Immobilization of Lecitase® Ultra onto the Organic Modified SBA-15 for Soybean Oil Degumming

Wenyi Chen¹,²#, Maomao Kou²#, Lin Li¹, Bing Li¹, Jianrong Huang², Shudong Fan³, Li Xu⁴,⁵, and Nanjing Zhong²,³*

¹ Guangdong Province Key Laboratory for Green Processing of Natural Products and Product Safety, CHINA
² School of Food Science, Guangdong Pharmaceutical University, Zhongshan 528458, CHINA
³ Shandong Yuwang Ecological Food Co., Ltd, Yucheng 251200, CHINA
⁴ School of Chemistry and Chemical Engineering, Guangdong Pharmaceutical University, Zhongshan, 528458, CHINA
⁵ Guangdong Pharmaceutical University–University of Hong Kong Joint Biomedical Innovation Platform, Zhongshan, 528437, CHINA

Abstract: In this study, SBA-15 was functionalized by organic groups (-CH₃, -C₄H₉, -C₈H₁₇, -CH₂CH₂NH₂, -C₆H₅, et al.), and then Lecitase® Ultra (LU) was immobilized onto the modified SBA-15 for soybean oil degumming. The hydrolysis activity, degumming performance, reusability in degumming, and the composition of phospholipids in the gum, of the immobilized LU samples, were carefully studied. Hydrolysis activities over 1800 U/g were obtained from all the immobilized LU samples. The highest activity of up to 4554.17 U/g was observed from the 3-ureidopropyl group-modified SBA-15-supported LU. Most of the immobilized LU samples removed the phospholipids effectively from crude soybean oil (initial phosphorous content 314.23 mg/kg), with a residual phosphorus content of less than 10 mg/kg. The reusability of the immobilized LU samples in the degumming process was evaluated. No loss of activity was observed from the methyl and N-(2-aminomethyl)-3-aminopropyl group-modified SBA-15-supported LU samples after five cycles of reuse. In addition, 3-aminopropyl and 3-glycidoxypropyl group-modified SBA-15-supported LU samples retained over 90% of their initial activity; N-phenylaminomethyl and 1-isocyanatopropane group-functionalized SBA-15-supported LU samples retained approximately 80% of their initial activity. The phospholipids in the gum were analyzed. The n-octadecyl and N-(2-aminomethyl)-3-aminopropyl group-functionalized SBA-15-supported LU samples were selective for lysophosphatidylethanolamine (LPE) preparation, and LPE percentages up to 37.14 and 38.80% were obtained, respectively. The N-phenylaminomethyl group-modified SBA-15-supported LU showed selectivity toward lysophosphatidylcholine (LPC) production, with an LPC percentage of up to 38.5%.

Key words: enzyme degumming, Lecitase® Ultra, organic modification, phospholipids, SBA-15

1 Introduction

Soybean oil is well known for its high content of unsaturated fatty acids. It is widely used in the food industry, including in salad dressings, frozen foods, imitation dairy products, mayonnaise and baked goods¹. To obtain light-colored, transparent, bland-tasting and oxidative stable products, crude soybean oil must be refined to remove undesirable compounds. Degumming is the single most important step in the refining process; it removes phospholipids and mucilaginous gums. If phospholipids and galactolipids are present in oil, the Maillard reaction occurs when they are heated above the “caramelization” temperature of sugar. This unwanted reaction darkens oil and causes a burnt flavor. In addition, phospholipids present in the oil will also increase the amount of 1) acid and sodium hydroxide chemicals required in the caustic refining process, 2) filter aid, bleaching clays and silica adsorbents in the bleaching process, and 3) sodium methoxide or

Abbreviations: LU; Lecitase® Ultra, FT-IR; Fourier transform infrared, TGA; Thermogravimetric analysis, TFE-SEM; thermal field emission-scanning electron microscopy, PE; phosphatidylethanolamine, LPE; lysophosphatidylethanolamine, PC; phosphatidylcholine, LPC; lysophosphatidylcholine, PI; phosphatidylinositol, LPI; lysophosphatidylinositol, BHT; 2,6-Di-tert-butyl-4-methylphenol, NHP; non-hydratable phospholipids, PLA; phospholipase A₁, IE; immobilization efficiency, HRTEM; high-resolution transmission electron microscope, TAG; triacylglycerols

¹These authors contributed equally to this work.
*Correspondence to: Nanjing Zhong, School of Food Science, Guangdong Pharmaceutical University, Zhongshan 528458, CHINA
E-mail: adong473@163.com
Accepted January 21, 2022 (received for review November 7, 2021)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jocs
enzymes in the interesterification and biodiesel process\textsuperscript{2}. Therefore, phospholipids must be reduced to levels that meet specifications for food and industrial applications (namely, a phosphorous content of less than 10 mg/kg). Conventional degumming processes, such as water, ultrafiltration, and acid degumming processes, cannot guarantee that the phosphorus levels are lower than 10 mg/kg, which is required for physical refining\textsuperscript{3}. These techniques are not suitable for oils that contain high levels of nonhydratable phospholipids (NHPs). Normally, approximately 90\% of phospholipids in soybean oil arehydratable phospholipids, and they can be easily removed by water degumming: they absorb water, swell and become insoluble in the oil phase and are removed. The residual approximately 10\% of NHPs can also be effectively eliminated during the later chemical refining process\textsuperscript{4}. However, high levels of NHP are found in green and damaged soybean seeds. For example, NHP levels of up to 50\% have been observed in seriously damaged soybean seeds\textsuperscript{5}. Although NHP can be removed simultaneously with free fatty acids in the chemical refining process, a larger amount of chemicals must be used, leading to more oil loss, a high cost for wastewater treatment, and a decrease in some beneficial minor components (such as tocopherols and sterols)\textsuperscript{6}.

