DHA from fish and thraustochytrid oils. *Food Chemistry*, *229*, 509–516.

Ando, Y., Saito, S., Oishi, S., Yamanaka, N., Hibi, M., Osaki, N., & Katsuragi, Y. (2016). Alpha linolenic acid-enriched diacylglycerol enhances postprandial fat oxidation in healthy subjects: A randomized double-blind controlled trail. *Journal of Oleo Science*, *65*(8), 685–691.

Basso, A., Froment, L., Hesseler, M., & Serban, S. (2013). New highly robust divinyl benzene/acrylate polymer for immobilization of lipase CALB. *European Journal of Lipid Science and Technology*, *115*(4), 468–472.

Bessesen, D. H., Vensor, S. H., & Jackman, M. R. (2000). Trafficking of dietary oleic, linolenic, and stearic acids in fasted or fed lean rats. *American Journal of Physiology. Endocrinology and Metabolism*, *278*(6), E1124–E1132.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

DeLany, J. P., Windhauser, M. M., Champagne, C. M., & Bray, G. A. (2000). Differential oxidation of individual dietary fatty acids in humans. *The American Journal of Clinical Nutrition*, *72*(4), 905–911.

Fernandez-Lafuente, R., Armisén, P., Sabuquillo, P., Fernández-Lorente, G., & Guisán, J. M. (1998). Immobilization of lipases by selective adsorption on hydrophobic supports. *Chemistry and Physic of Lipids*, *93*(1–2), 185–197.

Freitas, L., Paula, A. V., dos Santos, J. C., Zanin, G. M., & de Castro, H. F. (2010). Enzymatic synthesis of monoglycerides by esterification reaction using *Penicillium camembertii* lipase immobilized on epoxy SiO_2-PVA composite. *Journal of Molecular Catalysis. B-Enzymatic*, *65*(1–4), 87–90.

Hernández-Martín, E., Hill, C. G., & Otero, C. (2009). Selective synthesis of diacylglycerols of conjugated linoleic acid. *Journal of the American Oil Chemists' Society*, *86*(5), 427–435.

Leyton, J., Drury, P. J., & Crawford, M. A. (1987). Differential oxidation of saturated and unsaturated fatty acids in vivo in the rat. *British Journal of Nutrition*, *57*(03), 383–393.

Li, D. M., Wang, W. F., Liu, P. Z., Xu, L., Faiza, M., Yang, B., … Wang, Y. H. (2017). Immobilization of *candida antarctica* lipase B onto ECR1030 resin and its application in the synthesis of n-3 PUFA-rich triacylglycerols. *European Journal of Lipid Science and Technology*, *119*(12), 1700266.

Li, D. M., Wang, W. F., Qin, X. L., Li, X. X., Yang, B., & Wang, Y. H. (2016). A novel process for the synthesis of highly pure n-3 polyunsaturated fatty acid (PUFA)-enriched triglycerides by combined transesterification and ethanolysis. *Journal of Agricultural and Food Chemistry*, *64*(34), 6533–6538.

Liu, N., Fu, M., Wang, Y., Zhao, Q. Z., Sun, W. Z., & Zhao, M. M. (2012). Immobilization of Lecitase® Ultra onto a novel polystyrene DA-201 resin: Characterization and biochemical properties. *Applied Biochemistry and Biotechnology*, *168*(5), 1108–1120.

Liu, N., Wang, Y., Zhao, Q. Z., Cui, C., Fu, M., & Zhao, M. M. (2012). Immobilisation of lecitase® ultra for production of diacylglycerols by glycerolysis of soybean oil. *Food Chemistry*, *134*(1), 301–307.

Murase, T., Aoki, M., Wakisaka, T., Hase, T., & Tokimitsu, I. (2002). Anti-obesity effect of dietary diacylglycerol in C57BL/6J mice: Dietary diacylglycerol stimulates intestinal lipid metabolism. *Journal of Lipid Research*, *43*, 1312–1319.

Murase, T., Mizuno, T., Omachi, T., Onizawa, K., Komine, Y., Kondo, H., … Tokimitsu, I. (2001). Dietary diacylglycerol suppresses high fat and

high sucrose diet-induced body fat accumulation in C57BL/6J mice. *Journal of Lipid Research*, *42*, 372–378.

