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Enzymatically Catalyzed Synthesis of Low-Calorie Structured Lipid in a Solvent-free System: Optimization by Response Surface Methodology

Lu Han, Zijian Xu, Jianhua Huang, Zong Meng, Yuanfa Liu,* and Xingguo Wang*

School of Food Science and Technology, Jiangnan University, State Key Laboratory of Food Science and Safety, 1800 Lihu Road, WuXi 214122, Jiangsu, People's Republic of China

ABSTRACT: A kind of low-calorie structured lipid (LCSL) was obtained by interesterification of tributyrin (TB) and methyl stearate (St-ME), catalyzed by a commercially immobilized 1,3-specific lipase, Lipozyme RM IM from *Rhizomucor miehei*. The condition optimization of the process was conducted by using response surface methodology (RSM). The optimal conditions for highest conversion of St-ME and lowest content LLL-TAG (SSS and SSP; S, stearic acid; P, palmitic acid) were determined to be a reaction time 6.52 h, a substrate molar ratio (St-ME:TB) of 1.77:1, and an enzyme amount of 10.34% at a reaction temperature of 65 °C; under these conditions, the actually measured conversion of St-ME and content of LLL-TAG were 78.47 and 4.89% respectively, in good agreement with predicted values. The target product under optimal conditions after short-range molecular distillation showed solid fat content (SFC) values similar to those of cocoa butter substitutes (CBS), cocoa butter equivalent (CBE), and cocoa butters (CB), indicating its application for inclusion with other fats as cocoa butter substitutes.

KEYWORDS: reduced-calorie structured lipid, interesterification, Lipozyme RM IM, response surface methodology (RSM)

INTRODUCTION

Presently, the most familiar class of low-calorie structured lipids (LCSL) is SALATRIM (short and long acyl triacylglyceride molecules), which is characterized by a combination of short-chain (C_{2-4}) and long-chain (C_{16-22}) acyl residues into a single triacylglycerol structure. The caloric availability of the tested SALATRIM molecules was determined to be approximately 5 kcal/g[1] lower than that of other edible oils (9 kcal/g).

There are two types of triacylglycerol (TAG) structures in SALATRIM, one composed of two short-chain and one long-chain acyl moiety on the glycerol (SSL-TAG) and another composed of two long-chain and one short-chain acyl moiety (LLS-TAG). Varieties of products useful in food applications can be attained by designing the fatty acid composition and ratio of SSL- to LLS-TAG. For example, they can used in baking chips, coatings, dips, and baked products or as cocoa butter substitutes.[2]

In previous papers, Fumoso et al. synthesized a SALATRIM through the acidolysis of triolein by acetic acid and butyric acid in *n*-hexane media, whereas this organic was bad for health and increased industrial cost.[3] Two SALATRIM products were produced by Foglia et al. with a new biocatalyst, *Carica papaya* lipase, which is special for its sn-3 stereoselectivity and strong short-chain fatty acyl selectivity. In addition, it is very inexpensive and accessible.[4,5] Absorption of long-chain fatty acid by the human body is determined by its stereoposition on TAG and the presence of calcium and magnesium in the diet.[6,7] When stearic acid is located at the sn-2 position on TAG, the resultant sn-2 monostearin after hydrolysis by pancreatic lipase is well absorbed.[8,9] Because one of the raw material used in these papers is hydrogenated soybean oil, which composed mainly of long chain fatty acids, the interesterification product contains large quantities of triglycerides with long chain fatty acids in sn-2 position is negative for low calorie target. Xuelin et al. carried out esterification of glycerol with three types of fatty acid. Sodium methoxide was used as chemical catalyst, leading to random positional distribution of fatty acids and increased reaction temperature and energy consumption. The following detoxication and purification were also troublesome.[10]

Although some low-calorie fats were produced, the study of their application was not very common and few were obtained to simulate cocoa butter (CB) analogue fat. Vivienne et al. synthesized a low-calorie fat that had possible use in spreads or for inclusion with other fats in specialized blends.[11] In our study, a mixture of low-calorie triacylglycerols was produced in a solvent-free system by interesterification of tributyrin (TB) and methyl stearate (St-ME). Compared with stearic acid, St-ME accelerates the rate of interesterification and has a lower melting point, in which case the bad effect of high temperature on enzymatic activity can be avoided. Lipozyme RM IM was selected as the catalyst. It is an immobilized form of lipase from *Rhizomucor miehei* (RML) with high activity and good stability under different experimental conditions. It has been widely used in the food industry and in the energy and organic chemicals industries, especially in the modification of oils, fats, or free fatty acids.[12,13] The broad application in this area relies on its several advantages: the sn-1,3 specificity makes the production with expected features easy and reduces the amount of side products; also, the mild reaction condition reduces energy consumption.

