Immobilized lipase on core–shell structured Fe₃O₄–MCM-41 nanocomposites as a magnetically recyclable biocatalyst for interesterification of soybean oil and lard

Wenlei Xie*, Xuezhen Zang

School of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou 450001, PR China

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A core–shell structured Fe₃O₄–MCM-41 nanocomposite was prepared by means of a surfactant-directed sol–gel process. Candida rugosa lipase was then bound to the magnetic core–shell material by using glutaraldehyde as a cross-linking reagent. The as-prepared Fe₃O₄–MCM-41 support and the immobilized lipase were characterized in detail using enzyme activity assays, TEM, XRD, FTIR, VSM and nitrogen adsorption–desorption techniques. Results showed that the magnetite nanoparticles were coated with the MCM-41 silica with the formation of core–shell structured materials, and the lipase was successfully immobilized on the core–shell structured support. The catalytic performance of the bound lipase was tested in the interesterification of lard and soybean oil. It was shown that the immobilized lipase had a better catalytic activity towards the interesterification reaction. The slip melting point of the final product was lower than that of the original blend, and the interesterification led to an obvious variation in the microstructure of the product.

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1. Introduction

Most native vegetable oils have limited applications in their original forms. In order to enhance the physicochemical characteristics of vegetable oils, chemical modification can be employed to produce new oil products with special functions or usability (Lee, Akoh, Himmelsbach, & Lee, 2008; Xu, 2000). Industrially, plastic fats (such as shortening and margarine) are commonly prepared by partial hydrogenation of vegetable oils (Philippaerts et al., 2011). The hydrogenated product is a semi-solid fat with good shortening, tractility and plasticity property. Unfortunately, the hydrogenation process can yield high contents of trans fatty acids (FAs) in the end products, which are known to have detrimental effects on human health. It has been reported that trans FAs are able to raise low-density lipoprotein (LDL) and lower high-density lipoprotein (HDL) levels in human blood, thereby leading to several health problems, such as increased risk factors of coronary heart disease (CHD) (Dhaka, Gulia, Ahlawat, & Khatkar, 2011). Recently, interesterification, which can change the distribution of FAs on the glycerol backbone of triacylglycerol (TAG) molecules, has gained considerable attention as a promising alternative approach to modify the physicochemical properties of vegetable oils in the food industry, since no trans FAs are produced during the interesterification processes (Costales-Rodríguez, Gibon, Verhé, & De Greyt, 2009; Ferrari, Esteves, & Mukherjee, 1997). As a result, the interesterification process has been considered to be a feasible way for production of trans-free modified fats with appropriate crystallization behavior, satisfactory melting features, and a desirable texture (Ribeiro, Grimaldi, Gioielli, & Gonçalves, 2009; Soares et al., 2009).

Generally, the interesterification reaction can be conducted either chemically or enzymatically. The alkaline catalysts, such as sodium alkoxide and sodium hydroxide, are traditionally applied as homogeneous catalysts for the interesterification of vegetable oils (Rousseau, Marangoni, & Jeffrey, 1998). However, they are technically difficult to be separated from the product, and the undesirable wastewater is inevitably produced in the downstream purification processes. As compared to the chemical catalysts, a lipase catalyst has many merits including milder reaction conditions, selectivity and ease of product recovery (Meng, Liu, Shan, Jin, & Wang, 2010; Xu et al., 1998). However, the high prices of commercial lipases, together with their lack of long-term operational stability, and difficulty in recycling and reusing, are the major hurdles to their wide applications for the interesterification reaction in the food industry (Yang, Fruekilde, & Xu, 2003).

Considerable research endeavors have been made to circumvent the aforementioned problems associated with the utilization

* Corresponding author.

E-mail address: xwenlei@163.com (W. Xie).

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of free lipases. The immobilization of lipase onto a suitable support is a commonly utilized strategy to enhance the operational stability of commercially available biocatalysts (Jeyarani & Reddy, 2010; Pacheco, Palla, Capriste, & Carrin, 2013). The immobilized lipase can be easily separated from the reaction mixture at the end of the reaction and can be reused. In the past decade, various solid matrices employed for the immobilization of enzyme have been used. Among them, mesoporous silica materials have received significant interest due to their extraordinary properties, such as the controlled pore size, high surface area, low mass transfer limitation, and in particular sufficient silanol groups for surface modification (Lee, Lin, & Mou, 2009; Wang & Caruso, 2005). On the other hand, as an important class of materials, magnetic nanocomposites are also frequently utilized as a potential support material owing to their advantages of fast separation by applying a magnetic field (Xie & Ma, 2009). However, magnetic nanoparticles tend to aggregate due to their magnetic attraction, and thus they are required to be chemically stable and well-dispersed in a liquid media (Xie & Wang, 2014). The silica coating has been employed to effectively suppress the aggregation of magnetic nanoparticles thanks to its non-toxicity, good chemical inertness, and ease of further surface modification. More recently, core–shell structured particles have attracted increasing attention because of their low cytotoxicity, and chemical liable surface (Rosenholm, Zhang, Sun, & Gu, 2011). Of the various core–shell structured microspheres, magnetic mesoporous silica microspheres consisting of a magnetic core and a mesoporous silica shell are of particular interest (Rosenholm et al., 2011; Yang, Li, & Ma, 2012). The integration of functionalized mesoporous silica materials with magnetically responsive magnetite composites can form porous magnetic nanocomposites, which have the advantages of both mesoporous silica and magnetic nanoparticles. The mesoporous silica-coated magnetic nanoparticles possess large surface areas, and the mesoporous silica shell can be functionalized easily by organosilanes. With this respect, the abundant silanol groups on the surface of the core–shell structured materials can react with alkoxysilane regents to form Si–O bonds, which support terminal functional groups available for the immobilization of lipase (Chen & Mu, 2014; Jeong, Kim, Kim, Park, & Kim, 2011; Yang et al., 2012). Accordingly, the magnetic core–shell structured material seems to be an excellent carrier for the immobilization of lipases.