Enzymatic degumming has attracted attention in recent years and offers a green route and eco-friendly solution for industrial processes. The phospholipases employed in the enzymatic process hydrolyze the phospholipids and generate lysophospholipids, which are water-hydratable and therefore easier to remove\textsuperscript{7}. Enzymatic degumming techniques are advantageous over conventional processes in terms of mild reaction conditions, reduced acid and alkali use, less water consumption and higher oil yields\textsuperscript{8,9}. Nevertheless, free phospholipases are nonrecyclable, which increases industrial operation costs. Immobilization of phospholipases facilitates the separation of products and the recovery of phospholipases for reuse. Due to their reusability, stability and, in turn, lower operation costs, immobilized phospholipases have shown potential in enzymatic degumming\textsuperscript{10}. Immobilization of enzymes facilitated their repeated uses and reduced the operation costs. In addition, immobilization is also an efficient strategy for enzyme feature improvement. With proper design, enzyme features, including stability, activity, selectivity, specificity, and resistance to inhibitors and chemical reagents, could be improved efficiently after immobilization\textsuperscript{10–12}. Moreover, immobilization is also an efficient way to purify enzymes, especially for lipases\textsuperscript{13}. Lipases are very special enzymes, having a peculiar mechanism of interfacial activation. The structure of most lipases includes a mobile polypeptide chain, called lid. The lid has a hydrophobic face and a hydrophilic face. In the aqueous medium, the hydrophobic area of the lid interacts with the hydrophobic areas surrounding the active center, secluding it from the medium. And in this case, the lipase is in the closed form. While in the hydrophobic medium, the lid moves and the hydrophobic pocket and active center is exposed (the lipase in the open form), lipases become adsorbed on the hydrophobic surface. Therefore, lipases can attach insoluble drops of substrates (oils and fats)\textsuperscript{14}. Currently, strategies developed for enzyme immobilization include physical adsorption, covalent binding to activated polymers, entrapment and occlusion, and crosslinking\textsuperscript{14}. Among which, physical adsorption is expected to be potential, because the process is simple with low cost, and the physical adsorbed enzymes can retain high activity\textsuperscript{15,16}.

Of the phospholipases, phospholipase \textit{A\textsubscript{1}} (PLA\textsubscript{1}) hydrolyzes the ester bonds of phospholipids at the sn-1 position and produces 2-acyl-lysophospholipids and free fatty acids. PLA\textsubscript{1} has been successfully applied in vegetable oil degumming\textsuperscript{17–19}. The immobilization of PLA\textsubscript{1} for oil degumming has been studied recently. Yu\textit{et al.} found that\textsuperscript{20}, in a plant degumming trial, calcium alginate-chitosan-supported PLA\textsubscript{1} (PLA\textsubscript{1}-CAC) was able to reduce the residual phosphorus content to 9.7 mg/kg after 6.5 h of degumming. The recovery of soybean oil was 99.1\%, and the PLA\textsubscript{1}-CAC could be recycled at least 4 times. To enhance the recovery and isolation of immobilized enzyme particles from reaction systems, these authors employed magnetic Fe\textsubscript{3}O\textsubscript{4}/SiO\textsubscript{x}-g-P-(GMA) nanoparticles for PLA\textsubscript{1} immobilization\textsuperscript{21}. The results indicated that the obtained immobilized PLA\textsubscript{1} could retain more than 80\% of its initial activity after 10 cycles of soybean oil degumming at 55°C, and the residual phosphorus content was less than 10 mg/kg. More recently, these authors immobilized PLA\textsubscript{1} onto magnetic particles and found that magnetically immobilized PLA\textsubscript{1} possessed more than 80\% of its initial activity after 5 cycles of soybean oil degumming, and the residual phosphorus content was 9.8 mg/kg\textsuperscript{22}. Despite the progress that has been achieved, studies regarding phospholipase immobilization are still increasing.

Lecitase\textsuperscript{8} Ultra (LU) is a commercial chimeric enzyme, it is produced by the fusion of the genes of lipase from \textit{Thermomyces lanuginosus} and the PLA\textsubscript{1} from \textit{Fusarium oxysporum}. LU was first designed for oil degumming, and it was commercialized only as an enzyme solution by Novo Nordisk A/S\textsuperscript{23}.

SBA-15 is a typical mesoporous silicate; its average pore diameter is approximately 8 nm, and it is an ideal candidate for enzyme immobilization. Organic functionalization of SBA-15 was not a new idea; series of organic groups had been used to modify the SBA-15, and then the organic functionalized SBA-15 was used to support lipases for monoacylglycerols and diacylglycerols production in our previous studies\textsuperscript{24,25}. However, studies on the organic functionalized SBA-15 supported lipases/phospholipases for oil degumming have not been seen. In addition, activi-
Immobilization of Lecitase® Ultra for Oil Degumming

2 Materials and Methods

2.1 Materials and reagents

Mesoporous SBA-15 was purchased from Nanjing XFNANO Materials Tech Co., Ltd. (Nanjing, China). Crude soybean oil was kindly provided by Shandong Yuwang Ecological Food Co., Ltd. (Yucheng, China). Soybean lecithin was supplied by Shenzhen Siwei Tongchuan Trading Co., Ltd. LU solution with protein concentration at 10.04 μg/mL was obtained from Novozymes (Beijing, China). The activity of the LU solution was measured to be at 3581.67 ± 75.35 U/mL (through hydrolysis of soybean lecithin). Organosilane compounds of (3-aminopropyl)triethoxysilane (>98%), 1-[3-(trimethoxysilyl)propyl]urea (>94%), N-octadecyliethoxysilane (>85%), N-dodecyltrimethoxysilane (>98%) and 3-mercaptopropyltriethoxysilane (>98%) were respectively purchased from Sigma-Aldrich (Shanghai, China), Tokyo Chemical Industry Co., Ltd., Aike Reagent (Chengdu, China), Xiya Reagent (Shandong, China) and Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China); triethoxysilylethylpropylmethacrylate (>97%), 3-(trimethoxysilyl)propyl methacrylate (>97%), triethoxymethylsilane (>98%), N-[3-(trimethoxysilyl)propyl]ethylenediamine (>95%), 3-(trimethoxysilyl)propyl isocyanate (>95%), 3-glycidoxypropyltrimethoxysilane (>97%), triethoxyceryl silane (>97%), and trimethoxyphenylsilane (>95%) were purchased from Aladdin Reagents Co., Ltd. (Shanghai, China); in addition, hexadecyltrimethoxysilane, N-phenylaminomethyltriethoxysilane and N-butyltrimethoxysilane with purity of more than 95% were purchased from Gelest, Inc. (Shanghai, China). Glycerol with a purity of more than 99.0% was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Phospholipid standards of phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylinositol (PI) and lysophosphatidylinositol (LPI) were purchased from Sigma-Aldrich (Shanghai, China). 2,6-Di-tert-butyl-4-methylphenol (BHT) was from Aladdin Reagents Co., Ltd. (Shanghai, China). All other solvents and reagents were of analytical or chromatographic grade.

2.2 Surface modification of SBA-15

SBA-15 was organically functionalized through silanization according to our previously published procedure. Typically, 2 g of SBA-15 powder was dispersed in 60 mL dry methylbenzene in a 150-mL round-bottom flask, and the mixture was refluxed under a nitrogen atmosphere. Then, 10 mmol of silane coupling agent was added drop-wise into the dispersion, and the mixture was reacted at 95°C for 8 h. After the reaction, the mixture was filtered by centrifugation (10 min at 3000 rpm), and the modified SBA-15 was washed with ethanol (50 mL×3) and diethylene (50 mL×3). After that, the modified SBA-15 was dried under vacuum (pressure at 0.093 MPa) at 80°C for 6 h. The obtained organic group-modified SBA-15 was named R-SBA-15 (R represents the functional group).