According to some studies, the Lipozyme RM IM-catalyzed interesterification could be adjusted to a ping-pong Bi−Bi mechanism as shown in Figure 1.[14]

The enzyme first binds on substrate. The resulting enzyme−substrate complex then releases the first product species and is

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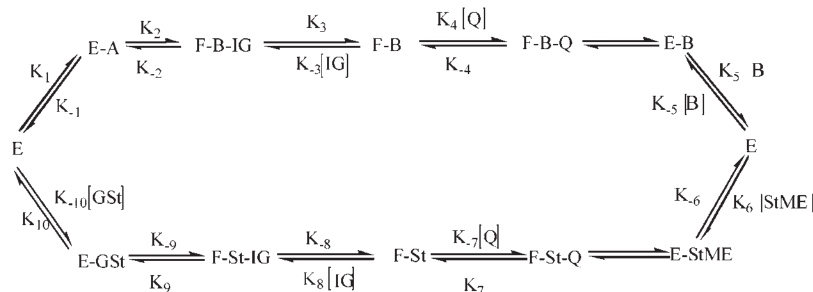

Figure 1. Schematic representation of the Michaelis−Menten mechanism for interesterification. A, native ester bond in tributyrin; B, fatty acid methyl ester formed from a residue liberated from the original tributyrin; Q, methanol; IG, low-acylglycerol intermediate; St, stearic acid; St-ME, methyl ester of St; GSt, acylglycerol containing the new ester bond formed with the acyl group of St; E, uncomplexed nonacylated form of enzyme; F, acylated form of enzyme; E-X, complexed form of the nonacylated form of the enzyme with species X; F-Y-Z, complex of species Z with the form of the enzyme acylated by species Y.

