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Optimization of oligoglycerol fatty acid esters preparation catalyzed by Lipozyme 435

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ABSTRACT: Oligoglycerol fatty acid esters (OGEs) are an important kind of polyglycerol fatty acid esters (PGEs) which have been widely used as emulsifiers in food, medicine and cosmetic industries. The aim of this study was to investigate the preparation of OGEs by the esterification of oligoglycerol with linoleic acid in a solvent-free system using Lipozyme 435 as the catalyst. The effects of substrate molar ratio, reaction time, reaction temperature, enzyme dosage, and water addition on the efficiency of esterification (EE) were studied. Single factor experiments and response surface methodology (RSM) were employed to optimize the reaction parameters. The optimum conditions were obtained as follows: reaction time 4.52 h, reaction temperature 90 °C, enzyme dosage 2 wt% (based on the total substrate mass), the molar ratio of oligoglycerol to linoleic acid 1.59:1 and no water addition. Under these conditions, the experimental EE (95.82±0.22%) fitted well with that predicted by RSM (96.15%). Similar results were obtained when the process was scaled up to a production of 500 g in a pilot bubble column reactor (BCR). The enzyme maintained 98.2% of the relative activity after 10 batches of reaction in the BCR. Electrospray ionization mass spectrum was employed to rapidly analyze the esterification products, and most species of OGEs have been identified.

KEYWORDS: *Biocatalysis; Bubble column reactor; Esterification; Immobilized enzyme; Lipase; Oligoglycerol fatty acid esters*

RESUMEN: ***Optimización de la preparación de ésteres grasos de oligoglicerol catalizada por Lipozyme 435***. Los ésteres grasos de oligoglicerol (OGEs) son una clase importante de ésteres de ácidos grasos de poliglicerol (PGE) que han sido ampliamente utilizados como emulsionantes en alimentación, medicina y en la industria cosmética. El objetivo de este estudio fue investigar la preparación de OGEs mediante la esterificación de oligoglicerol con ácido linoleico en un sistema libre de disolvente utilizando Lipozyme 435 como catalizador. Se estudiaron los efectos en la eficiencia de esterificación (EE) de la relación molar de sustratos, de los tiempos de reacción, las temperaturas de reacción, la dosis de la enzima, y de la adición de agua. Se realizaron ensayos factoriales individuales y metodología de superficie de respuesta (RSM) para optimizar los parámetros de la reacción. Las condiciones óptimas se obtuvieron como sigue: tiempo de reacción 4,52 h, temperatura de reacción 90 °C, dosis de enzima 2% en peso (basado en la masa total del sustrato), la relación molar de ácido linoleico:oligoglicerol fue de 1,59:1 sin adición de agua. Bajo estas condiciones, el EE experimental (95,82±0,22%) se corresponde bien con lo predicho mediante RSM (96,15%). Se obtiene un resultado similar cuando el proceso se escaló a una producción piloto de hasta 500 g en un reactor de columna de burbujas (BCR). La enzima mantiene el 98,2% de su actividad relativa después de 10 lotes de reacción en el BCR. Se empleó un espectrómetro de masas de ionización mediante electrospray para analizar rápidamente los productos de esterificación, y se han identificado la mayoría de las especies de OGEs.

***PALABRAS CLAVE*:** *Biocatálisis; Enzima inmovilizada; Ésteres grasos de oligoglicerol; Esterificación; Lipasa; Reactor de columna de burbujas*

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1. INTRODUCTION

Natural fats and oils are attractive feed stocks for the chemical industry as they are renewable and more eco-friendly compared with fossil resources. This makes it very interesting for them to be manufactured into surfactants. Polyglycerol fatty acid esters (PGEs) are one such new type of safe and effective non-ionic surfactant, with extensive applications in food, cosmetic, detergents, and pharmaceuticals (Richardson *et al*., 2004, Shima *et al*., 2004, Takatori *et al*., 2004). PGEs are amphiphilic molecules which show good properties for emulsification, crystallization adjustment, viscosity modifier, moisturizing, antimicrobial, etc. Their amphiphilic nature makes them suitable to be used for the stabilization of food emulsion (Curschellas *et al*., 2013) and reverse microemulsion (Ding *et al*., 2007). Recently, PGEs have been used as a crystal inhibitor to retard precipitation in the diacylglycerol-rich oil (Saitou *et al*., 2014). Since PGEs have a higher content of hydroxyl groups, their emulsifying properties are considered to be superior to that of monoglycerides (Yamagata *et al*., 2000).

According to Griffin (1954), the emulsifying properties of esters depend on their hydrophilic lipophilic balance (HLB) values; a higher HLB value indicates a higher hydrophilicity. To meet different applications, PGE products with different HLB values (2 to 16) can be prepared by changing the degree of polymerization (n), the degree of esterification and the chain length of fatty acids (Márquez-Alvarez *et al*., 2004). Oligoglycerols (di- and/or triglycerol, n=2 or 3) are more hydrophilic than higher ones (n≥4) therefore have higher solubility in polar solvents. With the increase in n, the viscosity of polyglycerol increases with its color changing from white to deep yellow (Martin and Richter, 2011). Oligoglycerols are considered to have more promising applications than high polyglycerol (n≥4). Among PGEs, oligoglycerol fatty acid esters (OGEs) are of great importance due to their outstanding emulsifying properties (Márquez-Álvarez *et al*., 2004).

PGEs can be prepared either chemically or enzymatically. The enzymatic method is preferred as enzymes are generally more specific, react under mild conditions, and produce less side products (Panpipat *et al*., 2012, Blasi *et al*., 2007). The lipase-catalyzed synthesis of PGEs through the esterification or transesterification of polyglycerol with fatty acids or methyl esters has been studied thoroughly (Ortega *et al*., 2014, Ortega *et al*., 2013, Xiao *et al*., 2012, Charlemagne and Legoy, 1995). All of these processes are solvent-free systems. These processes are regarded as greener alternatives to those using organic solvents, as the latter may leave undesirable solvent residues in food products. However, so far the synthesis of OGEs by lipase with oligoglycerols as substrate has received little attention, and the optimal condition for the industrial preparation of OGEs has not been reported. This undoubtedly hindered the application of OGEs. Therefore, in this study, determination of the optimal conditions for the enzymatic synthesis of OGEs is included.