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

The protein concentration of the commercial LU solution was measured by the Bradford assay. Immobilization of LU onto (R)-SBA-15 was carried out according to our previously published method. Briefly, the LU solution was dissolved in 40 mL phosphate buffer (25 mM, pH 6.0) with an LU concentration of 200.8 μg/mL. Then, 100 mg of SBA-15 was added to the solution and magnetically stirred at 25°C for 30 min. After that, the suspensions were filtered and washed with phosphate buffer. The supernatant and the washing solutions were collected for protein determination. Protein content measurement was carried out according to the Bradford method using bovine serum albumin as the standard. The immobilization efficiency (IE) was calculated according to the following equation:

\[
IE(\%) = \left( \frac{X_i - X_f}{X_i} \right) \times 100
\]

where \(X_i\) is the amount of initial protein in the enzyme suspension (mg) and \(X_f\) is the amount of unbound protein in the filtrate after immobilization (mg).

In addition, the amount of enzyme that was immobilized could be calculated, and enzyme loading (μg LU per mg of R-SBA-15) was used to indicate the loading capacity of LU onto R-SBA-15. Lipase loading was calculated based on the following equation:

\[
\text{Lipase loading (μg/mg)} = \left( \frac{c \times v \times IE}{m} \right)
\]

where \(c\) is the LU concentration (μg/mL), \(v\) is the volume of the LU solution (LU dissolved in phosphate buffer, mL), \(40\) mL in the present study), and \(m\) is the amount of support (mg; 100 mg in the present study).

The immobilized LU samples were dried in a vacuum oven (pressure at -0.093 MPa) at 30°C for 6 h and designated as R-SBA-15-LU. For example, the \(n\)-hexadecyl group-functionalized SBA-15-supported LU was labeled as Scheme 1a.
2.4 Characterization

Fourier transform infrared (FT-IR) spectra were obtained on a Nicolet iS5 FT-IR spectrophotometer (Thermo Scientific) in the 4000-400 cm$^{-1}$ wavenumber range using the standard KBr disk method. Thermogravimetric analysis (TGA) was performed using a TA SDT 650 thermogravimetric analyzer (TA Instruments, USA) in the range of 30-800°C under N$_2$ flow (heating rate of 10°C/min). The morphology was analyzed by thermal field emission-scanning electron microscopy (TFE-SEM) on a GeminiSEM500 and high-resolution transmission electron microscopy (HRTEM) on a JEM-2100F microscope.

2.5 Enzyme activity assay

The activity of the immobilized LU samples was assayed by hydrolysis of a deoiled soy lecithin emulsion, according to the method of Jiang et al. with some modifications.$^{19}$ One unit (U) of LU was defined as the amount of the immobilized LU or LU solution releasing 1 µmol of titratable free fatty acid per minute under the described conditions. The soybean lecithin mixture consisting of 4 g deoiled soy lecithin and 1 g polyvinyl alcohol was dissolved in 100 mL of phosphate buffer (25 mM, pH 5.0), and the substrate was then emulsified by stirring at 10,000 rpm for 10 min. The emulsion was maintained at 50°C, and then 1 mL of the free LU solution or 10 mg of the immobilized LU was added. After a 10 min reaction at 180 rpm, 10 mL of 95% ethanol was added to end the reaction, and the mixture was continuously titrated with 0.05 M NaOH solution for 10 min to maintain a constant pH. Blank experiments, without the addition of LU, were performed using the same procedures.

2.6 Enzymatic degumming process

Enzymatic degumming of crude soybean oil was performed according to the method of Jiang et al. with minor modifications.$^{19}$ Crude soybean oil samples (200 g) were heated to 80°C in a water bath, and 0.24 mL of the 45% citric acid solution was added and mixed at 10,000 rpm for 1 min. After that, the oil mixture was kept at 80°C and stirred at 500 rpm for 20 min. Then, the temperature was decreased to 50°C, 4 mL of distilled water was added, and the required amount of NaOH solution (4%, w/w) was added to adjust the mixture at pH 6.0. After that, immobilized LU (0.04%, based on soybean oil weight) was added, and the mixture was stirred at 180 rpm for 3 h. After the degumming reaction, the LU was inactivated by incubating at 95°C for 10 min, and the oil mixture was quickly centrifuged at 4000 rpm for 10 min. The supernatant was collected for phosphorus analysis. The crude gums precipitated at the bottom were collected for phospholipid analysis. The phosphorus content was analyzed according to the standard method.$^{28}$ The degumming rate was calculated according to the following equation:

\[
\text{Degumming rate} (\%) = \left( \frac{Y_1 - Y_2}{Y_1} \right) \times 100
\]

where $Y_1$: phosphorus content of crude oil; $Y_2$: phosphorus content of oil after degumming.
2.7 Phospholipid extraction and analysis by HPLC-ELSD

The phospholipids were extracted from the collected crude gums by chloroform and methanol solvent mixtures (2:1, v/v, containing 0.01% BHT). The amount of the solvent mixtures was tenfold the mass of the crude gums. After solvent addition, the whole mixture was homogenized by stirring at 10,956 × g (10,000 rpm) for 0.5 min and then left for 1 h. Afterward, the mixture was centrifuged at 1753 × g (4000 rpm) for 10 min, and the supernatant organic phase was gathered. Then, solvents in the gathered supernatant were evaporated through a rotary vacuum evaporator. The obtained concentrated phospholipids were purified by the addition of cold acetone. The phospholipids were washed with cold acetone 2-3 times and then freeze-dried for 16 h. Finally, the obtained phospholipids were stored at −20°C before HPLC analysis.

HPLC (LC-2010A HT, Shimadzu Corporation, Japan) equipped with an evaporative light scattering detector (ELSD-LT II, Shimadzu Corporation, Japan) was used to determine the phospholipid composition. The separation was performed in a Chromolith® Performer-ce-Si column (100 mm × 4.6 mm, 2 μm; Merck, Germany). Gradient elution was carried out by the mobile phases of A (isopropanol), B [n-hexane with 0.04% (v/v) trimethylamine] and C (13% acetic acid). The gradient was performed as follows: initial, 57% A, 40% B and 3% C; 0-12 min, changed to 50% A, 40% B and 10% C; 12-17 min, maintained 50% A, 40% B and 10% C; 17-17.1 min, changed to 57% A, 40% B and 3% C; 17.1-24 min, maintained 57% A, 40% B and 3% C. The flow rate of mobile phase was 1.5 mL/min, and the column temperature was kept constant at 30°C. The effluent was monitored by an ELSD detector with evaporator temperature at 70°C, and the flow rate of the nitrogen was controlled by the pressure of valve, which was constant at 320 kPa.