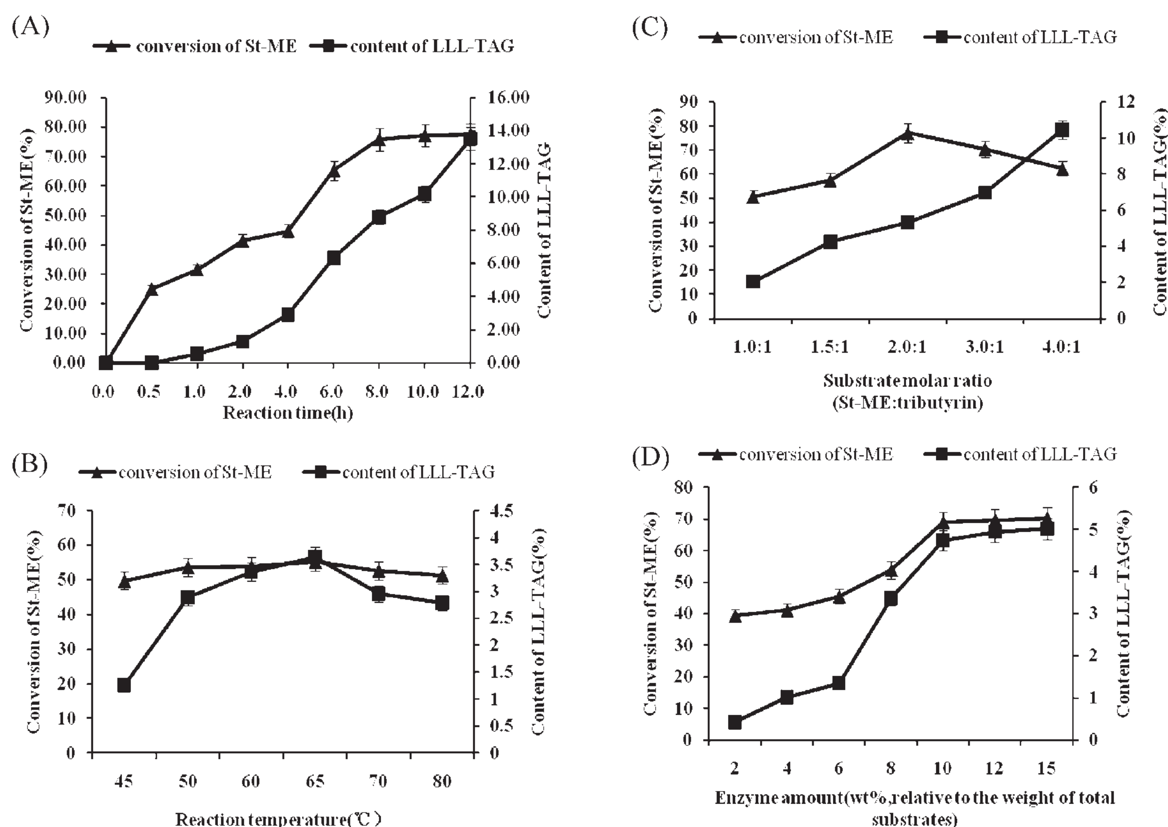

Figure 2. Effects of reaction time, reaction temperature, substrate molar ratio (St-ME: TB), and enzyme amount (relative to the weight of total substrates) on the conversion of St-ME (◆) and the content of LLL-TAG (■): (A) reaction temperature = 55 °C, substrate molar ratio (St-ME:TB) 2.0:1, enzyme amount (relative to the weight of total substrates) = 8%; (B) reaction time = 5 h, substrate molar ratio (St-ME:TB) = 2.0:1, enzyme amount (relative to the weight of total substrates) = 8%; (C) reaction time = 6 h, reaction temperature = 55 °C, enzyme amount (relative to the weight of total substrates) = 10%; (D) reaction time = 5 h, reaction temperature = 55 °C, substrate molar ratio (St-ME:TB) = 2.0:1.

simultaneously transformed to another form of enzyme−substrate complex. The next step involves binding of the second substrate to the transformed enzyme−substrate complex to form another complex. Subsequent breakdown of the complex leads to release of a second product species and the free enzyme.[15] Monoglycerides and diglycerides are present during the interesterification. Acyl migration happens easily in them, which is why LLL-TAG (SSS and SSP; S, stearic acid; P, palmitic acid) were formed. From the point of Bloomer et al.[16,17] lipase load, temperature, acyl donor type and lipase type, water content, and reaction time may influence the product. Acyl migration can not be totally avoided in the present system, but it can be decreased to a relatively lower level. A higher enzyme load, lower temperature, and ethyl ester as the acyl donor will favor the reduction of acyl migration.

Response surface methodology (RSM) was applied to reduce the experimental number and help optimize the process.[18] The solid fat content (SFC) of the target product after short-range molecular distillation was studied to evaluate their possible industrial applications.

Table 1. Experimental Data for the Three-Factor, Three-Level Surface Analysis

treatment[a]	reaction time, X_1 (h)	substrate molar ratio,[b] X_2	enzyme amount,[c] X_3(%)	conversion of St-ME (%)	content of LLL-TAG (%)
1	1 (8)[d]	0 (2.0)	−1 (8)	78.11	8.54
2	0 (6)	0 (2.0)	0 (10)	77.17	5.37
3	1 (8)	1 (2.5)	0 (10)	70.99	12.03
4	−1	0 (2.0)	−1 (8)	45.83	3.04
5	−1 (4)	0 (2.0)	1 (12)	69.74	4.53
6	0 (6)	0 (2.0)	0 (10)	77.94	5.21
7	0 (6)	−1 (1.5)	1 (12)	63.96	4.38
8	−1 (4)	1 (2.5)	0 (10)	50.43	3.73
9	0 (6)	−1 (1.5)	−1 (8)	49.75	4.27
10	−1 (4)	−1 (1.5)	0 (10)	54.94	2.54
11	1 (8)	−1 (1.5)	0 (10)	74.45	4.55
12	0 (6)	1 (2.5)	1 (12)	58.65	9.34
13	0 (6)	0 (2.0)	0 (10)	77.03	5.35
14	0 (6)	0 (2.0)	0 (10)	76.89	5.19
15	1 (8)	0 (2.0)	1 (12)	80.04	9.34
16	0 (6)	1 (2.5)	−1 (8)	49.47	7.24
17	0 (6)	0 (2.0)	0 (10)	76.98	5.29

[a] Treatments were run in random order. [b] Substrate molar ratio (St-ME:tributyrin). [c] Enzyme amount (relative to the weight of total substrates). [d] Numbers in parentheses represent actual experimental amounts.

Table 2. Regression Analysis of Variance for Response Surface Quadratic Model (ANOVA) after Backward Elimination Pertaining to the Predicted Conversion of St-ME

source	degrees of freedom	sum of squares	mean square	F value	Prob > F[a]
model	9	2439.98	271.11	639.46	<0.0001
X_1	1	853.88	853.88	1523.40	<0.0001
X_2	1	22.98	22.98	54.21	0.0002
X_3	1	302.95	302.95	714.56	<0.0001
X_1X_2	1	0.28	0.28	0.65	0.4466
X_1X_3	1	120.78	120.78	284.88	<0.0001
X_2X_3	1	6.33	6.33	14.92	0.0062
X_1X_1	1	2.45	2.45	5.79	0.0470
X_2X_2	1	794.43	794.43	1873.82	<0.0001
X_3X_3	1	270.05	270.05	636.95	<0.0001
residual	7	2.97	0.42		
lack of fit	3	2.25	0.75	4.15	0.1015[b]
pure error	4	0.72	0.18		
cor total	16	2442.95			
CV = 1.14%		adj R^2 = 0.9988			

[a] $P < 0.05$ indicates statistical significance. [b] $P > 0.05$ indicates the lack of fit is not significant.