Lipozyme 435 is a commercially available immobilized lipase from *Candida antarctica* with high activity, operational stabilities and stereospecificity. This lipase is highly robust and presents activity under a wide variety of conditions so it has enormous importance for hydrolysis and synthesis reactions (Ortega *et al*., 2014). Thus, it has been extensively used as a biocatalyst to catalyze esterification reactions (Duan *et al*., 2013, Souza *et al*., 2009).

In this study, Lipozyme 435 was utilized to catalyze the esterification of oligoglycerol with linoleic acid in the solvent-free system to prepare OGEs. The effects of reaction conditions investigated include reaction temperature, reaction time, substrate molar ratio, enzyme dosage and water content. Single factor experiments and response surface methodology (RSM) were used to optimize the process at a laboratory scale. Usually, the optimized conditions obtained by RSM are only valid under the experimental setting where the optimization was performed. Here the possibility of using the lab-scale optimum conditions in a pilot-scale plant setting was attempted to study the potential of the enzymatic reaction in industrial application. So the lab-scale-optimized conditions were transferred to pilot-scale in a bubble column reactor (BCR) (Mollenhauer *et al*., 2010). The operational stability of Lipozyme 435 under pilot-scale conditions was also investigated by reusing it in the BCR. Electrospray ionization mass spectrum (ESI-MS) which was considered as a quick and convenient tool for a qualitative analysis was employed to analyze the composition of esterification products (Khemchyan *et al*., 2013).

2. MATERIALS AND METHODS

2.1. Chemicals

Linoleic acid (95%) was purchased from Anqing biological engineering Co., Ltd. (Anhui, China). Lipozyme 435 (formerly named Novozym® 435 before 2010, declared activity 10000 PLU/g) was obtained from Novozymes A/S (Bagsvaerd, Denmark). Sodium hydroxide and glycerol were purchased from Fuyu chemical Co. Ltd. (Tianjin, China). Acetone and methanol were brought from MREDA technology Inc. (USA). All chemicals were of pure, analytical grade, and all solvents used were of HPLC grade.

2.2. Preparation and purification of oligoglycerols

Glycerol (150.0 g) and sodium hydroxide (5.25 g, 3.5 wt% based on the glycerol) were added to a 250 mL three-neck flask equipped with a condenser. The reaction was performed under nitrogen to prevent the dehydration of glycerol to acrolein. The mixture was reacted for 3.5 h at 250 °C and 400 rpm. After reaction, it was cooled down to room temperature, and the crude oligoglycerols were purified using molecular distillation (MD). The glycerol was removed and the heavy phase was collected under the following conditions: evaporator temperature 120 °C, condenser temperature 50 °C, evaporator vacuum pressure 5.0 Pa, and roller speed 300 rpm. The high polyglycerol (n≥4) removed from the heavy phase of MD has been collected, and the light phase containing oligoglycerols was obtained using the following conditions: evaporator temperature 170 °C, condenser temperature 60 °C, evaporator vacuum pressure 1.0 Pa, and roller speed 300 rpm.

2.3. Analysis of the oligoglycerol composition by gas chromatography (GC)

The composition of oligoglycerols was analyzed by gas chromatography (GC) according to a modified method described by Xiao *et al*. (2012). The GC analysis was carried out on an Agilent GC 7820A instrument (Agilent Technologies Inc., America), equipped with a flame ionization detector (FID) and a capillary column of AT-Wax (15 m×0.32 mm, 0.25 μm). The injector temperature was 280 °C and was used in the split mode with a split ratio of 20:1. FID was kept at 280 °C and the flow rate of the carrier gas (N_2) was kept at 30 mL·min^{-1}. In each measurement, 1 μL of sample was injected. The oven temperature was programmed from 120 °C to 260 °C as follows: rising from 120 °C to 165 °C at a rate of 60 °C·min^{-1} and held for 4 min, to 230 °C at a rate of 60 °C·min^{-1} and held for 2 min, to 260 °C at a rate of 10 °C·min^{-1} and held for 3 min. The percentage content of each oligoglycerol was calculated assuming the same response factor and the results are shown in Table 1.

TABLE 1. Composition (means±SD) of oligoglycerols after purification by molecular distillation.[a]

Component	Percent (wt%)
Glycerol	0.54±0.06
PG2	62.22±2.67
PG3	32.83±1.91
PG4	4.41±0.74

[a]PG2: diglycerol; PG3: triglycerol; PG4: tetraglycerol.

2.4. Lipase-catalyzed synthesis of OGEs in a solvent free system

Enzymatic esterification was carried out in a solvent-free system. Lipozyme 435, oligoglycerols and linoleic acid were added to a 25 mL airtight flask and reacted at 300 rpm under vacuum conditions (4 kPa) to remove water and avoid the oxidation of the reagents. The effects of different molar ratio of oligoglycerol to linoleic acid (2:1~1:2), enzyme dosage (0.5~3.0 wt% of the total substrate mass), reaction temperature (60~120 °C), reaction time (2~6 h), and water addition (0~12 wt% of the total substrate mass) on the EE were studied.

At the end of each reaction, 8 mL of reaction mixture were taken and centrifuged at 3000 rpm for 10 min to be separated into three layers. The immobilized enzyme was present in the middle level. It can be isolated by filtration and used repeatedly. The upper oil phase was titrated with a 0.1 M NaOH standard solution using phenolphthalein as the endpoint indicator to obtain the acid value. The EE of the reaction was calculated according to the following formula:

$$\mathrm{EE}(\%) = \frac{AV_0 - AV}{AV_0} \times 100$$

Where AV_0 is the acid value of linoleic acid, and AV is the acid value of the upper oil phase. All determinations were carried out in duplicate and the average values were reported.

2.5. Determination of optimal reaction conditions by RSM

To achieve maximal EE, a three-level three-factor Box-Behnken design was adopted to optimize the reaction conditions. The factors studied were enzyme dosage (E_d, wt%), the molar ratio of substrate (S_r, mol/mol) and the reaction time (T_i, h). The response studied was the efficiency of esterification (EE, %). The ranges of settings for factors were based on the results of single factor experiments and shown in Table 2. The initial reaction temperature was fixed at 90 °C for all experiments. All of the experiments

TABLE 2. Independent variables and levels used in the Box-Behnken design

	Levels		
Independent variables	**1**	**0**	**−1**
T_i (time, h)	5	4	3
S_r (molar ratio of substrate, mol/mol)	2	1.5	1
(enzyme dosage, wt%)	2	1.5	1

were performed in triplicate and the average values were reported. Design Expert software (version 7.0, Stat-ease, Inc.) was used for the analysis of variance (ANOVA) and regression analysis of the experimental data.