In the pursuit of more efficient and environmentally benign solid catalysts for the modification of vegetable oils (Xie & Chen, 2014; Xie & Qi, 2013), we herein wish to synthesize core–shell magnetic Fe3O4–MCM-41 nanocomposites, in which lipase from Candida rugosa is covalently immobilized onto these magnetic core–shell materials using glutaraldehyde as a cross-linking reagent. For this purpose, the core–shell magnetic materials composed of the Fe3O4 core and MCM-41 silica shell, were first prepared by using a surfactant-directed sol–gel method. Thereafter, the magnetic core–shell nanocomposites were amino-modified using 3-aminopropyltriethoxysiane (APTES), and then the C. rugosa lipase was covalently bound onto the amino-functionalized nanocomposites to afford the immobilized lipase. The successful immobilization of lipase was confirmed by magnetic measurements, Fourier transform infrared (FT-IR) spectra, and enzyme activity assays. The morphology, microstructure and magnetic properties of the magnetic core–shell structured nanocomposites before and after lipase binding were investigated in detail by means of transmission electron microscopy (TEM), vibrating-sample magnetometer (VSM), X-ray powder diffraction (XRD), and nitrogen adsorption–desorption techniques. The catalytic performance of the bound lipase was tested in the heterogeneous interesterification of lard and soybean oil in a batch reactor. The physicochemical characteristics of the initial blends and the final products were also determined in the present study in terms of FA profiles, TAG compositions, slip melting points, and microstructures.

2. Materials and methods

2.1. Materials

Commercial liquid C. rugosa lipase, 3-aminopropyl-triethoxysiane (APTES, >98%), cetyltrimethylammonium bromide (CTAB, >98%), and tetraethyl orthosilicate (TEOS, >98%) were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Commercial soybean oil and lard used in the present study were purchased from a local grocery store (Zhengzhou, China). Other materials were commercially available and used as received without further purification.

2.2. Preparation of core–shell structured Fe3O4–MCM-41 nanocomposites

Initially, the magnetic Fe3O4 cores were prepared by a chemical coprecipitation method (Shaw, Chen, Ou, & Ho, 2006). Typically, 3.9 g of FeSO4·7H2O and 8.1 g of FeCl3·6H2O were completely dissolved in 150 ml deionized water. Thereafter, ammonia solution was dropped into the solution under a nitrogen atmosphere with vigorous stirring. The final pH value of the resulting mixture was maintained at about 10. The addition of the ammonia solution resulted in the formation of black precipitates immediately. After aging in the mother solution for 1 h, the formed magnetite precipitates were magnetically separated, washed thoroughly with deionized water, and finally dried at 60 °C under vacuum for 24 h.

The core–shell structured Fe3O4–MCM-41 nanocomposites were prepared using the magnetite nanoparticles as cores via the Stöber method (Stöber, Fink, & Bohn, 1968). Briefly, 0.5 g of the magnetic Fe3O4 nanoparticles were dispersed in a solution composed of 60 ml ethanol and 120 ml deionized water. The resulting mixture was homogenized by ultrasonication for 30 min, and then 1.2 ml of ammonia aqueous solution was introduced into the solution and allowed to stir for 1 h. Afterward, to the solution, 10 ml of CTAB solution (100 mmol/l) as a structure directing agent to form the framework of MCM-41 was added dropwise. After stirring at room temperature for 1 h, 1.05 g of TEOS used as a silica source, was then added slowly to the solution and continued to stir for 24 h. The solid products were collected by magnetic separation, washed with plenty of ethanol/deionized water, and subsequently dried in a vacuum oven at 60 °C for 24 h. The magnetic core–shell structured Fe3O4–MCM-41 carriers were obtained by calcining the materials in air at 550 °C for 6 h. The core–shell structured Fe3O4–MCM-41 nanocomposites obtained here displayed a strong magnetic responsiveness, and the abundant silanol groups on the framework surface of the nanocomposites can react with the lipase to form the immobilized lipase by covalent bonds.

2.3. Lipase immobilization

Lipase from C. rugosa was immobilized on the magnetic core–shell nanocomposites following the procedure described in Fig. 1. Prior to the immobilization process, the magnetic core–shell nanoparticles were firstly derivatized with APTES to afford 3-aminopropyl-functionalized core–shell magnetic nanoparticles. Typically, the magnetic core–shell nanoparticle (1.0 g) and APTES (6 ml) were dispersed in anhydrous toluene to give a mixture solution (50 ml), and then the reaction mixture was continuously refluxed for 12 h under nitrogen atmospheres. Thereafter, the amino-functionalized nanocomposites were separated by applying a magnetic field and subsequently washed thoroughly with...
ethanol. Finally, the solid was dried in a vacuum at 60 °C for 24 h to obtain the 3-aminopropyl-functionalized magnetic nanoparticles.

The covalent binding of lipase was conducted by treatment of a lipase solution with the 3-aminopropyl-functionalized magnetic nanoparticles by using glutaraldehyde as a coupling reagent. In a typical assay, 10 ml of lipase solution (0.1 g/ml, 0.1 mol/l phosphate buffer, pH 7.0) and 10 ml of 10% glutaraldehyde, were mixed with 0.5 g of the 3-aminopropyl-functionalized magnetic nanoparticle, and then the resultant mixture was incubated at 35 °C, while shaking at 100 rpm for 18 h. Afterward, the immobilized lipase microspheres were recovered by magnetic separation, washed carefully with phosphate buffer (0.1 mol/l phosphate buffer, pH 7.0), and finally freeze-dried and stored at 4 °C for future use.

The content of lipase protein in the lipase solution and the washing solution was estimated by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976). The immobilization efficiency of lipase onto the magnetic Fe₃O₄–MCM-41 support was calculated from the following equation:

\[ q = \frac{(C_i - C_f)V_1}{C_iV_2} \times \frac{1}{C_f} \]  

where \( q \) is the immobilization efficiency (%), \( C_i \) and \( C_f \) represent the initial protein concentrations in the lipase solution before the immobilization and the final protein concentrations in the supernatant after the immobilization, respectively (mg/ml). \( V_1 \) and \( V_2 \) are the initial solution and the final solution volume of the supernatant, respectively (ml). All data used in this formula are the average of duplicated experiments.