Qin, X. L., Huang, H. H., Lan, D. M., Wang, Y. H., & Yang, B. (2014). Typoselectivity of crude *Geobacillus sp*. T1 lipase fused with a cellulose-binding domain and its use in the synthesis of structured lipids. *Journal of the American Oil Chemists' Society*, *91*(1), 55–62.

Saito, S., Fukuhara, I., Osaki, N., Nakamura, H., & Katsuragi, Y. (2016). Consumption of alpha-linolenic acid-enriched diacylglycerol reduces visceral fat area in overweight and obese subjects: A randomized, double-blind controlled, parallel-group designed trial. *Journal of Oleo Science*, *65*(7), 603–611.

Saito, S., Mori, A., Osaki, N., & Katsuragi, Y. (2017). Diacylglycerol enhances the effects of alpha-linolenic acid against visceral fat: A double-blind randomized controlled trial. *Obesity*, *25*(10), 1667–1675.

Steinhagen, M., Gräbner, A., Meyer, J., Horst, A. E. W., Drews, A., Holtmann, D., & Ansorge-Schumacher, M. B. (2016). Bridging the bridge—Stabilization of CalB against H_2O_2 and its application in epoxidation reactions. *Journal of Molecular Catalysis. B-Enzymatic*, *133*, S179–S187.

Tang, Q. Y., Popowicz, G. M., Wang, X. P., Liu, J. S., Pavlidis, L. V., & Wang, Y. H. (2016). Lipase-driven epoxidation is a two-stage synergistic process. *Chemistry Select*, *1*(4), 836–839.

Wang, W. F., Li, T., Qin, X. L., Ning, Z. X., Yang, B., & Wang, Y. H. (2012). Production of lipase SMG1 and its application in synthesizing diacylglycerol. *Journal of Molecular Catalysis. B-Enzymatic*, *77*, 87–91.

Wang, X. M., Li, D. M., Qu, M., Durrani, R., Yang, B., & Wang, Y. H. (2017). Immobilized MAS1 lipase showed high esterification activity in the production of triacylglycerols with n-3 polyunsaturated fatty acids. *Food Chemistry*, *216*, 260–267.

Wang, Y. H., Mai, Q. Y., Qin, X. L., Yang, B., Wang, Z. L., & Chen, H. T. (2010). Establishment of an evaluation model for human milk fat substitutes. *Journal of Agricultural and Food Chemistry*, *58*(1), 642–649.,

Wang, W. F., Xu, Y., Qin, X. L., Lan, D. M., Yang, B., & Wang, Y. H. (2014). Immobilization of lipase SMG1 and its application in synthesis of partial glycerides. *European Journal of Lipid Science and Technology*, *116*(8), 1063–1069.

Watanabe, Y., Shimada, Y., Yamauchi-Sato, Y., Kasai, M., Yamamoto, T., Tsutsumi, K., ... Sugihara, A. (2002). Synthesis of MAG of CLA with *Penicillium camembertii* lipase. *Journal of the American Oil Chemists' Society*, *79*(9), 891–896.

Watanabe, Y., Yamauchi-Sato, Y., Nagao, T., Negishi, S., Terai, T., Kobayashi, T., & Shimada, Y. (2005). Production of MAG of CLA by esterification with dehydration at ordinary temperature using *Penicillium camembertii* lipase. *Journal of the American Oil Chemists' Society*, *82*(9), 619–623.

Watanabe, Y., Yamauchi-Sato, Y., Nagao, T., Yamamoto, T., Ogita, K., & Shimada, Y. (2004). Production of monoacylglycerol of conjugated linoleic acid by esterification followed by dehydration at low temperature using *Penicillium camembertii* lipase. *Journal of Molecular Catalysis. B-Enzymatic*, *27*(4–6), 249–254.

Yamanaka, N., Saito, S., Osaki, N., Kamei, S., Nakamura, H., & Katsuragi, Y. (2016). Alpha-linolenic acid-enriched diacylglycerol oil suppresses the postprandial serum triglyceride level — a randomized, double-blind, placebo-controlled, crossover study. *Journal of Nutritional Science and Vitaminology*, *62*(6), 402–408.

How to cite this article: Liu N, Li D, Wang W, et al. Production and immobilization of lipase PCL and its application in synthesis of α-linolenic acid-rich diacylglycerol. *J Food Biochem*. 2018; 42:e12574. https://doi.org/10.1111/jfbc.12574