Our investigations found that the reacted St-ME mainly esterified to tributyrin (TB) to replace butyric acid, producing SSL-, SLL-, and LLL-TAGs of high melting point, which was undesired for it tastes like wax when the content of LLL-TAG is >5%. Therefore, the reaction course could be indirectly detected by the values of St-ME conversion and LLL-TAG content.

MATERIALS AND METHODS

Materials. Lipozyme RM IM (from *R. miehei*), a commercially immobilized 1,3-specific lipase, was obtained from Novozymes A/S (Bagsvaerd, Denmark). Tributyrin (purity > 98%) was purchased from J&K Scientific Ltd. (New Jersey). Methyl stearate (purity > 98%, containing 6% methyl palmitate) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). *n*-Hexane, isopropanol, and acetonitrile purchased from J&K Scientific Ltd. were of HPLC purity. All other reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co. Ltd.

Interesterification. Interesterification reactions were performed in 50 mL round-bottom flasks. TB and St-ME at different substrate molar ratios weighed precisely were put into the flasks. Then Lipozyme RM IM was added to the flasks, and this mixture was melted. Flasks were placed in a rotary evaporator (IKA) at 85 rpm and at a certain temperature, which was controlled by a water bath. The rotary evaporator was coupled to a vacuum pump. After a given time, product was recovered after removal of the enzyme. All reactions were duplicated.

Table 3. Regression Analysis of Variance for Response Surface Quadratic Model (ANOVA) Pertaining to the Predicted Content of LLL-TAG

source	degrees of freedom	sum of squares	mean square	F value	Prob > F^a
model	9	104.69	11.63	854.12	<0.0001
X_1	1	53.15	53.15	3902.62	<0.0001
X_2	1	34.44	34.33	2529.27	<0.0001
X_3	1	2.53	2.53	185.87	<0.0001
X_1X_2	1	9.89	9.89	726.29	<0.0001
X_1X_3	1	0.12	0.12	8.74	0.0212
X_2X_3	1	0.99	0.99	72.70	<0.0001
X_1X_1	1	0.25	0.25	18.22	0.0037
X_2X_2	1	0.15	0.15	10.90	0.0131
X_3X_3	1	2.96	2.96	216.99	<0.0001
residual	7	0.095	0.014		
lack of fit	3	0.069	0.023	3.54	0.1268[b]
pure error	4				
cor total	16		6.520E−003		
CV = 1.99%	adj R^2 = 0.9979				

[a] $P < 0.05$ indicates statistical significance. [b] $P > 0.05$ indicates the lack of fit is not significant.

Analysis of Interesterification Product. Analysis of product was performed using a HPLC system (Waters, America) equipped with an Alltech 3300 (Grace Davision Discovery Sciences, America) evaporative light-scattering detector (ELSD). The ELSD was set to 55 °C at an air gas rate of 1.8 mL/min and a gain of 1. The interesterification reaction product was withdrawn and diluted with chloroform, making the solutions 5−10 mg/mL. Mixtures were analyzed by a Waters 2996 HPLC system on a C18 reverse phase column (Waters Corp., Milford, MA) (5 μm, 150 × 4.6 mm) column. Separations were performed with acetonitrile (solvent A) and *n*-hexane/isopropanol (solvent B; 1:1, v/v) as eluent according to the following gradient profile: initial condition 65:35 (A/B), hold for 14 min at a flow rate of 1.0 mL/min, decrease linearly to 40:60 (A/B) over 11 min, and hold for 5 min at a flow rate of 1 mL/min. Total run time was 30 min.

Purification of Interesterification Product. Molecular distillation equipment (KDL1, UIC, Germany) was used to purify the reaction product. The major part of the equipment was constructed from stainless steel. The vacuum system includes a diffusion pump and two vamp pumps. The heating of the evaporator was provided by the jacket circulating heated oil from an oil bath. Repeated distillations at a constant temperature were conducted. The process variables were as follows: distillation temperature, 100 °C; rotate speed of the wiped film, 120 rpm; feed speed, 2 mL/min; absolute pressure, 2 Pa; preheating temperature, 50 °C; condensate temperature, 50 °C. Heavy phase was the target product.

SFC Determination of the Target Product. SFC profiles were determined with an AM4000 MQC NMR Analyzer (Oxford, U.K.). Nuclear magnetic resonance tubes with a 10 mm diameter were filled with approximately 20−25 mm of the target product. The tubes were capped and tempered according to IUPAC method 2.150,[19] which included holding samples at 80 °C for 30 min, at 0 °C for 90 min, at 26 °C for 40 h, and at 0 °C for 90 min.