The hydrolytic activity of the immobilized lipase was assayed using olive oil emulsion hydrolysis (Cho & Rhee, 1993). A certain amount of the immobilized lipase was added to 4 ml of the olive oil emulsion and 5 ml of phosphate buffer (0.025 mol/l, pH 7.5). Hydrolysis reactions were carried out at 35 °C for 15 min in a shaking water bath. The quantity of FAs liberated was measured by titration with 0.05 mol/l NaOH solution. One unit of lipase activity (U) was defined as the amount of lipase required to hydrolyze olive oil liberating 1 μmol of FAs per minute under the assay conditions.

For the immobilized lipase, the MCM-41 silica is considered as a capping shell to stabilize the magnetite nanoparticles by preventing their aggregation, since the magnetic nanoparticles usually suffer from coagulation in the liquid–phase reactions. Besides, the binding of lipase can lead to increased enzyme rigidity and reduced structural flexibility.

### 2.4. Characterization

The TEM photographs of the samples were taken on a JSM-6390LV transmission electronic microscopy with an accelerating voltage of 200 kV. The XRD patterns were obtained on a Rigaku D/max-3B X-ray diffractometer (Tokyo, Japan) employing Cu Kα radiation (\( \lambda = 0.1542 \) nm). The N₂ adsorption–desorption analysis was performed at −196 °C using a Quantachrome NOVA 1000e instrument. The specific surface areas (S_BET) were calculated by using the Brunauer–Emmett–Teller (BET) method. The pore size distribution and pore volume were estimated from the desorption branch of the N₂ adsorption–desorption isotherms by applying the Barrett–Joyner–Halenda (BJH) method. The FT-IR spectra were recorded on a Shimadzu IR-Prestige-21 spectrometer by using KBr pellet techniques. The magnetic measurements of the samples were carried out on a Shimadzu IR-Prestige-21 spectrometer by using KBr pellet techniques. The magnetic measurements of the samples were carried out on a vibrating sample magnetometer (LakeShore model 7304).

### 2.5. Enzymatic interesterification procedures

A reaction mixture of lard and soybean oil at different substrate ratios was charged into a screw-capped flask. The binary formulated blends were completely melted and homogenized at 80 °C for 40 min. After the temperature had been lowered to 45 °C, the immobilized lipase was added as a biocatalyst to the reaction mixture. The loading of the immobilized lipase was maintained at 15 wt.%. The batch enzymatic interesterification of lard and soybean oil blend was allowed to proceed at 45 °C for 48 h in a shaking incubator on a reciprocal shaker. The weight of lard to soybean oil used in the present study was set at 80:20, 60:40, 40:60, and 20:80, respectively. At the end of the interesterification reaction, the
immobilized lipase was separated using a magnetic field from the reaction mixture, and the interesterified product was then used for subsequent analysis. All interesterification reactions were carried out in triplicate.

2.6. Analytical methods

The FA profiles of the samples were analyzed by a gas chromatography (GC) method, as described in the official AOAC method (AOCS, 2009). Initially, the methylation of the interesterified products was performed through transesterification catalyzed by 2 mol/l methanolic potassium hydroxide solution. Typically, 50 mg of the sample, 2 ml of hexane and 3 ml of potassium hydroxide solution in methanol were added in a 25-ml screw-capped test tube, and the mixture was shaken vigorously for 10 min. Thereafter, the resultant mixture was centrifuged, and subsequently the separated upper layer was decanted and dried over anhydrous sodium sulfate for GC analysis.

After conversion of FA residues into fatty acid methyl esters (FAMEs), the concentrations were determined using an Agilent 6890 N gas chromatograph equipped with a split-injection port, a flame-ionization detector (FID), and a fused silica capillary column (60 m x 0.25 mm) coated with 0.25 μm of BPX-70 (SGE, Australia). The carrier gas used was nitrogen, and the gas flow rate was 1.2 ml/min. The injector and detector temperatures were maintained at 260 °C and 300 °C, respectively. 1 μl of sample was injected, and a split ratio of 1:20 was employed. The initial temperature was increased to 160 °C and held for 5 min, then programmed to increase to 200 °C at a rate of 5 °C/min, and held isothermally for another 42 min at the final temperature. The FA composition was identified by comparison with the relative retention time of the peaks with those of the respective FAME standards. The percentage of FAs was calculated as the ratio of partial area to the total peaks area in accordance with the AOAC Official Method Ce 1-62 (AOCS, 2009).

The FA compositions at the sn-2 position of the products were assessed by using pancreatic lipase analysis according to the official AOAC method (AOCS, 2009). The TAGs can be hydrolyzed selectively by the pancreatic lipase to afford 2-monoacylglycerol (2-MAG). The formed 2-MAG was used for the sn-2 positional analysis of FA profiles in the TAGs after methylation.

The TAG species was analyzed using reversed phase high-performance liquid chromatography (HPLC) fitted with an evaporative light-scattering detector (ELSD) (AOCS, 2009). In brief, 50 mg of interesterified products was initially dissolved in 5 ml of hexane. Then, the resulting mixture was spotted on thin-layer chromatography (TLC), and developed with acetic acid/ethyl ether/petroleum ether (1:10:90, v/v/v). Bands corresponding to TAGs were scrapped from the silica plates, and extracted using chloroform. Next, 20 μl aliquots were injected into a commercially packed Genesis C18 column (150 mm x 4.6 mm). The mobile phase was a binary solvent of dichloromethane and acetonitrile at a gradient composition from 35% acetonitrile increasing to 55% acetonitrile in 45 min. The carrier gas used was nitrogen, and the gas flow rate was 1.2 ml/min. The injector and detector temperatures were maintained at 260 °C and 300 °C, respectively. 1 μl of sample was injected, and a split ratio of 1:20 was employed. The initial temperature was increased to 160 °C and held for 5 min, then programmed to increase to 200 °C at a rate of 5 °C/min, and held isothermally for another 42 min at the final temperature. The FAME composition was identified by comparison with the relative retention time of the peaks with those of the respective FAME standards. The percentage of FAs was calculated as the ratio of partial area to the total peaks area in accordance with the AOAC Official Method Cd 1c-85 (AOCS, 2009). The slip melting point (SMP) was measured using the capillary tube method, as described in the AOCS Official Method Cc 3–25 (AOCS, 2009).