Prior to HPLC analysis, the phospholipid sample was dissolved in a chloroform/methanol mixture (2/1, v/v) and then filtered through a microfilter (0.22 μm). The injection volume was 10 μL, and all samples were determined in duplicate. Phospholipids were identified based on the peak time of the standard phospholipids (PC, LPC, PE, LPE, PI) and LPI. The compounds were quantified based on the calibration curves of the standards. The percentage of the phospholipid species was calculated according to the following equation:

\[
\text{Phospholipid percentage (wt%) } = \frac{m}{m_o} \times 100
\]

where \(m\) is the amount of the individual phospholipid species (mg) (for example, \(m_{PE}\) is the amount of PE), and \(m_o\) is the total amount of phospholipids (mg), including PE, LPE, PI, PC and LPC (that is, \(m = m_{PE} + m_{LPE} + m_{PI} + m_{PC} + m_{LPC}\)). The LPI content was not calculated because the LPI peak partially overlapped with unidentified compounds; therefore, it was not easy to obtain an accurate quantification.

2.8 Reusability of immobilized LU for oil degumming

The reusability of the immobilized LU was studied in five consecutive cycles under the following procedure: crude soybean oil samples (200 g) were heated to 80°C in a water bath, and 0.24 mL of the 45% citric acid solution was added and mixed at 10,000 rpm for 1 min. After that, the oil mixture was maintained at 80°C and stirred at 500 rpm for 20 min. Then, the temperature was decreased to 50°C, 4 mL distilled water was added, and the required amount of NaOH solution (4%, w/w) was added to adjust the mixture to pH 6.0. After that, immobilized LU (0.04%, based on soybean oil weight) was added, and the mixture was stirred at 180 rpm for 3 h. After the degumming reaction, the oil mixture was quickly centrifuged at 4000 rpm for 10 min. The supernatant was collected for phosphorus content determination. The immobilized LU samples were deposited with phospholipids at the bottom in gel form. The gel was washed with n-hexane, and the obtained immobilized LU samples were dried under vacuum for 6 h for the next run under the selected conditions. The relative activity of the immobilized LU was defined as the ratio of the degumming rate obtained from each cycle to the degumming rate obtained from the first cycle.

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

2.9 Statistical analysis

SPSS 13.0 statistical analysis software was used for data analysis by one-way ANOVA. Tukey’s test was used to detect the differences, and \(p < 0.05\) was considered significant.

3 Results and Discussion

3.1 Characterization

Samples of the parent SBA-15 and the N-phenylamino-methyl group-modified SBA-15 before and after LU immobilization were characterized by FT-IR, and the spectra are presented in Fig. 1a. There were three typical Si-O-Si peaks in all samples, respectively centered at 1080 cm⁻¹ (asymmetric stretching), 800 cm⁻¹ (symmetric stretching) and 460 cm⁻¹ (bending vibration); the three peaks were assigned to the condensed silica network. There was a broad envelope band located at approximately 3450 cm⁻¹, it was attributed to the O-H stretching vibration which was caused by physisorbed water and surface hydroxyls. In addition, the IR band near 1640 cm⁻¹ was ascribed to the O-H deformation vibration. Moreover, the band situated at approximately 970 cm⁻¹ was assigned to the bending vibration of the framework Si-OH group in SBA-15.

Peaks centered at 1500 and 1600 cm⁻¹ could be attribut-
ed to the C=C symmetrical stretching vibration in the benzene ring, indicating the successful functionalization of the N-phenylaminomethyl group. In addition, a decrease in the intensity of the bands located at 3450 and 970 cm⁻¹ was observed after N-phenylaminomethyl group modification and the subsequent LU immobilization, indicating that some of the silanol group had reacted with the N-phenylaminomethyl group through silanization.

Bands centered at 1472 cm⁻¹ were assigned to the CH₃ asymmetric in plane bending vibration from lipase molecules. In addition, bands at 1427 and 1577 cm⁻¹ could be attributed to the COO⁻ asymmetric in plane bending vibration from lipase molecules. This result suggested that the LU was successfully immobilized onto the supports.

TGA curves (Fig. 1b) further indicated the successful immobilization of lipase, and the 2.56% weight loss of the N-phenylaminomethyl-modified SBA-15 after LU immobilization further indicated the successful immobilization of lipase.
Immobilization of Lecitase® Ultra for Oil Degumming

J. Oleo Sci. 71, (5) 721-733 (2022)

The parent SBA-15, the n-(2-aminoethyl)-3-aminopropyl group-modified SBA-15 and its later LU immobilization, were also characterized by SEM and TEM, images were presented in Fig. 2. The SEM images (Figs. 2a, 2b and 2c) all exhibited wavy or short, rod-shaped morphologies. The uniform morphology indicated that the textural properties of the mesoporous SBA-15 were retained after organic group functionalization and subsequent LU immobilization. The TEM images (Figs. 2d, 2e and 2f) showed straight arrays of long-range regular mesopore channels. Straight arrays of mesoporous channels were reserved and could be observed clearly after organic functionalization and after LU immobilization. This result indicated that the ordered structure and the Si-O-Si network in SBA-15 were not disturbed by organic functionalization and the lipase immobilization process.

3.2 Enzymatic activity of the immobilized LU for deoiled soy lecithin hydrolysis

The enzymatic activity of the present immobilized LU samples was measured by hydrolysis of deoiled soy lecithin. As presented in Table 1, all samples exhibited considerable activity (over 1800 U/g). Parent SBA-15-supported LU(SBA-15-LU) exhibited hydrolysis activity at 1852.5 U/g. After organic modification, the immobilized LU samples presented hydrolysis activities ranging from approximately 2000 to 4000 U/g. Activities from 3000 to 4000 U/g were observed from n-hexadecyl, 3-aminopropyl, N-(2-aminoethyl)-3-aminopropyl, 3-glycidoxypropyl, and N-phenylaminomethyl group-modified SBA-15-supported LU (Schemes 1a, 1b, 1c, 1d and 1e). The highest activity of up to 4554.17 U/g was obtained from 3-ureidopropyl group-modified SBA-15-supported LU (Scheme 1f). The activities of the immobilized LU samples for the hydrolysis of oil were also studied in our previous study[25]. The highest activity of up to 4777.78 U/g was observed from propyl methacrylate group-modified SBA-15-supported LU (Scheme 1g), followed by the 3-aminopropyl and N-(2-aminoethyl)-3-aminopropyl group-modified SBA-15-supported LU (Schemes 1b and 1c), with activities of 3555.56 and 3444.44 U/g, respectively. The results indicated that the activities of hydrolysis of deoiled soy lecithin and hydrolysis of oil were different. This phenomenon was not strange; with different substrates, the activity varied. This has also been observed in the different activities between hydrolysis and glycerolysis[23,32]. Interestingly, Yu and coauthors found that magnetic Fe3O4/SiO2-g-P(GMA) nanoparticles supported by PLA displayed activity of up to 2066.67 U/g/activity for soybean lecithin hydrolysis)41. Li et al. reported that the activity of PLA for oil degumming was enhanced by bioimprinting and immobilization, and an activity of 1288 U/g was obtained in their study[31].