2.6. Scaling-up synthesis of OGEs in the BCR

The scaling-up synthesis of OGEs was performed in a 2 L pilot-scale BCR (Figure 1) manufactured by Handway Technology Co., Ltd. (Foshan, China) and the Department of Food Science and Engineering, Jinan University (Guangzhou, China). Oligoglycerols (300.00 g), linoleic acid (267.85 g) and Lipozyme 435 (11.36 g) were added to the reactor vessel and reacted under the optimum conditions (as obtained from section 2.5). N_2 gas was bubbled through the sieve at the bottom of the reactor where it entered into the reaction system and formed a boiling-like mixture. With this gas bubbling, the heterogeneous reaction system was vigorously agitated. Therefore the immiscible reactants and immobilized enzymes were efficiently mixed by the N_2 bubbling to enhance the reaction rate. The water formed by the reaction can be efficiently removed by the N_2 flow (2.5 $L \cdot min^{-1}$) and collected by the condenser. The removal of water was beneficial to shift the thermodynamic equilibrium to the esterification. The N_2 gas pumped through the reactor was recycled to save on its consumption. After the reaction, the liquid reaction mixture was collected through the sieve. The immobilized enzyme was retained in the reactor, washed by anhydrous ethanol, and used for the next batch reaction under the same conditions. The product collected was centrifuged and separated into two layers. The upper layer was the OGEs phase which was analyzed by HPLC and ESI-MS.

2.7. The reusability of Lipozyme 435 in BCR

A distinct advantage of the BCR is the lower mechanical shear force which allows for maintaining a considerably longer half-life of enzyme. In this study, 10 consecutive batches of OGEs production were conducted under the optimized conditions to assess the reusability of Lipozyme 435. The relative activity of enzyme was defined as follows:

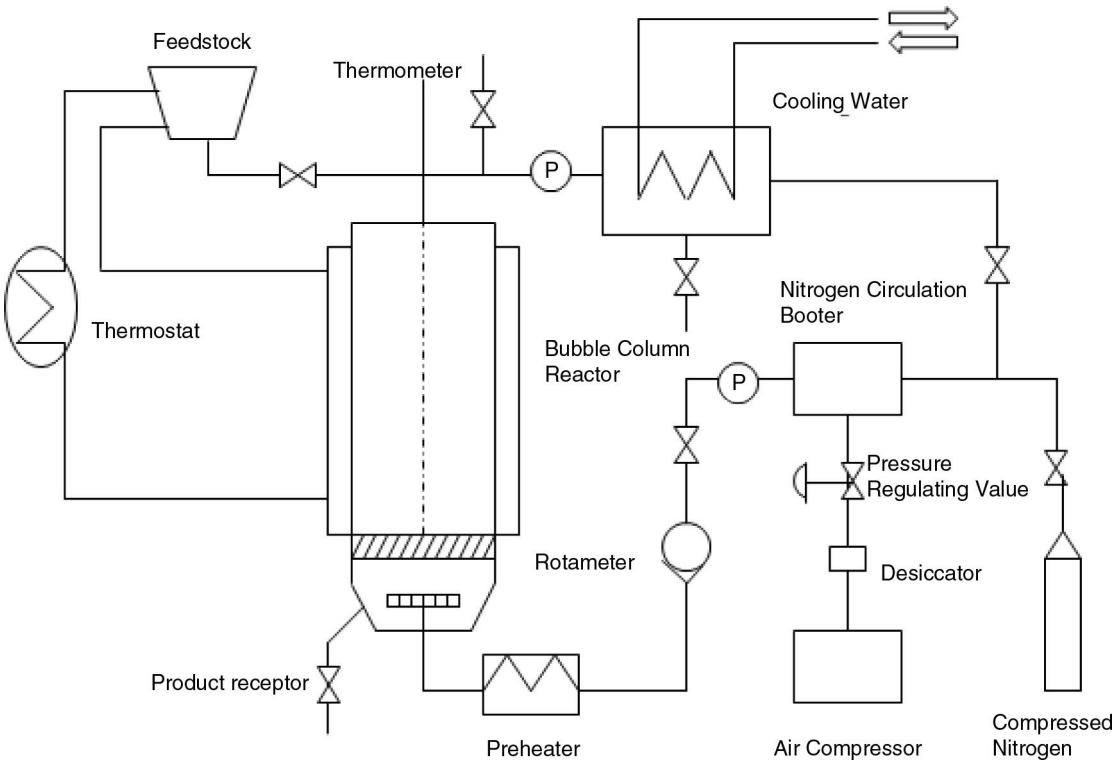

FIGURE 1. Simplified flow diagram of the bubble column reactor equipment.

$$\text{Relative activity (\%)} = \frac{\text{EE of each batch}}{\text{EE of the 1st batch}} \times 100$$

2.8. Composition of the esterification products analyzed by HPLC and ESI-MS

High performance liquid chromatography (HPLC) and ESI-MS were employed to analyze the composition of the esterification products. The HPLC system was composed of a Shimadzu HPLC system (LC-20AD pump, SIL-20A auto injector) equipped with a 150 mm ×4.6 mm Diamonsil C18(2) column (DIKMA, Beijing, China) and a Sedex 55 evaporative light scattering detector (ELSD) (SEDERE, France). The ELSD was set to 70 °C with a carrier air flow rate of 1.7 $L \cdot min^{-1}$. The column temperature was set to 40 °C. A mixture of acetonitrile/isopropyl alcohol (55/45, v/v) was delivered as the mobile phase at a flow rate of 1.0 $mL \cdot min^{-1}$. The samples were dissolved in isopropyl alcohol with a concentration of 5.0 $mg \cdot mL^{-1}$. About 1 mL of this sample solution was filtered through a nylon membrane filter (0.22 μm), transferred to a 1.5 mL sample vial, and analyzed by HPLC. The injection volume of each analysis was 0.5 μL. Each sample was analyzed twice and the average value was reported. The content of each ester compound was estimated by calculating the peak areas obtained from the ELSD chromatogram assuming the same response factors.

