Four-Factor Response Surface Optimization of the Enzymatic Modification of Triolein to Structured Lipids

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ABSTRACT: The ability of an immobilized lipase to modify the fatty acid composition of (88.8% $C_{18:1}$, 4.3% $C_{16:0}$, 3.1% $C_{18:0}$, and 3.8% $C_{18:2}$ as determined by gas chromatography, and approximately 90% triolein) in hexane by incorporation of a medium-chain fatty acid, capric acid (C_{10}), to form structured triacylglycerol was studied. Response surface methodology was used to evaluate the effect of synthesis variables, such as reaction time (12–36 h), temperature (25–65°C), molar substrate ratio of capric acid to triolein (2:1–6:1), and enzyme amount (10–30% wt% of triacylglycerol), on the yield of structured lipid. Optimization of the transesterification was attempted to obtain maximum yield of structured lipid while using the minimum molar substrate ratio and enzyme amount as much as possible. Computer-generated contour plot interpretation revealed that a relatively high molar substrate ratio (6:1) combined with low enzyme amount (10%) after 30 h of reaction at 25°C gave optimum incorporation of capric acid. A total yield for combined mono- and dicaproolein of up to 100% was obtained.
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KEY WORDS: Acidolysis, capric acid, Lipozyme IM60, optimization, response surface methodology, structured lipid, transesterification, triolein.

Medium-chain triacylglycerol (MCT) is a saturated fat composed mainly of C_8 (caprylic) and C_{10} (capric) fatty acids (1). MCT is the basis of a new group of fats known as "structured lipids" that have advantages in clinical nutrition and the treatment of disease (2). The nutritional needs of hospitalized patients and those with special dietary needs have required such structured lipids for years (2).

Lipases are currently being used as biocatalysts for the hydrolysis, synthesis, and modification of fats and oils (3). Lipases may be suitable to catalyze the incorporation of medium-chain fatty acids into triacylglycerols. Although considerable research has been conducted with enzymes, the development of enzymatic lipid modification processes remains to be widely adopted by industry (4). Despite the obvious advantages of enzymatic transesterification and interesterification, the economics of this option still need to be improved (4). There are many factors that influence yield in the enzyme-catalyzed synthesis or modification of lipids. The parameters that influence the reaction equilibrium are reasonably well understood, but knowledge about the control and kinetics of the reaction is still rather limited (5).

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes and it has been successfully adapted to food science research (6,7). Hill and Hunter (7) and Myers *et al.* (8) reviewed its applications in processing, experimental design, and data analysis. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. It is a faster and a less expensive method for gathering research results than classical one-variable-at-a-time or full-factorial experimentation (6). In this study, RSM was used to evaluate the effect of several variables on the formation of structured lipid.

The objectives of this study were to better understand the relationships between the factors (reaction time, reaction temperature, molar substrate ratio, and enzyme amount) affecting a structured lipid synthesis, and to determine the optimum conditions for transesterification of Trisun 90 (90% triolein) with capric acid.

MATERIALS AND METHODS

Experimental design. A three-level and four-factor fractional factorial design was adopted in this study (6,9). The independent variables (x_i) and their levels are presented in Table 1. To avoid bias, 27 runs were performed in a totally random order.

Materials. Trisun 90 (88.8% $C_{18:1}$, 4.3% $C_{16:0}$, 3.1% $C_{18:0}$, and 3.8% $C_{18:2}$ as determined by gas chromatography, and approximately 90% triolein) was obtained from SVO Enterprises (Eastlake, OH). Capric acid (C_{10}) was purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized 1,3-specific lipase, Lipozyme IM60 from *Rhizomucor miehei,* was kindly provided by Novo Nordisk Bioindustrial, Inc. (Danbury, CT). All organic solvents were from Fisher Scientific (Norcross, GA).