Table 1 also listed the IE, which reflects the bound enzyme protein percentage. As presented, IE over 50% was obtained from all the samples. In addition, IE over 90% was observed from 6 samples. It indicated that the present supports were capable of absorbing protein. Nevertheless, it should be stated out that, the use of protein is valid only if the enzyme is pure. Therefore, the IE cannot fully reflect the expressed activity[41]. For example, a final activity of 50% of the initial one may be because only 50% of the enzyme has been immobilized but the immobilized enzyme maintain intact its activity, or because 100% of the enzyme activity is immobilized but decrease the activity to 50% (and many other situations are also possible).

Modulation of enzymes by immobilization on different supports was reported in some studies. For example, Thermomyces lanuginosus lipase was modulated by adsorption on hydrophobic supports, and the enzymatic properties for ethanolysis of oil was improved in solvent-free systems[17]. In addition, the LU properties (stabilization and inversion of enantiospecificity) were modulated via immobilization on differently activated Immobead-350. And the catalytic properties of the obtained LU were greatly influenced by the supports[35].

3.3 Enzymatic degumming of crude soybean oil by the immobilized LU samples

The immobilized LU samples were then used for crude soybean oil degumming, and the results are listed in Table 2. Except for the n-butyl, n-dodecyl, n-hexadecyl, 3-ureidopropyl and phenyl group-functionalized SBA-15-supported LU samples (Schemes 1h, 1i, 1a, 1f and 1j), all other immobilized LU samples could remove the phospholipids effectively, with a residual phosphorus content of less than 10 mg/kg. Unexpectedly, the degumming performance of the immobilized LU samples was not related to their hydrolysis activity (hydrolysis of deoiled soy lecithin, not hydrolysis of triacylglycerols (TAG) oil). For example, the 3-ureidopropyl group-modified SBA-15-supported LU (Scheme 1f) exhibited the highest hydrolysis activity, while its degumming performance was unacceptable, with a residual phosphorus content of 12.25 mg/kg (entry 9). Moreover, the n-hexadecyl group-modified SBA-15-supported LU (Scheme 1a) also exhibited considerable hydrolysis activity (3702.50 U/g), while its degumming performance was quite poor, with a residual phosphorus content of up to 31.01 mg/kg (entry 5). The reasons for this could be due to the different substrates. The substrate for hydrolysis activity determination was deoiled soy lecithin, while in the oil degumming performance procedure, the substrates included both phospholipids (small amounts) and TAG oils (large amounts). Since LU can hydrolyze both phospholipids and TAG oils[36], some of the immobilized LU samples may prefer to hydrolyze large amounts of TAG.
oils, and thus, their degumming performance is poor.

Enzymatic degumming by immobilized PLA$_1$ has been studied by some authors. With magnetic nanoparticles or calcium alginate-chitosan as carriers, the immobilized PLA$_1$ samples have been reported to be able to reduce the phospholipids effectively, and a final phosphorous level of less than 10 mg/kg was obtained$^{20, 27, 30}$. In addition, immobilized bioimprinted PLA$_1$ was reported to be capable of reducing the phosphorous content from 406.9 to 7.3 mg/kg$^{39}$. Gelatin hydrogel-supported PLA$_1$ can decrease the phosphorous level to less than 10 mg/kg$^{4, 20, 37, 38}$.

Table 1 Immobilization of LU onto the (organic groups modified) SBA-15$^a$.

| Entry | Immobilized LU samples $^b$ | Activity (U/g) $^c$ | IE (%) $^d$ | Lipase (μg/mg) |
|-------|-----------------------------|---------------------|------------|----------------|
| 1     | SBA-15-LU                   | 1852.50±297.83      | 86.00±0.66 | 69.08±0.53     |
| 2     | $\text{H}_3\text{C}^{\text{SBA-15-LU}}$ | 2117.50±450.85      | 81.99±0.66 | 65.86±0.53     |
| 3     | $\text{C}_6\text{H}_4^{\text{SBA-15-LU}}$ | 2710.83±418.63      | 87.70±1.11 | 70.44±0.90     |
| 4     | $\text{C}_4\text{H}_6^{\text{SBA-15-LU}}$ | 2393.33±172.22      | 90.75±0.47 | 72.89±0.37     |
| 5     | $\text{C}_6\text{H}_5^{\text{SBA-15-LU}}$ | 2629.17±107.51      | 99.78±0.20 | 80.14±0.16     |
| 6     | $\text{C}_6\text{H}_5^{\text{SBA-15-LU}}$ | 3702.50±5.00        | 91.46±1.39 | 73.46±1.11     |
| 7     | $\text{C}_8\text{H}_5^{\text{SBA-15-LU}}$ | 2003.33±329.13      | 90.18±3.30 | 72.43±2.65     |
| 8     | $\text{H}_2\text{N}^{\text{SBA-15-LU}}$  | 3981.67±319.34      | 94.16±1.86 | 75.62±1.49     |
| 9     | $\text{H}_2\text{N}^{\text{SBA-15-LU}}$  | 3840.00±511.72      | 71.84±2.38 | 57.70±1.91     |
| 10    | $\text{H}_2\text{N}^{\text{SBA-15-LU}}$  | 4554.17±164.04      | 57.76±2.66 | 46.39±2.14     |
| 11    | $\text{HS}^{\text{SBA-15-LU}}$          | 2485.83±528.28      | 87.10±0.83 | 69.96±0.67     |
| 12    | $\text{O}^{\text{SBA-15-LU}}$           | 2006.67±683.68      | 84.07±1.29 | 67.52±1.03     |
| 13    | $\text{O}^{\text{SBA-15-LU}}$           | 2930.00±522.05      | 86.06±0.13 | 69.12±0.10     |
| 14    | $\text{O}^{\text{SBA-15-LU}}$           | 3181.67±170.98      | 80.22±1.38 | 64.43±1.11     |
| 15    | $\text{H}_2\text{C}^{\text{SBA-15-LU}}$ | 2973.33±199.44      | 82.59±0.86 | 66.34±0.69     |
| 16    | $\text{O}^{\text{SBA-15-LU}}$           | 2145.83±445.06      | 95.28±2.98 | 76.53±2.40     |
| 17    | $\text{H}_2\text{C}^{\text{SBA-15-LU}}$ | 3825.00±435.97      | 55.65±3.34 | 44.70±2.68     |

$^a$ Immobilization conditions: The LU solution was dissolved in 40 mL phosphate buffer (25 mM, pH 6.0) with LU concentration at 200.8 μg/mL. Then, 100 mg of SBA-15 was added into the solution and magnetically stirred at 25°C for 30 min. LU, Lecitase® Ultra. Note: Standard deviation values were calculated from triplicate experiments.