The MS spectrum was recorded with an AB 4000 QTRAP mass spectrometer (USA) equipped with an ESI source. The samples were dissolved in methanol and were delivered to the ESI source with a syringe pump at a flow rate of 10 $\mu L \cdot min^{-1}$. The operating parameters were as follows: ions spray voltage 5500 V, capillary voltage 40 V, nebulizing gas pressure 30 psi, and capillary temperature 250 °C. Mass spectra within a mass range of 100~2000 m/z were acquired in the positive ion mode. The compositions of OGEs were identified by measuring their m/z values.

3. RESULTS AND DISCUSSION

3.1. Effect of reaction temperature

The effect of reaction temperature on the EE was studied at the range of 60~120 °C for 5 h with an oligoglycerol/linoleic acid molar ratio at 1.5:1 and an enzyme dosage of 3 wt%. As seen from the Figure 2a, The EE soared from 76.6% at 60 °C to 95.0% at 90 °C and plateaued between 90 °C and 110 °C, suggesting that the reaction temperature had a dramatic influence on the esterification reaction. This may be explained by the effect of temperature on the enzyme activity and the affinity of the enzyme for substrate (Eom *et al*., 2010). The appropriately elevated reaction temperature was very effective in improving the mass transfer by increasing the activity of the enzyme and the diffusion of the substrate. However, the EE declined rapidly above 110 °C, indicating that thermal deactivation of the enzyme had taken place. This deactivation was attributed to the destroying of the spatial conformation and structure of the enzyme at high temperatures.

According to the literature (Duan *et al*., 2013, Pan *et al*., 2013), the optimal reaction temperature of Lipozyme 435 is between 60~70 °C, and above 70 °C the stability of the enzyme is reduced as a result of changing the secondary and tertiary structure of the protein. Few data are found in the literature regarding the reaction temperature at an excess of 80 °C in enzyme catalyzed reactions. Nevertheless, here the reaction was performed above 90 °C, and high EEs (>95%) were achieved. The result indicated that Lipozyme 435 is a thermostable enzyme and can react at above 90 °C. This can be explained by the fact that the immobilization of the lipase makes the enzyme conformation stable and hence enhances its thermal stability. On the other hand, due to the high viscosity of oligoglycerol, it was necessary to raise the reaction temperature in order to reduce the viscosity of the reaction system and to increase the solubility of oligoglycerol in the fatty-acid phase. However, with the increase in temperature, the possibility of water-stripping from the enzyme surface was increased. So the optimal reaction temperature was selected at 90 °C for subsequent experiments.

3.2. Effect of reaction time

To determine the optimal reaction time, esterification reactions with different reaction times (2~6 h) were conducted at 90 °C and an enzyme dosage of 3 wt% with an oligoglycerol/linoleic acid molar ratio of 1.5:1. As shown in Figure 2b, the EE increased from 91.90% at 2 h to 95.51% at 3 h, and it did not improve significantly afterwards, indicating that the equilibrium state had been reached.

Compared to the data reported by Pan *et al*. (2013), although here the reaction was carried out at a lower enzyme dosage (3 wt% vs. 6.1 wt%), the equilibrium was reached in a shorter time period (3 h vs. 24 h) and the EE obtained was even higher (95.51% vs. 92.4%). Several reasons could explain this: (a) the esterification reaction here was carried out in a solvent-free system. This increased substrate concentrations and avoided the enzyme deactivation caused by organic solvents (e.g. methanol) which were beneficial for achieving a fast reaction rate (Shimada *et al*., 2002). (b) A higher reaction temperature (90 °C) was adopted in this study. This decreased the viscosity of the reaction mixture, improved the diffusion, and hence enhanced the accessibility of the reagents to the active-site of the enzyme. (c) It has been reported that Lipozyme 435 is more selective to fatty acids with a certain chain length and degree of