Statistical Analysis. The experimental data were analyzed by the response surface procedure (Design Expert, State-Ease Inc., Statistics Made Easy, Minneapolis, MN; ver. 5.0.7.1997) to fit the following second-order polynomial model predicted for optimization of St-ME conversion and LLL-TAG content:

$$Y \underset{i=1}{\overset{3}{=}} \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

Y is one of the two responses, X_i and X_j are the coded independent variables, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept, linear, quadratic, and interactive terms, respectively.

Box−Behnken design for three independent variables was used to obtain the combination of optimization, which allows one to design a minimum number of experimental runs. For the present study, a total of 17 tests were necessary to estimate the coefficients.

RESULTS AND DISCUSSION

Selection of Independent Variables and Their Levels. Figure 2 showed the effects of four independent variables on St-ME conversion and LLL-TAG (SSS and SSP; S, stearic acid; P, palmitic acid) content in the interesterification product. There are two steps in interesterification. First, triacylglycerols are hydrolyzed to monoglycerides and diglycerides; second, new triglycerides are synthesized by the esterification of acyl donors with monoglycerides and diglycerides.[20] Acyl migration happens easily in monoglycerides and diglycerides, so undesired products LLL-TAG formed inevitably. The acyl migration can be treated as linear increases with time.[21] The conversion of St-ME was increased quickly with the reaction time in the first 6 h and then the rate of increase became very slow as the reaction process was brought to equilibrium gradually. The content of LLL-TAG kept increasing slowly with reaction time (Figure 2A). The conversion of St-ME and the content of LLL-TAG both showed increasing−decreasing patterns as the reaction temperature increased (Figure 2B). Obviously, high temperature increases the reaction rate as it reduces the viscosity of the lipid mixture and certainly increases the substrate and product transfer on the surface or inside the enzyme particles. High temperature greatly reduced the enzyme stability and its half-life.[22] In this study, the changes of St-ME conversion or LLL-TAG content were slight, which means the reaction was not influenced much by temperature in the range between 45 and 80 °C. The conversion of St-ME showed increasing−decreasing patterns, whereas the content of LLL-TAG showed increasing pattern as St-ME moles increased. The reason was that higher St-ME moles would raise the reaction equilibrium and increase the ratio of the collision between