$^b$ R-SBA-15, the organic functionalized SBA-15, and R was the modified organic group.

$^c$ Hydrolysis activity of the immobilized LU. The conditions for the enzymatic hydrolysis of deoiled soy lecithin emulsion were: pH 5.0, reaction at 50°C for 10 min.

$^d$ Immobilization efficiency.
Immobilization of Lecitase® Ultra for Oil Degumming

Table 2 Enzymatic degumming of crude soybean oil by the immobilized LU$^a$.

| Entry | Immobilized LU samples $^b$ | Residual P (mg/kg) $^c$ | Degumming rate (%) $^d$ |
|-------|-----------------------------|------------------------|-------------------------|
| 1     | H$_2$C$_{12}$H$_{25}$-SBA-15-LU | 5.14 ± 1.49           | 95.69 ± 1.25           |
| 2     | C$_6$H$_{12}$SBA-15-LU          | 14.22 ± 5.27          | 88.06 ± 4.42           |
| 3     | C$_6$H$_{12}$-SBA-15-LU         | 4.94 ± 2.80           | 95.86 ± 2.35           |
| 4     | C$_{12}$H$_{25}$-SBA-15-LU      | 25.87 ± 6.92          | 78.28 ± 5.81           |
| 5     | C$_{12}$H$_{25}$-SBA-15-LU      | 31.01 ± 3.04          | 73.97 ± 2.55           |
| 6     | C$_{12}$H$_{25}$-SBA-15-LU      | 6.32 ± 5.34           | 94.70 ± 4.49           |
| 7     | H$_2$N$_{12}$-SBA-15-LU         | 6.32 ± 4.29           | 94.70 ± 3.60           |
| 8     | H$_2$N$_{12}$-SBA-15-LU         | 4.74 ± 2.58           | 96.02 ± 2.17           |
| 9     | H$_2$N$_{12}$-SBA-15-LU         | 12.25±4.53            | 89.72 ± 3.80           |
| 10    | H$_2$N$_{12}$-SBA-15-LU         | 8.89 ± 3.60           | 92.54 ± 3.03           |
| 11    | O$_{12}$C$_{12}$-SBA-15-LU      | 7.70 ± 3.60           | 93.53 ± 3.03           |
| 12    | SBA-15-LU                       | 9.28 ± 2.08           | 92.21 ± 1.75           |
| 13    | O$_{12}$O$_{12}$-SBA-15-LU      | 5.93 ± 2.72           | 95.03 ± 2.28           |
| 14    | H$_2$C$_{12}$O$_{12}$-SBA-15-LU | 8.30 ± 4.85           | 93.04 ± 4.07           |
| 15    | SBA-15-LU                       | 13.43 ± 1.90          | 78.28 ± 5.81           |
| 16    | SBA-15-LU                       | 2.57 ± 2.92           | 97.84 ± 2.45           |

$^a$ Degumming conditions: Crude soybean oil samples 200 g were heated to 80°C in a water bath, and 0.24 mL of the 45% citric acid solution was added and mixed at 10,000 rpm for 1 min. After that, the oil mixture was kept at 80°C and stirred at 500 rpm for 20 min. Then, the temperature was decreased to 50°C, and 4 mL distilled water was added, and required amount of NaOH solution (4%, w/w) was added to adjust the mixture at pH 6.0. After that, 0.04% immobilized LU (based on soybean oil weight) was added, and the mixture was stirred at 180 rpm for 3 h. After degumming reaction, the LU was inactivated by incubating at 95 ºC for 10 min, and the oil mixture was quickly centrifuged at 4000 rpm for 10 min. The supernatant was collected for phosphorus analysis. LU, Lecitase® Ultra. Note: Standard deviation values were calculated from triple experiments.

$^b$ R-SBA-15 represents the organic functionalized SBA-15 and R was the modified organic group; R-SBA-15-LU, LU immobilized onto the R-SBA-15.

$^c$ Residual P represents the residual phosphorus content.

$^d$ Degumming rate represents the amount of phosphorus removal, which was calculated according to Equation 3 in Materials and Methods.
phorous content from 400 mg/kg to 50-70 mg/kg, and a final phosphorous content of less than 5 mg/kg was obtained with a second enzymatic treatment after charcoal treatment and dewaxing.

3.4 Reusability of the immobilized LU samples in the oil degumming process

The reusability of the immobilized LU samples in the degumming process was studied, and the results are demonstrated in Fig. 3. Encouragingly, nearly no loss of activity was observed from methyl and N-(2-aminoethyl)-3-aminopropyl group-modified SBA-15-supported LU (Schemes 1k and 1c) after five cycles of reuse. In addition, 3-aminopropyl and 3-glycidoxypropyl group-modified SBA-15-supported LU (Schemes 1b and 1d) retained over 90% of their initial activity after five cycles of reuse. N-Phenylaminomethyl and 1-isocyanatopropane group-functionalized SBA-15-supported LU (Schemes 1e and 1l) retained approximately 80% of their initial activity. However, relatively poor reusability was observed from n-octadecyl group-modified SBA-15-supported LU (Scheme 1m), with 53.25% of its initial activity retained after five cycles of reuse. The results indicated that organic modification of the carriers was effective in improving the stability of the immobilized lipase and that quite a few of the studied immobilized LU samples have potential for crude oil degumming in practice.

Interestingly, calcium alginate-chitosan- and calcium alginate-gelatin-supported PLA1 was reported to be able to retain 80% of their initial activity after 4 cycles of reuse. In addition, magnetic nanoparticle-supported PLA1 can retain more than 80% of its initial activity after 10 cycles of reuse. Moreover, the gelatin hydrogel-supported PLA1 remains quite stable in a spinning basket bioreactor for rice bran oil degumming, with no activity loss observed after 6 cycles of reuse.

3.5 Composition analysis of the phospholipids in the gum

The phospholipids were analyzed by the HPLC-ELSD. HPLC chromatograms of the phospholipid standards and the phospholipid samples in the gum are presented in Figs. S1a and S1b, respectively. Calibration curves of the phospholipid standards are presented in Figs. S2a. The ELSD detector response was linear over the concentration range of 0.4 to 1.2 mg/mL for PE (Fig. S2a) and the range of 0.4 to 2.0 mg/mL for LPE, PC, LPC, and PI (Figs. S2b, S2c, S2d and S2e), as indicated by the high correlation coefficients ($R^2>0.99$).