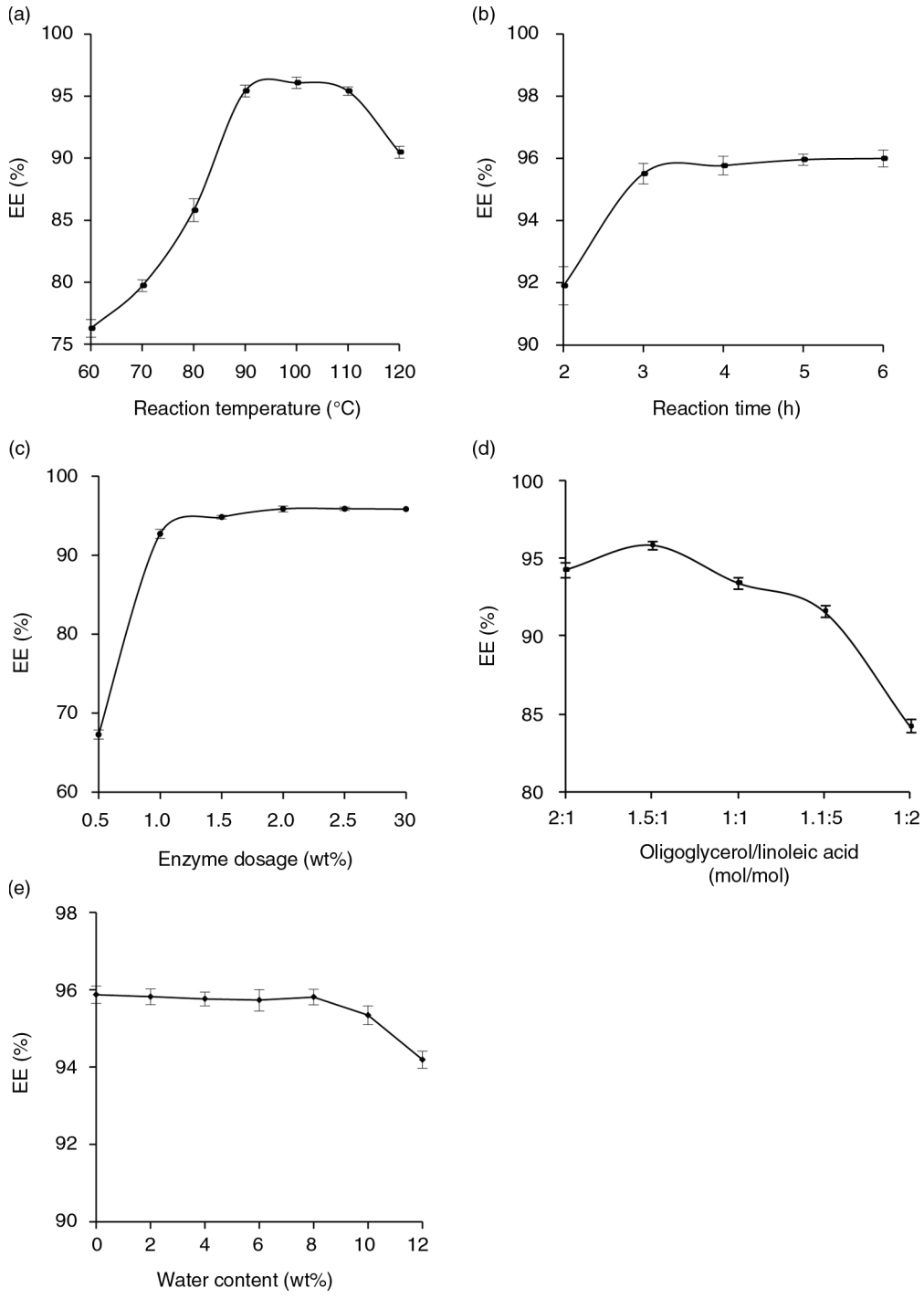

FIGURE 2. Single factor experiments of lipase-catalyzed esterification of oligoglycerol with linoleic acid. The efficiency of esterification (EE) (means±SD, n=3) was effected by: (a) Reaction temperature (oligoglycerol/linoleic acid molar ratio at 1.5:1, enzyme dosage 3 wt%, reaction time 5 h); (b) Reaction time (oligoglycerol/linoleic acid molar ratio at 1.5:1, enzyme dosage 3 wt%, reaction temperature 90 °C); (c) Enzyme dosage (oligoglycerol/linoleic acid molar ratio at 1.5:1, reaction temperature 90 °C, reaction time 3 h); (d) Mole ratio of oligoglycerol to linoleic acid (reaction temperature 90 °C, reaction time 3 h, enzyme dosage 2 wt%); (e) Initial water content (oligoglycerol/linoleic acid molar ratio at 1.5:1, reaction temperature 90 °C, reaction time 3 h, enzyme dosage 2 wt%).

unsaturation (Zou and Akoh, 2013), thus it might be possible that the enzyme could show a higher activity to linoleic acid. (d) The use of vacuum conditions to avoid linoleic acid oxidation also removed the water formed by the reaction, which drove the equilibrium to form the ester products.

3.3. Effect of enzyme dosage

To investigate the effect of the enzyme dosage on the EE, experiments with various enzyme dosage (0.5~3 wt%) were performed for 3 h at 90 °C with an oligoglycerol/linoleic acid molar ratio of 1.5:1 (Figure 2c). It can be seen that the EE soared from 67.29% to 92.69% when the enzyme dosage was increased from 0.5 wt% to 1 wt%, and it plateaued at 95.85% with the enzyme dosage of 2 wt%.

Higher enzyme dosages enhanced the effective contact area between enzyme and substrate molecules and provided abundant active sites as a result of increasing the probability of substrate enzyme collision, which were conducive to the reaction occurring (Zhong *et al*., 2013). However, the contacting area between the enzyme and substrates reached saturation when an excessive amount of enzyme was added. It led to the incomplete exposure of the lipase to the substrates at the active site. So a further increase in the enzyme dosage had little effect on the EE. Thus, 2 wt% was chosen as the optimal enzyme dosage in this esterification reaction.

3.4. Effect of oligoglycerol/linoleic acid molar ratio

The esterification of oligoglycerol with different molar ratios of linoleic acid (2:1~1:2) has been carried out for 3 h at 90 °C at the enzyme dosage of 2 wt%. It can be seen from Fig. 2d that the maximum EE (95.87%) was obtained at a molar ratio of oligoglycerol/linoleic acid of 1.5:1.

In previous studies, the effect of glycerol/fatty acid molar ratio on acylglycerol synthesis has been reported. According to Byun *et al*. (2007), a large excess of glycerol could improve the yield of monoacylglycerol (MAG) (68 wt%) by the esterification of fish oil fatty acids with glycerol, because the esterification equilibrium was displaced toward product formation. Brady *et al*. (1988) pointed out that increasing the concentration of glycerol can greatly improve enzyme stability. However, in this study, the EE was overall decreased with the increasing amount of oligoglycerol. This is possibly due to the higher viscosity of oligoglycerol compared with that of glycerol. High viscosity of the reaction mixture prevented the diffusion of substrates and hindered the removal of water formed by the esterification. As will be discussed in the next section, high amounts of water had a negative effect on EE. On the other hand, high amounts of linoleic acid also decreased EE. This might be explained by the acidification of the micro-aqueous interface of the enzyme which would inhibit the enzyme activity (Chowdary *et al*., 2000). Previous investigations have reported that free fatty acids had a strong inhibition effect on enzyme activity when the substrate acid concentration rose above a critical level (Duan *et al*., 2013). Therefore, in this research the optimal oligoglycerol/linoleic acid molar ratio was 1.5:1.

3.5. Effect of water content

To explore the effect of water content (0–12 wt% of the substrate) on the EE, experiments were carried out under the optimum conditions established from the above discussion (90 °C, 3 h, oligoglycerol/fatty acid molar ratio 1.5:1, enzyme dosage 2 wt%). As displayed in Figure 2e, the EE had a tendency to decline with increased water content.

Theoretically, the appropriate amount of water is essential to maintaining the enzyme activity (Wang *et al*., 2009). However, as Lipozyme 435 is a type of immobilized lipase that already has a certain content of water, and it can maintain activity without the addition of extra water (Zhong *et al*., 2013). On the contrary, the addition of extra water reduced the enzyme concentration and affected the hydration of the enzyme directly, or indirectly, by changing the nature of lipase support materials (Freitas *et al*., 2007), which resulted in a decrease in enzyme activity. On the other hand, esterification is the reverse reaction of hydrolysis. Excessive water accumulated during the progression of the reaction is favorable for hydrolysis rather than esterification. Therefore the removal of water shifted the equilibrium toward the formation of desired products. So in this study, the vacuum condition was applied to remove water and ensure a high EE.