The crystal network morphology of the product was evaluated with a polarized light microscope (PLM, XP-203) equipped with a digital video camera. The samples were initially heated at 80 °C and kept at this temperature for 30 min to destroy the crystal nuclei. After this, 10 mg of the molten sample was placed on a microscope slide that had been heated to 80 °C, and then allowed to cool to 25 °C and held for 20 h before the measurements. The crystal morphology was captured at room temperature.

The analytical data were expressed as the mean ± standard deviations, and were analyzed using one-way analysis of variance (ANOVA). The level of statistical significance was set at P < 0.05.

3. Results and discussion

3.1. Characterization of the immobilized lipase

As illustrated in Fig. 1, core–shell structured Fe3O4-MCM-41 nanocomposites were readily prepared and subsequently employed for the immobilization of lipase. In the first step, the chemical coprecipitation of Fe2+ and Fe3+ ions in ammonia solution afforded the magnetite nanoparticles. Then, to avoid magnetic aggregation, a layer of MCM-41 silica was coated on the magnetite nanoparticles, since the magnetite has a strong affinity for MCM-41 silica. Through the sol-gel process, a mesoporous MCM-41 layer could be formed on the Fe3O4-MCM-41 nanocomposites, in which CTAB was employed as a structure-directing agent and TEOs as a silica source. Thereafter, the resultant core–shell structured Fe3O4–MCM-41 nanocomposites were chemically aminopropyl-modified with APTES to produce the aminopropyl-functionalized magnetic nanoparticles. After this, the condensation of the terminal amino groups of lipase with aminopropyl-functionalized Fe3O4–MCM-41 nanocomposites was performed using glutaraldehyde as a cross-linking reagent. It can be expected that the lipase possesses a rigid structure, as it is covalently bound to the magnetic core–shell nanocomposites. By using the Bradford method, it was found that the immobilization efficiency (q) of lipase was 76% under the assay conditions. The hydrolytic activity of the immobilized lipase was also evaluated according to the olive oil emulsion hydrolysis method, and the specific activity of the immobilized lipase was determined to be 228.2 U/g of immobilized lipase.

The immobilization of lipase on the magnetic core–shell support could be demonstrated by FT-IR techniques. Fig. 2 shows the comparative FT-IR spectra of the magnetite core, naked Fe3O4–MCM-41 nanocomposite, and the lipase-bound nanoparticle. For the magnetite nanoparticle (Fig. 2(a)), an IR band at 3375 cm⁻¹, attributed to O–H stretching, was observed, and the adsorption peak at around 564 cm⁻¹ was ascribed to the characteristic absorption of an Fe–O bond (Lee, Isobe, & Senna, 1996). Besides, the IR band at 1656 cm⁻¹ was mainly owing to the bending vibration of water in the Fe3O4 nanoparticles (Xie & Ma, 2009). After coating with a mesoporous MCM-41 silica shell, the Fe3O4–MCM-41 nanocomposite showed characteristic IR bands of both Fe3O4 magnetite and mesoporous MCM-41 silica (Fig. 2(b)). As observed, three characteristic IR peaks at 1080 cm⁻¹, 807 cm⁻¹, and 452 cm⁻¹ were clearly seen for the Fe3O4–MCM-41 nanocomposites, which were responsible for the anti-symmetric, symmetric Si–O stretching and the deformation mode of SiO2 tetrahedral in
the MCM-41 silica. Moreover, the IR band in the mid-infrared region at 970 cm\(^{-1}\) for the Fe\(_3\)O\(_4\)–MCM-41 nanocomposites was mainly assignable to the bending vibration of Si–OH group in MCM-41 silica (Jeong et al., 2011; Khorshidi & Shariati, 2014). The appearance of these characteristic IR bands confirmed that the MCM-41 silica was practically incorporated and coated onto the magnetite nanoparticles. After immobilization of the lipase on the magnetic core–shell nanocomposites (Fig. 2(c)), there were additional IR bands at 1647 cm\(^{-1}\) and 1545 cm\(^{-1}\), which were ascribed to amide I and II, respectively (Liu, Guan, Shen, & Liu, 2005). Moreover, as compared with the IR spectrum of the Fe\(_3\)O\(_4\)–MCM-41 nanocomposites, new IR bands centered at 2864 cm\(^{-1}\) and 2938 cm\(^{-1}\), attributed to the CH\(_2\) symmetric and asymmetric stretching vibration in the propyl chain of silylating reagents, appeared in the immobilized lipase sample (White & Tripp, 2000). Such results suggested that during the immobilization process, Schiff base linkages are formed between the amino groups of the enzyme and the aldehyde groups of glutaraldehyde. The glutaraldehyde, as a cross-linking reagent, has two aldehyde groups, which can react with both the terminal amine group of the enzyme and the magnetic core–shell nanocomposites. By drawing on the IR results, the lipase has been successfully immobilized on the surface of the magnetic core–shell nanocomposites.