Table 3 lists the composition of the phospholipids in the gum. No lysophospholipids (LPE, LPC or LPI) were detected from the water degumming process (entry 1) since water degumming cannot remove the attached fatty acids in the phospholipid molecules. On the other hand, phospholipases can cleave fatty acids from phospholipid molecules through hydrolysis; therefore, lysophospholipids of LPC and LPE were determined from the enzymatic degumming process (entries 2 to 10). LU is related to PLA1, which removes the fatty acid attached to the phospholipids in the sn-1 position and produces 2-acyl-lysophospholipids. Interestingly, the present organic modification of SBA-15 seemed to afford some selectivity toward lysophospholipids for the supported LU samples. The n-octadecyl and N-(2-aminoethyl)-3-aminopropyl group-functionalized SBA-15-supported LU samples are selective for LPE preparation, with LPE percentages up to 37.14 and 38.80% obtained (entries 5 and 7). The N-phenylaminomethyl group-modified SBA-15-supported LU showed selectivity toward LPC production, with an LPC percentage up to 38.5% obtained (entry 10). In addition, over 60% for LPE and LPC (LPE + LPC) was obtained from the three mentioned immobilized LU samples (entries 5, 7 and 10), indicating that there was potential for lysophospholipid production.

Fig. 3   Reusability of the immobilized LU samples. LU, Lecitase Ultra; R-SBA-15-LU, LU immobilized on the organic functionalized SBA-15, and the R was the modified organic group; SBA-15-LU, LU immobilized on the parent SBA-15.

J. Oleo Sci. 71, (5) 721-733 (2022)
4 Conclusions

The present immobilized LU samples exhibited considerable activities, with over 1800 U/g observed for all the samples. Most of the immobilized LU samples could effectively remove phospholipids from crude soybean oil, with a residual phosphorus content of less than 10 mg/kg. In addition, their operational stability was good after five cycles of reuse: methyl and N-[(2-aminooethyl)-3-aminopropyl group-modified SBA-15-supported LU samples maintained their activity (no activity loss); 3-aminopropyl and 3-glycidyloxypropyl group-modified SBA-15-supported LU samples retained over 90% of their initial activity; N-phenylamino-
methyl and 1-isocyanatopropane group-functionalized SBA-15-supported LU samples retained approximately 80% of their initial activity. Therefore, they have potential for crude oil degumming in practice.

In addition, they also have potential for lysophospholipid production. The n-octadecyl and N-(2-aminooethyl)-3-aminopropyl group-functionalized SBA-15-supported LU samples were selective for LPE preparation; the N-phenylaminomethyl group-modified SBA-15-supported LU showed selectivity toward LPC production. In addition, over 60% of LPE and LPC (LPE + LPC) was obtained from the three mentioned immobilized LU samples.
Author Contributions
Wenyi Chen, performed research and analyzed data; Maomao Kou, performed research and analyzed data; Lin Li, methodology and contributed analytic tools; Bing Li, methodology and contributed analytic tools; Jianrong Huang, analyzed data; Shudong Fan, resources; Li Xu, methodology and analyzed data; Nanjing Zhong, conceptualization, designed research, methodology and wrote the manuscript.

Acknowledgements
This work was supported by the National Natural Science Foundation of China (31772000), the Open Project Fund from Guangdong Province Key Laboratory for Green Processing of Natural Products and Product Safety (KL-2018-13), the Key Projects of Social Welfare and Basic Research of Zhongshan City (2021B2007) and the Science and Technology Planning Project of Guangzhou (No. 202002030089).

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

Supporting Information
This material is available free of charge via the Internet at doi: 10.5650/jos.ess21353

References
1) More, N.S.; Gogate, P.R. Intensified degumming of crude soybean oil using cavitation reactors. J. Food Eng. 218, 33-43 (2018).
2) Dayton, C.L.G.; Galhardo, F. Enzymatic degumming in Green Vegetable Oil Processing (Farr, W.E.; Proctor, A. eds.), Academic press and AOCS press (2013). https://doi.org/10.1016/C2015-0-04080-1.
3) Jiang, X.; Chang, M.; Jin, Q.; Wang, X. Application of phospholipase A and Phospholipase C in the degumming process of different kinds of crude oils. Process Biochem. 50, 432-437 (2015).
4) Yu, D.; Ma, Y.; Xue, S.J.; Jiang, L.; Shi, J. Characterization of immobilized phospholipase A on magnetic nanoparticles for oil degumming application. LWT-Food Sci. Technol. 50, 519-525 (2013).
5) Rossell, J.B.; Pritchard, J.L.R. Analysis of oilseeds, fats, and fatty foods in London. London: Elsevier Applied Science (1991).
6) Jiang, F.; Wang, J.; Ju, L.; Kaleem, I.; Dai, D.; Li, C. Optimization of degumming process for soybean oil by phospholipase B. J. Chem. Technol. Biotechnol. 86, 1081-1087 (2011).
7) Germinati, S.; Paoletti, L.; Aguirre, A.; Periú, S.; Menez, H.G.; Castelli, M.E. Industrial uses of phospholipases: Current state and future applications. Appl. Microbiol. Biot. 103, 2571-2582 (2019).
8) Jiang, F.; Wang, J.; Kaleem, I.; Dai, D.; Zhou, X.; Li, C. Degumming of vegetable oils by a novel phospholipase B from Pseudomonas fluorescens BIT-18. Bioresour. Technol. 102, 8052-8056 (2011).
9) Qu, Y.; Sun, L.; Li, X.; Zhou, S.; Zhang, Q. Enzymatic degumming of soybean oil with magnetic immobilized phospholipase A. LWT-Food Sci. Technol. 73, 290-295 (2016).
10) Arana-Peña, S.; Rios, N.S.; Carballares, D.; Gonçalves, L.R.B.; Fernandez-Lafuente, R. Immobilization of lipases via interfacial activation on hydrophobic supports: Production of biocatalysts libraries by altering the immobilization conditions. Catal. Today 362, 130-140 (2021).
11) Silva, E.A.; Moreno-Perez, S.; Baso, A.; Serban, S.; Pestana-Mamede, R. et al. Biocatalyst engineering of Thermomyces lanuginosus lipase adsorbed on hydrophobic supports: Modulation of enzyme properties for ethanalysis of oil in solvent-free systems. J. Biotechnol. 289, 126-134 (2019).
12) Arana-Peña, S.; Rios, N.S.; Carballares, D.; Mendez-Sanchez, C.; Loka, Y. et al. Effects of enzyme loading and immobilization conditions on the catalytic features of lipase from Pseudomonas fluorescens immobilized on octyl-agarose beads. Front. Bioeng. Biotech. 8, 1-13 (2020).
13) Rodrigues, R.C.; Virgen-Ortiz, J.J.; dos Santos, J.C.S.; Berenguer-Murcia, A.; Alcantara, A.R. et al. Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions. Biotechnol. Adv. 37, 746-770 (2019).
14) Bahaki, M.; Yousefi, M.; Habibi, Z.; Mohammadi, M.; Yousefi, P. et al. Enzymatic production of biodiesel using lipases immobilized on silica nanoparticles as highly reusable biocatalysts: Effect of water, t-butanol and blue silica gel contents. Renew. Energ. 91, 196-206 (2016).
15) He, J.; Song, Z.; Ma, H.; Yang, L.; Guo, C. Formation of a mesoporous bioreactor based on SBA-15 and porcine pancreatic lipase by chemical modification following the uptake of enzymes. J. Mater. Chem. 16, 4307-4315 (2006).
16) Gao, S.; Wang, Y.; Yang, G.; Liu, G.; Dai, Y. Immobilization of lipase on methyl-modified silica aerogels by physical adsorption. Bioresour. Technol. 100, 996-999 (2009).
Immobilization of Lecitase® Ultra for Oil Degumming

17) Manjula, S.; Jose, A.; Divakar, S.; Subramanian, R. Degumming rice bran oil using phospholipase-A1, *Eur. J. Lipid Sci. Tech.* 113, 658-664 (2011).