3.6. Model fitting of RSM

Based on the above individual studies, RSM using the Box-Behnken design (Table 2) was applied to obtain the optimal reaction conditions. The EE obtained from all the experiments are given in Table 3. Model fitting and ANOVA results (Table 4) show that the quadratic polynomial model was suitable (F-value=84.9 at $p<0.001$, $R^2=0.9909$) for representing the actual relationship of reaction parameters. The insignificant lack of fit test (F-value=0.26) with $p>0.05$ also indicated that the model represented the experimental data well. The coefficient of determination (R^2) and adjusted-R^2 were 0.9909 and 0.9793, respectively, suggesting that the polynomial model used was accurate. Multiple regression coefficients obtained by the method of least squares to predict a second-order polynomial model for the EE are presented in Table 4. The corresponding p values of coefficients suggested that T_i (reaction time), S_r (substrate molar ratio), E_d (the amount of enzyme), $T_i \times E_d$ (substrate molar ratio×the amount of enzyme), S_r^2 (square of substrate molar ratio), E_d^2 (square of the enzyme dosage) were significant variables with $p<0.05$. The remaining terms had no significant effects on EE. Furthermore, from Table 4 it can be concluded that EE was influenced

TABLE 3. Three-level three-factor Box-Behnken design test results[a]

Experiment No.	Factors			EE (%)
	T_i	S_r	E_d	
1	−1	−1	0	91.75
2	−1	1	0	94.26
3	0	−1	−1	89.80
4	1	−1	0	92.61
5	0	0	0	95.36
6	−1	0	1	95.85
7	1	0	−1	93.68
8	0	0	0	94.86
9	0	0	0	95.12
10	1	1	0	94.82
11	0	0	0	94.97
12	1	0	1	96.06
13	0	1	1	95.05
14	0	0	0	95.56
15	0	−1	1	93.64
16	−1	0	−1	92.81
17	0	1	−1	93.69

[a]EE: efficiency of esterification.

in the following order of significance: $E_d > S_r > T_i$. The second-order polynomial model for the EE (Y, %) is shown in the following equation:

$$Y=95.17+0.31A+1.25B+1.33C-0.075AB-0.16AC-0.62BC-0.13A^2-0.168B^2-0.44C^2$$

Where Y is the EE; A, B, and C are the independent variables. Here A is T_i, B is S_r, and C is E_d.

3.7. Optimization of the reaction

The optimal conditions for the synthesis of OGEs by Lipozyme 435 were predicted by Design-Expert software. When esterification was catalyzed by Lipozyme 435 under the optimal conditions (90 °C, 4.52 h, enzyme dosage 2 wt%, oligoglycerol/linoleic acid =1.59 in moles), the predicted EE based on the model was 96.15%. The small deviation between the optimal conditions and those local optimal ranges from contour plots (Figure 3) is possibly due to the interaction effects of various experimental factors. To further verify the reliability of the regression model, three independent experiments were performed at the predicted optimum conditions, and the actual EE was 95.82±0.22%, proving the high accuracy of the RSM model used.

3.8. Production in BCR

According to Mollenhauer *et al*. (2010), for a heterogeneous reaction system with high viscosity, a BCR is more suitable than a conventional stirred tank or a fixed bed reactor. In this study, a BCR as shown in Figure 1 was employed to scale-up the synthesis of OGEs, and the bubbling of N_2 gas was used as a means of agitation for the reaction mixture. The optimal reaction conditions obtained by RSM were applied to BCR. As expected, the EE achieved was higher than that of a conventional magnetic stirred reactor (97.23% vs. 95.82%). In the

TABLE 4. Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	Sum of Squares	*df*	Mean Square	F Value	Pr>F	Significant
Model	42.51	9	4.72	84.93	<0.0001	*
T_i	0.78	1	0.78	14.05	0.0072	*
S_r	12.55	1	12.55	225.68	<0.0001	*
E_d	14.10	1	14.10	253.52	<0.0001	*
$T_i \times S_r$	0.023	1	0.023	0.40	0.5449	
$T_i \times E_d$	0.11	1	0.11	1.96	0.2044	
$S_r \times E_d$	1.54	1	1.54	27.65	0.0012	*
T_i^2	0.071	1	0.071	1.27	0.2970	
S_r^2	11.95	1	11.95	214.85	<0.0001	*
E_d^2	0.83	1	0.83	14.96	0.0061	*
Residual	0.39	7	0.056			
Lack of Fit	0.063	3	0.021	0.26	0.8545	
Pure Error	0.33	4	0.082			
Cor Total	42.90	16				
R^2	0.9909					
Adj. R^2	0.9793					