The TEM micrographs for the magnetite nanoparticles, the magnetic core–shell nanocomposites, and the immobilized lipase microsphere, are displayed in Fig. 3. As revealed in this figure, the naked Fe\(_3\)O\(_4\) nanoparticles are nearly spherical in shape, with an average size of 10.3 nm (Fig. 3(a)), and the aggregates of Fe\(_3\)O\(_4\) particles with a tight structure were clearly present due to the strong magnetic dipole–dipole interactions between Fe\(_3\)O\(_4\) nanoparticles. After coating with MCM-41 silica, the magnetic composite particles prepared as supports for the lipase showed spherical morphologies and well-defined core–shell structures with a dark core of Fe\(_3\)O\(_4\) magnetite and a gray silica shell. Moreover, their surface became smoother than that of the magnetite nanoparticles (Fig. 3(b)). Besides, the separate core–shell magnetic nanocomposites were discernible, implying that the formation of an MCM-41 silica layer on the surface of the Fe\(_3\)O\(_4\) nanoparticles could weaken the aggregation of the magnetic nanoparticles. This result revealed that the MCM-41 silica was indeed encapsulated onto the magnetic nanoparticles and the magnetic Fe\(_3\)O\(_4\) nanoparticles were successfully coated with the MCM-41 shell. The formed core–shell magnetic structure not only offers magnetic behaviors of the materials, but also provides a large number of accessible surface silanol groups for lipase immobilization. As indicated in Fig. 3(c), the morphology and size for the lipase-bound microspheres were similar to those of the unbound ones. These observations suggested that the morphology of magnetic core–shell nanoparticles remained unchanged after the lipase binding.

The magnetite nanoparticle, the magnetic core–shell nanocomposite, and the immobilized lipase microsphere were characterized.
by XRD techniques. The Fe$_3$O$_4$ nanoparticles exhibited six diffraction peaks of Fe$_3$O$_4$ with 2θ of 30.1°, 35.5°, 43.1°, 53.4°, 57.0°, and 62.6°, corresponding to the typical reflections of (220), (311), (400), (422), (511), and (440) crystallographic planes of Fe$_3$O$_4$ (Fig. 1S, Supplementary material), respectively, indicative of a cubic spinel crystal structure of pure Fe$_3$O$_4$ magnetite (JCPDS database file, No. 85-1436) (Xie & Ma, 2009; Xie & Wang, 2014). After coating of the MCM-41 shell, the formed Fe$_3$O$_4$–MCM-41 nanocomposite had six similar characteristic peaks to those of Fe$_3$O$_4$ magnetite core, though the intensity of the related XRD peaks was reduced slightly, thus implying that the magnetic cores were well preserved after MCM-41 silica coating. When the lipase was covalently bound onto the Fe$_3$O$_4$–MCM-41 nanocomposites, no obvious change of XRD peaks in the position occurred in the crystalline structure of the Fe$_3$O$_4$ core. Besides, there was no XRD peak for MCM-41 silica to be detected obviously owing to the amorphous structure of the silica layer. The decrease in the intensity of the characteristic XRD peaks is expected mainly due to the formation of an amorphous silica shell around the magnetite core as well as the immobilization of the lipase. Therefore, the immobilized lipase maintained the magnetic properties and could be recovered easily from the product with an external magnet, which would be favorable for application in bioseparations.

Fig. 4 presents the N$_2$ adsorption–desorption isotherms of the core–shell Fe$_3$O$_4$–MCM-41 nanocomposite and the immobilized lipase microsphere. For the two selected samples, the isotherm curves could be mainly ascribed to a type IV isotherm with a clear H1-type hysteresis loop, according to IUPAC nomenclature, which was characteristic of ordered mesoporous materials (Hassan et al., 2014). Besides, a sharp step at a relative pressure of about 0.49 was observed, corresponding to the filling of the mesopores.
were estimated to be 81 m²/g and 0.13 cm³/g, respectively, while lipase microsphere, the measured surface area and pore volume was 32 m²/g and 0.32 cm³/g, and mean pore size of 2.5 nm. As for the immobilized lipase microsphere, the measured surface area and pore volume were estimated to be 81 m²/g and 0.13 cm³/g, respectively, while the mean pore size calculated from the N₂ adsorption–desorption isotherm was 3.8 nm. Apparently, the textural parameters for the bound lipase tended to change in comparison with the Fe₃O₄–MCM-41 nanocomposite. This result was most likely resulted from the blockage of pores for the Fe₃O₄–MCM-41 carrier by the lipase. Although such a change in the textural properties occurred after the lipase binding, the immobilized lipase still remained the typical mesoporous structure during the immobilization process, which would ensure efficient diffusion of substrates and catalytic site accessibility during the interesterification reaction.

The BET surface area and pore-size distribution were calculated by N₂ adsorption–desorption isotherms. The Fe₃O₄–MCM-41 sample exhibited a high BET surface area of 545 m²/g, pore volume of 0.32 cm³/g, and mean pore size of 2.5 nm. As for the immobilized lipase microsphere, the measured surface area and pore volume were estimated to be 81 m²/g and 0.13 cm³/g, respectively, while the mean pore size calculated from the N₂ adsorption–desorption isotherm was 3.8 nm. Apparently, the textural parameters for the bound lipase tended to change in comparison with the Fe₃O₄–MCM-41 nanocomposite. This result was most likely resulted from the blockage of pores for the Fe₃O₄–MCM-41 carrier by the lipase. Although such a change in the textural properties occurred after the lipase binding, the immobilized lipase still remained the typical mesoporous structure during the immobilization process, which would ensure efficient diffusion of substrates and catalytic site accessibility during the interesterification reaction.

The magnetic properties of Fe₃O₄ nanoparticle, Fe₃O₄–MCM-41 nanocomposite, and the immobilized lipase were investigated by VSM techniques at room temperature. The typical magnetization curves for the samples with the applied magnetic field are shown in Fig. 2S, Supplementary material. The three magnetization hysteresis loops exhibited no remanence or coercivity, which demonstrated the typical superparamagnetic behavior of the materials for all selected samples (Khorshidi & Shariati, 2014; Xie & Wang, 2014). The saturation magnetization value of the Fe₃O₄ nanoparticle was measured to be 46.8 emu/g. After being coated with a layer of MCM-41 silica, the magnetization moment of the Fe₃O₄–MCM-41 nanocomposite appeared to decrease to 26.3 emu/g, mostly due to the shielding effect of a silica shell around the magnetite core. When the Fe₃O₄ nanoparticle is coated by the MCM-41 silica, the interfacial interactions between the magnetite nanoparticle and the MCM-41 shell could restrict the rotation of a magnetic moment, thus causing the decrease in saturation magnetization of the nanocomposites. Even so, the core–shell Fe₃O₄–MCM-41 nanostructure still possesses a satisfactory magnetically separable ability and can be facilely separated by an external magnetic field. For the immobilized lipase microsphere, the saturation magnetization value was found to be further reduced to 15.3 emu/g. Taking advantage of the magnetic properties, the immobilized lipase can be easily separated from the product as an external magnetic field is used. Indeed, when a magnet was placed near the glass bottle in which the immobilized lipase was suspended absolutely in the product, the particles were attracted completely by the magnet, and simultaneously the cloudy solution turned transparent rapidly. Once the magnetic field was removed, the immobilized lipase could be redispersed quickly with a slight shake. Hence, the immobilized lipase prepared in the present study can respond quickly to an external magnetic field, which is very useful for the rapid dispersion and separation of biocatalyst.