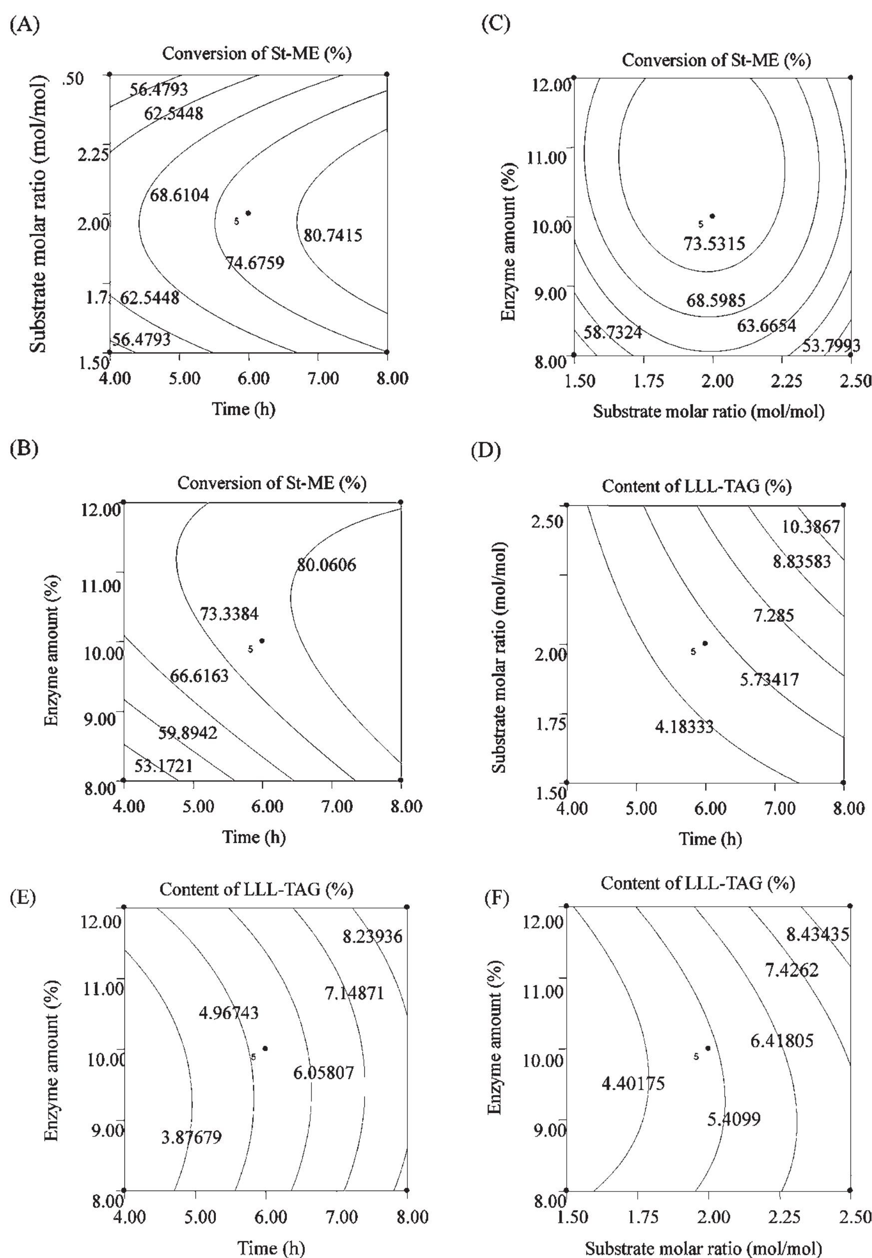

Figure 3. Contour plots of conversion of St-ME (A−C) and content of LLL-TAG (D−F), reaction temperature = 65 °C: (A) enzyme amount (relative to the weight of total substrates) = 10%; (B) substrate molar ratio (St-ME:TB) = 2; (C) reaction time = 6 h; (D) enzyme amount (relative to the weight of total substrates) = 10%; (E) substrate molar ratio (St-ME:TB) = 2; (F) reaction time = 6 h.

substrates and catalyst. When enzyme saturates the interface, there is no more increment (Figure 2C). With other variables fixed, both the conversion of St-ME and the content of LLL-TAG increased, with the enzyme amount increasing first, and then the tendency of increase became very slow at any further increase in enzyme amount for the saturation of enzyme in the interface (Figure 2D).

Overall, reaction time, substrate molar ratio, and enzyme amount had more influence on St-ME conversion and LLL-TAG content. With a set reaction temperature of 65 °C, the lower, middle, and upper levels of the three independent variables were chosen in Table 1.

Model Fitting. Table1 shows the independent variables, their levels, the experimental design, and the observed responses.

The response and variable settings in Table 1 were fitted to each other with multiple regression. The statistics of second-order models for two response variables were calculated (Tables 2 and 3). Y_1 and Y_2 are the predicted values for the conversion of St-ME (%) and the content of LLL-TAG (%), respectively. X_1, X_2, and X_3 are the coded variables as described in Table1.

$$Y_1\ (\%) = 77.20 + 10.33X_1 - 1.70X_2 + 6.15X_3 + 0.26X_1X_2 - 5.50X_1X_3 - 1.26X_2X_3 - 0.76X_1^2 - 13.74X_2^2 - 8.01X_3^2 \qquad (2)$$

$$Y_2\ (\%) = 5.28 + 2.58X_1 + 2.08X_2 + 0.56X_3 + 1.57X_1X_2 - 0.17X_1X_3 + 0.50X_2X_3 + 0.24X_1^2 + 0.19X_2^2 + 0.84X_3^2 \qquad (3)$$

All P values of the coefficient (β) except X_1X_2 in Y_1 for the two models were below 0.05, which implied that the models were