*P<0.05

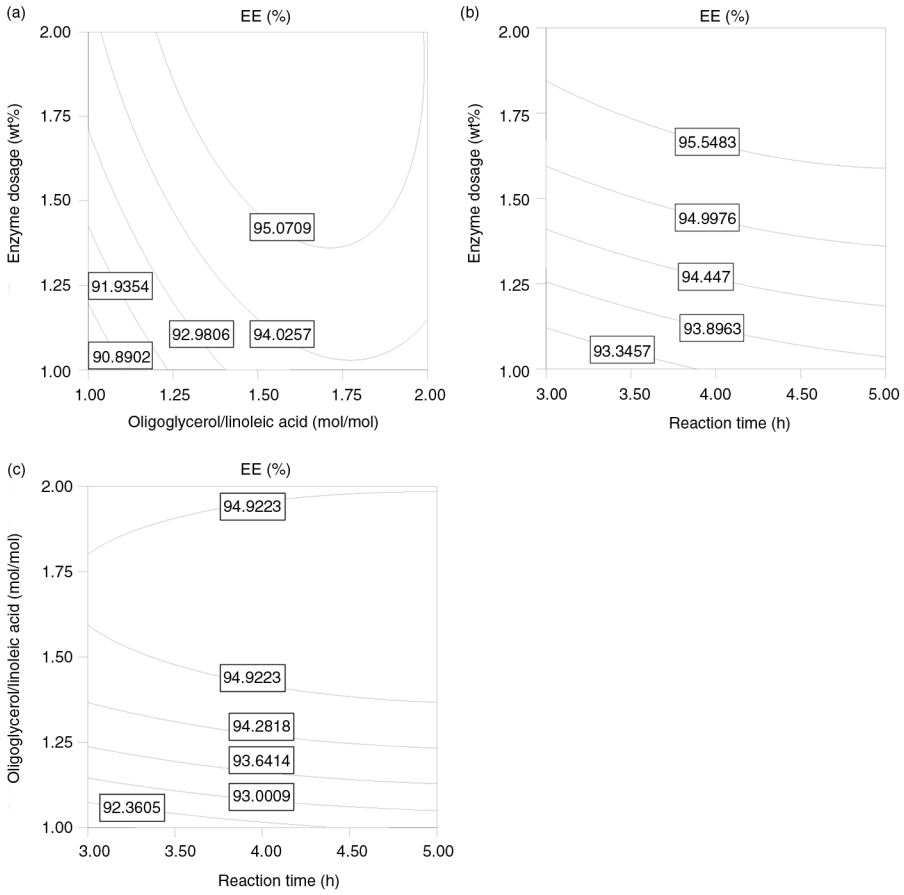

FIGURE 3. Contour plots of interactions among enzyme amounts, oligoglycerol/linoleic acid molar ratio and reaction time. Plot of EE as a function of (a) enzyme dosage and substrate molar ratio for reacting 4.0 h, (b) enzyme dosage and reaction time with a molar ratio of oligoglycerol/linoleic acid at 1.5, (c) oligoglycerol/linoleic acid molar ratio and reaction time with the amount of enzyme fixed at 1.5 wt%.

operational stability test, Lipozyme 435 still maintained 98.2% of the relative activity after 10 consecutive batch reactions (Figure 4). So in a high viscosity reaction system, agitation by N_2 bubbling can give higher reaction efficiency than by magnetic stirring. That may be because the bubbling of N_2 ensured a convective flow field and created an effective interaction in the multiple-phase reaction system to provide a high mass-transferring rate. The excellent operational stability of Lipozyme 435 also benefitted from the gentle agitation and the protective atmosphere provided by N_2 bubbling. In comparison, magnetic stirring often mechanically destroys the structure of the enzyme carrier and hence reduces the activity (Mollenhauer *et al*., 2010). Therefore, production in BCR is a preferred way to achieve a higher yield of OGEs.

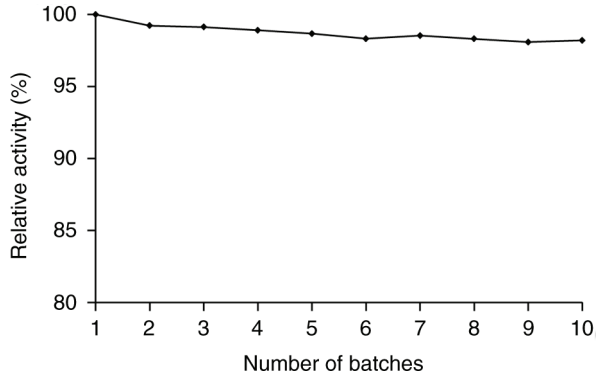

FIGURE 4. The reusability of Lipozyme 435 in bubble column reactor under the optimal conditions (4.52 h, 90 °C, 2 wt% based on the substrate mass, 1.59:1 of oligoglycerol/linoleic acid molar ratio).

3.9. Composition of the OGE product

The OGE product obtained under optimal conditions was analyzed by HPLC. As reported by Cassel *et al*. (2001), the retention time in the reversed-phase HPLC mode increased as more fatty acid chains were attached to polyglycerol. The four groups of peaks (A~D in Figure 5) represent the mono-, di-, tri-, and tetraesters of oligoglycerols according to

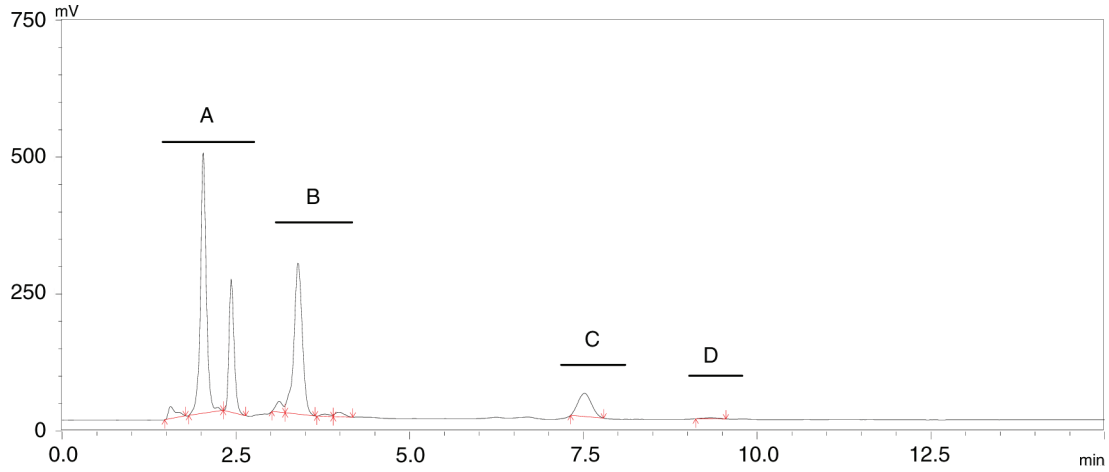

FIGURE 5. HPLC chromatogram of the OGE production catalyzed by Lipozyme 435 under the optimal conditions (4.52 h, 90 °C, 2 wt% based on the substrate mass, 1.59:1 of oligoglycerol/linoleic acid molar ratio). A: monoesters; B: diesters; C: triesters; D: tetraesters of oligoglycerols.

the reversed-phase HPLC theory and the polarity of products (Andersen *et al*., 2003). By integrating the peak areas in the ELSD chromatogram, it was found that the OGE product contained 56.92% monoesters, 34.94% diesters, 7.74% triesters, and 0.39% tetraesters of oligoglycerols.