### 3.2. Fatty acid composition and iodine value

The lard used in the present study contained a high amount of long-chain FAs, including oleic acid (34.6%), palmitic acid (30.4%), linoleic acid (14.5%), stearic acid (15.9%), palmitoleic acid (2.1%) and myristic acid (1.8%), with an IV of 58.7. In this case, about 40% of the FAs for the lard consisted of saturated FAs and accordingly the lard usually exhibited a solid form at room temperature. Conversely, the major FAs of the soybean oil were linoleic acid (53.6%), oleic acid (23.3%), linolenic acid (7.2%), palmitic acid (11.7%), and stearic acid (4.8%). The IV of the soybean oil was determined to be 131.7. Due to high contents of unsaturated FAs, soybean oil was indeed completely liquid at room temperature. The FA profile and IV of soybean oil and lard are in agreement with the FA profile of the initial blend and the interesterified product (Fig. 3S, Supplementary material), and concomitantly, the IV of the binary blends before and after the enzymatic reaction remained almost constant (data not shown here). Besides, no trans FA was detected in the interesterified blends catalyzed by the bound lipase. Hence, the enzymatic interesterification did not cause a significant alteration of the FA profile for the original blends. In spite of this, the positional distribution change of FAs in the TAGs can highly affect the nutritional and physicochemical properties.

The FA profile at the sn-2 position for the different blends before and after the enzymatic interesterification was investigated in the present investigation, and the results are presented in Table 1. Concerning the FA composition of soybean oil, 22.7% of oleic acid, 69.2% of linoleic acid and 6.4% of linolenic acid were primarily esterified at the sn-2 position of the glycerol backbone, while only small amounts of saturated FAs, such as 0.4% of stearic acid and 1.3% of palmitic acid, occupied this position. For the lard, the saturated FAs were mainly located at the sn-2 position. The sn-2 position of lard contained predominately 75.9% of palmitic acid, 8.3% of oleic acid, 4.7% of linoleic acid, 4.2% of myristic acid and 3.5% of stearic acid. Besides, only 3.0% of palmitoleic acid and 0.3% of

<table>
<thead>
<tr>
<th>LA/SO ratio</th>
<th>Myristic 14:0</th>
<th>Palmitic 16:0</th>
<th>Palmitoleic 16:1</th>
<th>Stearic 18:0</th>
<th>Oleic 18:1</th>
<th>Linoleic 18:2</th>
<th>Linolenic 18:3</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0 (before)</td>
<td>4.2 ± 0.2</td>
<td>75.9 ± 2.2</td>
<td>3.0 ± 0.1</td>
<td>3.5 ± 0.5</td>
<td>83.3 ± 0.3</td>
<td>4.7 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>58.7</td>
</tr>
<tr>
<td>80:20 (before)</td>
<td>3.9 ± 0.1</td>
<td>63.5 ± 0.4</td>
<td>2.7 ± 0.2</td>
<td>2.7 ± 1.3</td>
<td>10.3 ± 1.1</td>
<td>15.7 ± 0.3</td>
<td>1.2 ± 0.4</td>
<td>73.1</td>
</tr>
<tr>
<td>80:20 (after)</td>
<td>2.0 ± 0.0</td>
<td>37.0 ± 0.5</td>
<td>1.7 ± 0.0</td>
<td>15.6 ± 0.5</td>
<td>25.7 ± 0.6</td>
<td>16.7 ± 0.1</td>
<td>1.2 ± 0.3</td>
<td>72.3</td>
</tr>
<tr>
<td>60:40 (before)</td>
<td>3.0 ± 0.2</td>
<td>40.3 ± 1.3</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.0</td>
<td>13.1 ± 0.4</td>
<td>28.2 ± 0.5</td>
<td>2.4 ± 0.0</td>
<td>86.4</td>
</tr>
<tr>
<td>60:40 (after)</td>
<td>1.3 ± 0.0</td>
<td>32.7 ± 0.9</td>
<td>1.3 ± 0.1</td>
<td>11.8 ± 0.7</td>
<td>24.9 ± 0.3</td>
<td>25.8 ± 0.8</td>
<td>2.2 ± 0.1</td>
<td>84.9</td>
</tr>
<tr>
<td>40:60 (before)</td>
<td>2.8 ± 0.1</td>
<td>38.1 ± 0.7</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>14.5 ± 0.1</td>
<td>38.3 ± 1.0</td>
<td>3.4 ± 0.1</td>
<td>100.5</td>
</tr>
<tr>
<td>40:60 (after)</td>
<td>1.1 ± 0.1</td>
<td>26.5 ± 0.8</td>
<td>0.9 ± 0.3</td>
<td>8.7 ± 0.4</td>
<td>24.0 ± 0.2</td>
<td>35.2 ± 0.8</td>
<td>3.5 ± 0.1</td>
<td>99.9</td>
</tr>
<tr>
<td>20:80 (before)</td>
<td>1.9 ± 0.3</td>
<td>19.7 ± 0.6</td>
<td>0.5 ± 0.0</td>
<td>1.0 ± 0.3</td>
<td>18.5 ± 0.6</td>
<td>53.8 ± 0.8</td>
<td>4.6 ± 0.4</td>
<td>116.1</td>
</tr>
<tr>
<td>20:80 (after)</td>
<td>0.7 ± 0.1</td>
<td>22.6 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>7.9 ± 0.2</td>
<td>23.1 ± 0.1</td>
<td>40.8 ± 0.1</td>
<td>4.3 ± 0.2</td>
<td>114.9</td>
</tr>
<tr>
<td>0:100 (before)</td>
<td>ND</td>
<td>1.3 ± 0.5</td>
<td>ND</td>
<td>0.4 ± 0.2</td>
<td>22.7 ± 1.2</td>
<td>69.2 ± 1.1</td>
<td>6.4 ± 0.4</td>
<td>131.7</td>
</tr>
</tbody>
</table>