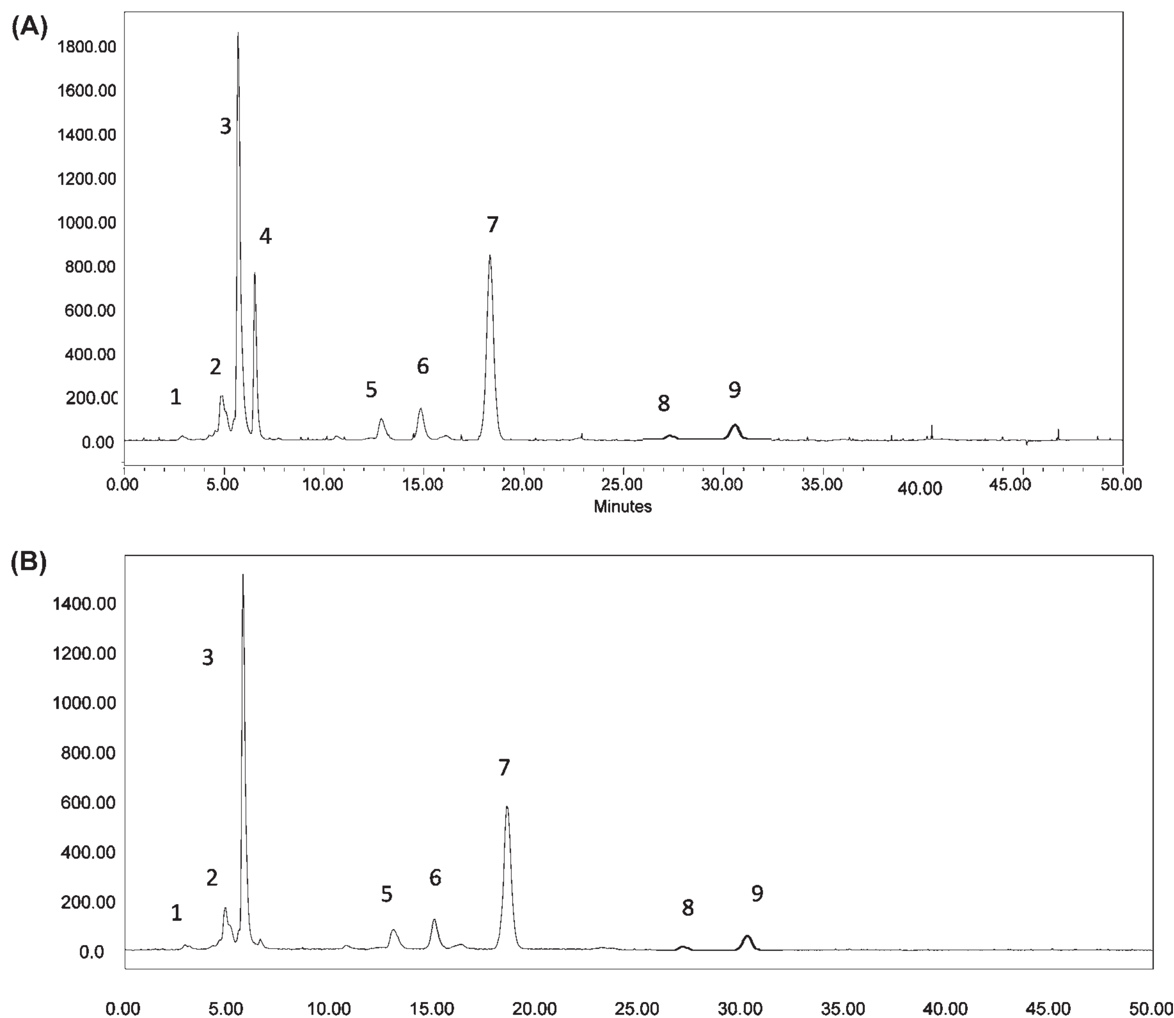

Figure 4. Purification of interesterification product produced under optimal conditions: (A) before purification; (B) after purification. Peaks: 1, BBB; 2, BBP; 3, BBS; 4, St-ME; 5, S diacylglycerols; 6, PBS; 7, SBS; 8, SSP; 9, SSS (B, butyric acid; P, palmitic acid; S, stearic acid).

statistically significant and adequate to explain most of the variability. To support hierarchy, X_1X_2 in Y_1, despite insignificance, was not eliminated from the model. The coefficients determination (R^2) of the models for conversion of St-ME and content of LLL-TAG were 0.9988 and 0.9991, respectively, indicating that the models adequately represented the real relationships among the selected parameters. According to analysis of variance, P values of lack of fit for the two models were both >0.05 (conversion of St-ME, 0.1015; content of LLL-TAG, 0.1268), which meant the models fit very well.

The mutual interaction of reaction time, substrate molar ratio, and enzyme amount is shown in Figure 3. The relationship between reaction factors and responses would be better understood by examining the three-dimensional response surface graphs (not given). As seen in Figure 3A−C, generally, an increment in reaction time and enzyme amount can increase the conversion of St-ME. The substrate molar ratio should be limited within the range 1.75−2.25, in which the maximal St-ME conversion can be gained. As to the content of LLL-TAG, the lower the three variables, the lower this response value (Figure 3D−F).

Optimization of Reaction and Model Verification. The optimal conditions were generated by using RSM with interactive calculations in the range selected. The two responses were selected at equal weight. Conversion of St-ME was used for maximization, whereas the content of LLL-TAG (SSS and SSP; S, stearic acid; P, palmitic acid) was opposite. Optimal conditions for these two responses at a temperature of 65 °C were determined to be a reaction time of 6.52 h, a substrate molar ratio (St-ME:TB) of 1.77:1, and an enzyme amount of 10.34%. Under the optimal conditions, conversion of St-ME and content of LLL-TAG are expected to be 78.30 and 4.93%, respectively. Production experiments were conducted according to the predicted optimal conditions. The measured conversion of St-ME was 78.47%, which is higher compared to the conditions before optimization, and the content of LLL-TAG was 4.89%. Both of the values were very near the predicted values above, which again proved the models fit very well.