To obtain more details about the OGE product, an ESI-MS spectrum recorded in the positive ion mode was used to analyze the product obtained under optimal conditions (Figure 6). The spectrum contains a wide range of peaks corresponding to the characteristic ions of OGEs. Over 10 OGE species were observed, including glycerol moieties ranging from n=2~4. Based on the ion intensities, the OGE species with n=2 and 3 were the most abundant, which was in agreement with the result of the oligoglycerol composition analyzed by GC. Due to the small vapor pressure difference between PG3 and PG4, after distillation there was still a little PG4 (4.41 wt%) remaining in the final products. Thus, OGE species with n=4 were also found with low ion intensity. Besides the OGE species as sodium and ammonium adducts, their water adducts and dehydrated forms were also detected in the spectrum (Figure 6). According to Orfanakis *et al*. (2013), the dehydrated species were not formed as the result of the

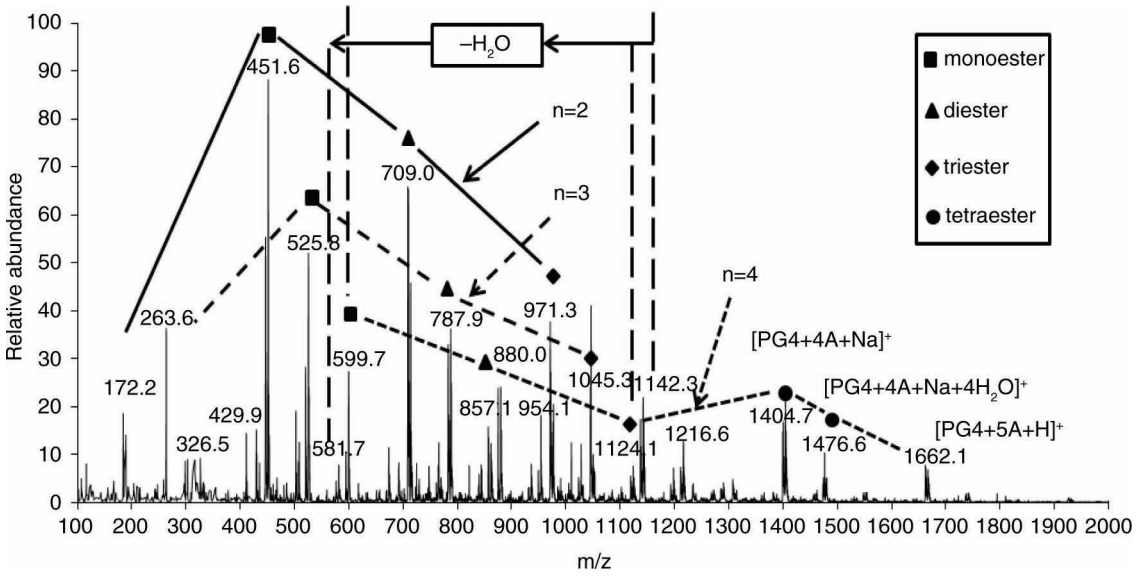

FIGURE 6. ESI-MS spectra of OGEs product in positive ion mode (degree of glycerol polymerization n=2~4). PG4: tetraglycerol; A: Linoleic acid.

ESI process. It is worth mentioning that the multiple water adducts of PG4-tetraester ($[M+Na+4H_2O]^+$, m/z=1476.6) were also observed. It indicated that the intermolecular hydrogen bonding was formed between the water molecules and hydroxyl groups due to the high density of the hydroxyl groups on the PG4-tetraester. The water molecules may come from the esterification or from the immobilized enzyme (as stripped in the carrier material).

Linoleic acid appears to react completely as its signal cannot be observed from the spectra. The mono-, di- and triester of oligoglycerol ions all showed high intensities in the spectrum. The intensity of monoesters was greater than the other ions of OGEs. According to the literature (Byun *et al*., 2007, Liu *et al*., 2007), the formation of triacylglycerol (TAG) was much less than that of MAG and diacylglycerol (DAG) when sn-1,3-specific lipases were used for the esterification of glycerol with fatty acids. The small amount of TAG might result from the acyl migration to the 2-position in 1,3-DAG, which requires higher amounts of energy to overcome the steric hindrance for further esterification (Liu *et al*., 2007). This isomerization reaction was considered to be the confined step of TAG synthesis. However, here the amount of triesters was significant. This may be due to the fact that Lipozyme 435 is a nonspecific lipase, thus the acyl donor can be esterified at any available hydroxyl groups in the oligoglycerol and shows higher activities in direct esterification. On the other hand, PG4-tetraester ($[PG4+4A+Na]^+$, m/z=1404.7, A=Linoleic acid) and PG4-pentaester ions ($[PG4+5A+H]^+$, m/z=1662.1) were detected with low intensities, while none of the PG2- and PG3-tetraester ions was observed in the spectra. It seems that the esterification occurred more easily between linoleic acid and PG4 than PG2 or PG3. This is possibly because PG4 has more hydroxyl groups so that the reaction with PG4 has a less prominent steric hindrance issue. However, it was clear that esterification was incomplete as none of the fully esterified OGE ions was detected.

4. CONCLUSION

The biosynthesis of OGEs was investigated using Lipozyme 435 in a solvent-free reaction system. The RSM based on a three-level three-factor Box-Behnken design was used to optimize the reaction parameters. An EE of 96.15% was achieved under the optimal reaction conditions: 90 °C, 4.52 h, enzyme dosage 2 wt% (based on the total substrate mass), oligoglycerol/linoleic acid molar ratio 1.59:1, with no water addition. These conditions were successfully applied in the BCR, and a higher EE was achieved compared with that from the magnetic stirring system. Moreover, the enzyme showed little reduction in activity after reacting for 10 batches. In the large-scale preparation of OGEs at high yield, short reaction time, less consumption of N_2 and excellent operational stability of Lipozyme 435 gave the BCR a promising potential for the industrial scale production of OGEs in a high-viscosity heterogeneous reaction system.

Due to the highly complex composition of OGE products, chromatographic analysis methods cannot quantify all these components directly. However, the product compositions were qualitatively determined by ESI-MS. The product analysis indicates that esterification occurred to a significant extent and the reaction product is mainly composed of mono- (56.92%), di- (34.94%) and triesters (7.74%) of oligoglycerols, which is preferred for giving good emulsifying properties. To further study the structure of OGEs, the separation and purification of esterification products need to be investigated in depth.

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