18) Yang, B.; Zhou, R.; Yang, J.; Wang, Y.; Wang, W. Insight into the enzymatic degumming process of soybean oil. *J. Am. Oil Chem. Soc.* 85, 421-425 (2008).

19) Jiang, X.; Chang, M.; Wang, X.; Jin, Q.; Wang, X. A comparative study of phospholipase A1 and phospholipase C on soybean oil degumming. *J. Am. Oil Chem. Soc.* 91, 2125-2134 (2014).

20) Yu, D.; Jiang, L.; Li, Z.; Shi, J.; Xue, J.; Kakuda, Y. Immobilization of phospholipase A1 and its application in soybean oil degumming. *J. Am. Oil Chem. Soc.* 89, 649-656 (2012).

21) Virgen-Ortíz, J.J.; dos Santos, J.C.S.; Ortiz, C.; Berenguer-Murcia, Á.; Barbosa, O. et al. Lecitase ultra: A phosphatide content. *Lipid Sci. Tech.* 85, 421-425 (2008).

22) Zhao, X.; Zhao, F.; Zhong, N. Production of diacylglycerols through glycerolysis with SBA-15 supported Thermomyces lanuginosus lipase as catalyst. *J. Sci. Food Agric.* 100, 1426-1435 (2020).

23) Zhong, N.; Chen, W.; Liu, L.; Chen, H. Immobilization of *Rhizomucor miehei* lipase onto the organic functionalized SBA-15: Their enzymatic properties and glycerolysis efficiencies for diacylglycerols production. *Food Chem.* 271, 739-746 (2019).

24) Arica, M.Y.; Soydogan, H.; Bayramoglu, G. Reversible immobilization of *Candida rugosa* lipase on fibrous polymer grafted and sulfonated p(HEMA/EGDMA) beads. *Bioproc. Biosyst. Eng.* 33, 227-236 (2010).

25) Kou, M.; Feng, S.; Zhong, N. Immobilization of Lecitase® Ultra onto the amino-functionalized SBA-15 and their applications in glycerolysis. *J. Oleo Sci.* 69, 347-358 (2020).

26) Miletić, N., Vuković, Z., Nastasović, A.,Loos, K. Effect of *Candida antarctica* lipase B immobilization on the porous structure of the carrier. *Macromol. Biosci.* 11, 1537-1543 (2011).

27) Wang, X.; He, L.; Huang, J.; Zhong, N. Immobilization of lipases onto the halogen & haloalkanes modified SBA-15: Enzymatic activity and glycerolysis performance study. *Int. J. Biol. Macromol.* 169, 239-250 (2021).

28) GB/T 5537-2008 Inspection of grain and oils-Determination of phosphatide content.

29) Xie, W.; Hu, L.; Yang, X. Basic ionic liquid supported on mesoporous SBA-15 silica as an efficient heterogeneous catalyst for biodiesel production. *Ind. Eng. Chem. Res.* 54, 1505-1512 (2015).

30) Phan, N.T.; Jones, C.W. Highly accessible catalytic sites on recyclable organosilane-functionalized magnetic nanoparticles: An alternative to functionalized porous silica catalysts. *J. Mol. Catal. A* 253, 123-131 (2006).

31) Barth, A. Infrared spectroscopy of proteins. *Biochim. Biophys. Acta* 1767, 1073-1101 (2007).

32) Li, Y.; Zhong, N.; Cheong, L.Z.; Huang, J.; Chen, H.; Lin, S. Immobilization of *Candida antarctica* Lipase B onto organically-modified SBA-15 for efficient production of soybean-based mono and diacylglycerols. *Int. J. Biol. Macromol.* 120, 886-895 (2018).

33) Li, Z.; Liu, H.; Zhao, G.; Wang, P.; Wang, L. et al. Enhancing the performance of a phospholipase A1 for oil degumming by bio-imprinting and immobilization. *J. Mol. Catal. B-Enzym.* 123, 122-131 (2016).

34) Bondrant, J.; Woodley, J.M.; Fernandez-Lafuente, R. Parameters necessary to define an immobilized enzyme preparation. *Process Biochem.* 90, 66-80 (2020).

35) Pinheiro, M.P.; Monteiro, R.R.C.; Silva, F.F.M.; Lemos, T.L.G.; Fernandez-Lafuente, R. et al. Modulation of Lecitase properties via immobilization on differently activated Immobead-350: Stabilization and inversion of enantiospecificity. *Process Biochem.* 87, 128-137 (2019).

36) Wang, Y.; Zhao, M.; Ou, S.; Xie, L.; Tang, S. Preparation of a diacylglycerol-enriched soybean oil by phospholipase A1 catalyzed hydrolysis. *J. Mol. Catal. B* 56, 165-172 (2009).

37) Yu, D.; Wang, Y.; Yu, B.; Wang, T.; Zhang, R. et al. Numerical simulation and application of nanomagnetic enzyme in a liquid-solid magnetic fluidized bed. *Process Biochem.* 75, 121-129 (2018).

38) Yu, D.; Ma, Y.; Jiang, L.; Elfalleh, W.; Shi, M.; Hu, L. Optimization of magnetic immobilized phospholipase A1 degumming process for soybean oil using response surface methodology. *Eur. Food Res. Technol.* 237, 811-817 (2013).

39) Sheelu, G.; Kavitha, G.; Fadnavis, N.W. Efficient immobilization of Lecitase in gelatin hydrogel and degumming of rice bran oil using a spinning basket reactor. *J. Am. Oil Chem. Soc.* 85, 739-748 (2008).

CC BY 4.0 (Attribution 4.0 International). This license allows users to share and adapt an article, even commercially, as long as appropriate credit is given. That is, this license lets others copy, distribute, remix, and build upon the Article, even commercially, provided the original source and Authors are credited.

733

*J. Oleo Sci.* 71, (5) 721-733 (2022)