* Lard; SO, soybean oil; IV, iodine value; ND, not detected.
linolenic acid were present at the sn-2 position in the lard. More importantly, as observed from Table 1, as compared with the initial blend, the FA profile at the sn-2 position was found to be varied after the interesterification for all selected samples, due to the redistribution of FAs on the glycerol backbone of TAGs molecule. For example, for the 60:40 blend of lard and soybean oil, it was shown that the amounts of palmitic acid, stearic acid, oleic acid, linoleic acid, and myristic acid at the sn-2 position were changed from 49.3%, 2.1%, 13.1%, 28.2% and 3.0% to 32.7%, 11.8%, 24.9%, 25.8%, and 1.3%, respectively, after the enzymatic interesterification. Accordingly, the immobilized lipase showed catalytic activities towards the interesterification of lard and soybean oil.

### 3.3. Triacylglycerol composition

The TAG profiles of soybean oil, lard as well as their blends before and after the enzymatic interesterification were determined and the results are shown in Table 2. As can be seen, the predominant TAG species of lard were POO (23.3%), POSt (22.8%), PLO (16.2%), PPO (11.1%), PLL (4.6%), PPL (3.4%) and PStSt (3.4%) (da Silva et al., 2011; Silva et al., 2009). Meanwhile, the soybean oil contained high levels of unsaturated TAG species such as LLL (26.6%), OLL (18.9%), PLL (16.1%), PLO (11.6%), OLO (7.4%), LLLn (7.4%) and POO (3.9%) (da Silva et al., 2011; Ribeiro et al., 2009).

As illustrated in Table 2, for the trials performed using different blends of lard and soybean oil, different TAG profiles of interesterified products were obtained. When compared with the initial blends, the interesterified products showed a significant change in the TAG species occurring after the interesterification. Specially, for experiments conducted using a 60:40 blend of lard and soybean oil, the interesterified product displayed an increased amount of TAG species, such as PLO, OLO, PLL, PPO, PPSt, and StOO from 15.5%, 4.6%, 2.3%, 9.7%, 1.7%, and 0.7% to 18.3%, 7.6%, 4.3%, 10.7%, 3.3%, and 2.5%, respectively. Some other TAG species on the contrary, were observed to be reduced, such as POO, LLL, PLL, POSt, and LLLn from 15.1%, 10.3%, 9.3%, 14.4%, and 2.8% to 14.0%, 4.7%, 4.3%, 10.7%, 3.3%, and 2.5%, respectively.

### 3.4. Slip melting point (SMP)

The melting properties of the interesterified products are highly dependent on their TAG compositions. The SMP was determined by the open capillary tube method, and the results are indicated in Table 1S, Supplementary material. The initial blends had SMPs ranging from 23.0 °C to 33.2 °C, and the SMP tended to increase upon increasing the proportion of lard in the blends, owing to the increase in the saturated FAs in the formulated blends. In particular, the interesterified products exhibited lower SMPs for all samples in comparison with their physical blends. This phenomenon was also reported in the previous literature (Neeharika et al., 2014), in which the enzymatic interesterification of rice bran oil with hydrogenated cottonseed oil resulted in trans-free fats with a lower-melting point. Clearly, the changes in the SMPs are attributed to the changes in the TAG compositions caused by the interesterification reaction, which was supported by the HPLC analysis. Hence, by varying the proportion of lard to soybean oil, the enzymatic interesterification could produce new tailored fats with desired melting properties that meet consumer preferences.

### 3.5. Crystal structure and crystal morphology

Crystal sizes are essential for the finished product acceptability. Smaller size crystals can yield firmer products, whereas larger size crystals can produce a sandy mouthfeel. The crystal microstructures of the samples are presented in Fig. 5S, Supplementary material. As observed, lard consisted of dense and asymmetrical