Purification of Product. Interesterification product contained the target product, unreacted substrate, a little monoglycerides and diglycerides (total amount < 5%), St-ME, and methyl butyrate, which can be pumped out directly at 0.1 MP. The amount of raw material TB in the interesterification product was rather low (total amount < 4%), and it can be absorbed easily by the body with beneficial functions. There is no need to remove it. This is the same for monoglycerides and diglyceridess for they are usually used as emulsifiers in the food industry, making food

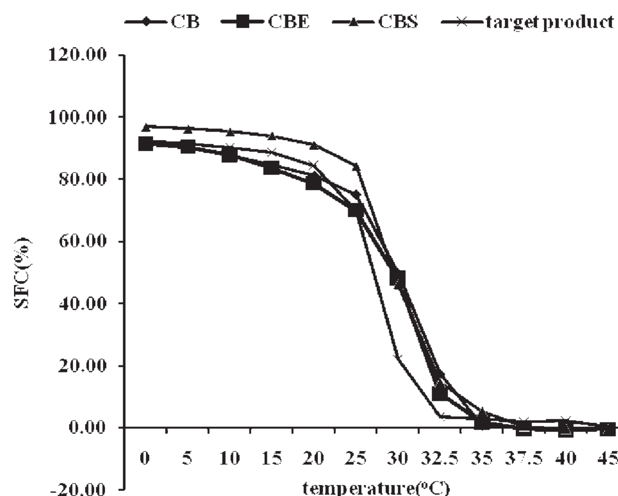

Figure 5. Solid fat contents of target product (×) and CB (●), CBE (■), and CBS (▲) (commercial products).

more homogeneous and easy to process.[23,24] After molecular distillation, St-ME at 6.5 min was eliminated almost totally, which is obvious by comparison of panels A and B of Figure 4.

Possible Industrial Application of Target Product. The SFC of the LCSL compared to those of cocoa butters (CB), cocoa butter equivalent (CBE), and cocoa butter substitutes (CBS) are shown in Figure 5. The amount of solid fat at 2−10 °C determines the spreadability at refrigerator temperature; SFC at 25 °C influences plasticity at room temperature, and SFC between 33 and 38 °C determines the mouthfeel.[25] Figure 5 shows the target product under optimal conditions had melting profiles similar to those of CB and CBE, which had a sharp transformation between 20 and 32.5 °C, decreasing from 84.50 to3.50%. Stored at room temperature, they are solid and crisp, whereas in the mouth, their SFC is <3%. This indicates the application of LCSL in baking chips, coatings, dips, and baked products or as cocoa butter substitutes.

In conclusion, a kind of LCSL, SALATRIM, composed of stearic acid and butyric acid, was successfully achieved with Lipozyme RM IM. On the basis of the single factor, RSM was used to model and optimize the process. The optimal conditions were as follows: temperature, 65 °C; reaction time, 6.52 h; substrate molar ratio (St-ME: TB), 1.77:1; enzyme amount, 10.34%. Under these conditions the actually measured conversion of St-ME and content of LLL-TAG (SSS and SSP; S, stearic acid; P, palmitic acid) were 78.47 and 4.89%, respectively. Target product under the optimal conditions after short-range molecular distillation showed similar SFC values with CBS, CBE, and CB, indicating its potential application for inclusion with other fats as cocoa butter substitutes. This is worthy of further research. Besides, some papers have reported that lipase properties are greatly influenced by immobilization.[26−30] Therefore, the interesterification activity and selectivity of Lipozyme RM IM could be improved by proper immobilization supports and suitable immobilization conditions. Petkar et al. concluded that Sepabeads, a methacrylate-based hydrophilic support with conjugated octadecyl chain, showed highest immobilized synthetic activity for *Humicola lanuginose* lipase B and *R. miehei* lipase.[26] In the study by Mateo, hydrophobic supports and proper detergents permit the hyperactivation of lipase.[27] Moreover, apart from protein engineering or directed evolution, the authors found that protein immobilization is a powerful technique to improve enzyme selectivity. Increasing the conversion of St-ME is possible if we improve the activity of the lipase from *R. miehei* (RML) by using more proper hydrophobic immobilization support and more suitable immobilization conditions. The study of the modulation of selectivity is a promising area of reasearch. More efforts may be expected in these areas.

AUTHOR INFORMATION

Corresponding Author

*Telephone/Fax: (086) 510-85876799. E-mail: yuanfa.liu@gmail.com (Y.L.); wxg1002@qq.com (X.W.).

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