### Table 2

<table>
<thead>
<tr>
<th>ECN</th>
<th>TAG</th>
<th>LA</th>
<th>80:20 Before</th>
<th>60:40 Before</th>
<th>40:60 After</th>
<th>20:80 Before</th>
<th>SO Before</th>
<th>After</th>
</tr>
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<tr>
<td>40</td>
<td>LLLn</td>
<td>0.6±0</td>
<td>1.6±0</td>
<td>4.6±0</td>
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<td>2.3±0</td>
<td>4.7±0</td>
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</tr>
<tr>
<td>42</td>
<td>LLL</td>
<td>ND</td>
<td>0.8±0</td>
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<td>1.9±0</td>
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</tr>
<tr>
<td>44</td>
<td>OLL</td>
<td>2.3±0</td>
<td>4.5±0</td>
<td>5.2±0</td>
<td>8.5±0</td>
<td>8.6±0</td>
<td>13.9±0</td>
<td>15.7±0</td>
</tr>
<tr>
<td>46</td>
<td>PLL</td>
<td>4.6±0</td>
<td>7.4±0</td>
<td>6.7±0</td>
<td>9.3±0</td>
<td>8.9±0</td>
<td>11.3±0</td>
<td>10.4±0</td>
</tr>
<tr>
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<td>PPO</td>
<td>2.6±0</td>
<td>3.7±0</td>
<td>7.8±0</td>
<td>4.6±0</td>
<td>7.6±0</td>
<td>5.4±0</td>
<td>8.7±0</td>
</tr>
<tr>
<td>50</td>
<td>PPO</td>
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<td>15.7±0</td>
<td>17.6±0</td>
<td>15.2±0</td>
<td>18.3±0</td>
<td>14.0±0</td>
<td>16.9±0</td>
</tr>
<tr>
<td>52</td>
<td>PPL</td>
<td>3.4±0</td>
<td>2.8±0</td>
<td>5.7±0</td>
<td>2.3±0</td>
<td>4.3±0</td>
<td>2.2±0</td>
<td>4.0±0</td>
</tr>
<tr>
<td>54</td>
<td>StPL</td>
<td>1.5±0</td>
<td>2.3±0</td>
<td>3.4±0</td>
<td>2.1±0</td>
<td>2.6±0</td>
<td>1.7±0</td>
<td>1.5±0</td>
</tr>
<tr>
<td>56</td>
<td>POO</td>
<td>23.3±0</td>
<td>20.2±0</td>
<td>17.6±0</td>
<td>15.1±0</td>
<td>14.0±0</td>
<td>9.6±0</td>
<td>10.3±0</td>
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<tr>
<td>58</td>
<td>PPO</td>
<td>11.1±0</td>
<td>10.9±0</td>
<td>12.6±0</td>
<td>9.7±0</td>
<td>10.7±0</td>
<td>6.5±0</td>
<td>6.8±0</td>
</tr>
<tr>
<td>60</td>
<td>PPP</td>
<td>1.1±0</td>
<td>0.4±0</td>
<td>1.2±0</td>
<td>0.1±0</td>
<td>0.8±0</td>
<td>0.1±0</td>
<td>0.1±0</td>
</tr>
<tr>
<td>62</td>
<td>PPSt</td>
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<td>2.2±0</td>
<td>3.1±0</td>
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</tr>
<tr>
<td>64</td>
<td>POST</td>
<td>22.8±0</td>
<td>20.4±0</td>
<td>9.1±0</td>
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<td>8.9±0</td>
<td>5.4±0</td>
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<tr>
<td>66</td>
<td>PLOO</td>
<td>2.6±0</td>
<td>1.0±0</td>
<td>2.3±0</td>
<td>0.7±0</td>
<td>1.6±0</td>
<td>0.4±0</td>
<td>0.5±0</td>
</tr>
<tr>
<td>68</td>
<td>SSO</td>
<td>1.5±0</td>
<td>0.8±0</td>
<td>1.0±0</td>
<td>0.6±0</td>
<td>1.0±0</td>
<td>0.2±0</td>
<td>1.1±0</td>
</tr>
<tr>
<td>70</td>
<td>PSSt</td>
<td>3.4±0</td>
<td>0.9±1</td>
<td>1.0±1</td>
<td>0.7±0</td>
<td>1.2±0</td>
<td>0.4±0</td>
<td>0.6±0</td>
</tr>
</tbody>
</table>

* ECN = equivalent carbon number, TAG = triacylglycerol; LA = lard; SO = soybean oil; L = linoleic acid; Ln = linolenic acid; O = oleic acid; P = palmitic acid; St = stearic acid; ND = not detected.
spherulitic crystals which were more tightly packed with less space. Meanwhile, soybean oil was completely liquid at a temperature of 25 °C, and thus no clear crystal was observed at this temperature. For the physical blends of lard and soybean oil, low density aggregates of fat crystals appeared, which was probably due to a dilution effect. After enzymatic interesterification, the interesterified products displayed obvious changes in their crystal morphologies. In comparison with the physical blend, the interesterified product exhibited a small crystal form, less aggregation, and a more homogeneous distribution of crystals. Accordingly, the interesterification reaction results in a reduction in spherulitic size and also a decrease in the number of spherulitic crystals. The variation in the crystal properties can be ascribed mainly to the change in TAG compositions resulted from the interesterification.

3.6. Reusability of the immobilized lipase

The reusability of the immobilized lipase was investigated through the determination of the residual activity towards the interesterification reaction. To achieve this, the spent immobilized lipase was recovered by magnetic separation, washed with phosphate buffer (0.1 mol/l phosphate buffer, pH 7.0) and tert-butanol, and finally freeze-dried. The recovered immobilized lipase was employed in the next batch of interesterification with fresh substrates under the same reaction conditions as described previously. Practically, it is difficult to evaluate quantitatively the progression of the interesterification process due to the complex compositions in the reaction mixture. As stated previously, after enzymatic interesterification, the immobilized lipase had possessed lower SMPs for all samples when compared with the physical blends due to the altered TAG compositions. Probably, the degree of interesterification could be tentatively evaluated depending on the reduction degree (RD) in the SMPs after interesterification. For the 60:40 blends of lard and soybean oil, as the immobilized lipase was used for 1, 2, 3, 4, and 5 cycles, the RD was 4.8, 4.4, 3.5 and 2.7 °C, respectively, suggesting that the immobilized lipase could be reused for four times without significant loss of catalytic efficiency. However, no obvious decrease in SMPs after the interesterification was observed if the immobilized lipase was reused for five cycles, which showed that it had lost its activity most likely due to the condensation of reactant or product species in the bound lipase.

4. Conclusion

A core–shell structural magnetically nano-composite, with Fe₃O₄ magnetite as the core and ordered MCM-41 silica as the shell layer, has been prepared by means of a surfactant-directed sol–gel process using cetyltrimethylammonium bromide as a template and tetraethyl orthosilicate as a silica precursor. The lipase from C. rugosa was covalently bound onto the core–shell structured Fe₃O₄–MCM-41 nanocomposites and then employed as a biocatalyst for the interesterification of lard and soybean oil. The characterization results showed that the core–shell structured Fe₃O₄–MCM-41 nanocomposites were fabricated and the lipase had been successfully anchored onto the surface of the magnetic core–shell nanoparticles. The immobilized lipase displayed good magnetic properties and could be facilely separated by applying an external magnetic field. The immobilized lipase showed promising biocatalytic activities for the interesterification of soybean oil and lard with advantages on good catalytic activity, and simple separation by magnetization. The physicochemical properties of the interesterified products were changed significantly after the enzymatic interesterification reaction.

Acknowledgements

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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.2015.09.009.

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