Transesterification. Transesterification was carried out in screw-capped test tubes. For the synthesis of structured lipid, 100 mg Trisun 90 was mixed with capric acid at different molar substrate ratios (2:1, 4:1, and 6:1) of capric acid to

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TABLE 1
Experimental Data for the Four-Factor, Three-Level Surface Response Analysis[a]

Experiment	Factors: Reaction time (h) (x_1)	Reaction temperature (°C) (x_2)	Molar substrate ratio (capric acid/Trisun 90) (x_3)	Enzyme amount (wt% Trisun 90) (x_4)	Percent yield of structured lipid: C_{49}[b]	C_{41}[c]	Total yield[d]
1	36	65	4:1	20	51.49	41.53	93.02
2	36	25	4:1	20	56.48	25.21	81.69
3	12	65	4:1	20	58.24	36.06	93.30
4	12	25	4:1	20	38.37	19.04	57.41
5	24	45	6:1	30	51.63	48.37	100.00
6	24	45	6:1	10	48.52	47.29	95.81
7	24	45	2:1	30	67.71	15.85	83.56
8	24	45	2:1	10	65.77	15.20	80.98
9	24	45	4:1	20	63.44	30.59	94.03
10	36	45	4:1	30	51.48	44.25	95.73
11	36	45	4:1	10	58.72	34.73	93.45
12	12	45	4:1	30	59.86	32.67	92.53
13	12	45	4:1	10	56.10	36.10	92.20
14	24	65	6:1	20	35.83	64.17	100.00
15	24	65	2:1	20	69.47	16.96	86.43
16	24	25	6:1	20	41.12	55.61	96.74
17	24	25	2:1	20	64.71	13.06	77.77
18	24	45	4:1	20	56.14	40.24	96.37
19	36	45	6:1	20	57.19	40.18	97.36
20	36	45	2:1	20	64.29	21.97	86.26
21	12	45	6:1	20	48.98	42.96	91.94
22	12	45	2:1	20	66.18	11.83	78.02
23	24	65	4:1	30	56.85	39.77	96.62
24	24	65	4:1	10	60.56	34.65	95.21
25	24	25	4:1	30	62.42	31.60	94.02
26	24	25	4:1	10	50.66	19.99	70.65
27	24	45	4:1	20	62.00	33.85	95.85

[a]Trisun 90 (SVO Enterprises, Eastlake, OH); capric acid (Sigma Chemical Co., St. Louis, MO).
[b]Triacylglycerol containing one capric acid molecule (monocaproolein); C_{49} = triacylglycerol total carbon number.
[c]Triacylglycerol containing two capric acid molecules (dicaproolein); C_{41} = triacylglycerol total carbon number.
[d]Total yield = sum of dicaproolein and monocaproolein.

Trisun 90 and lipase, Lipozyme IM60 (10, 20, and 30 wt% of Trisun 90) in 3 mL hexane. The mixture was stirred in an orbital shaking water bath at 200 revolutions/min and different temperatures (25, 45, and 65°C). They were sampled and analyzed at 12, 24, and 36 h.

Extraction and analysis. The enzyme was removed by passing reaction media through an anhydrous sodium sulfate column (10). To each aliquot of 75 μL reaction product was added 15 μL of internal standard solution (tricaprylin) before analysis by high-performance liquid chromatography (HPLC). HPLC was carried out with a Hewlett-Packard (Avondale, PA) Model 1090 Win liquid chromatograph equipped with a Vectra 486 computer and a Sedex 45 evaporative light-scattering mass detector (ELSD) (Richard Scientific, Novato, CA). The ELSD was set to 40°C at a nebulizer gas pressure of 2.1 and a gain of 5 for the nonaqueous reverse-phase system. A Hewlett-Packard 35900 digital A/D analog interface connected the mass detector electronically to the Vectra 486 computer. Triacylglycerol mixtures were analyzed by nonaqueous reversed-phase HPLC on a Beckman/Altex (San Ramon, CA) Ultrasphere ODS 5 μm (4.6 mm × 25 cm) column. The analysis procedure employed was based on a modification of the method of Foglia *et al.* (11). Separations were obtained with acetonitrile (solvent A) and acetone (solvent B) as eluent with the following gradient profile: initial condition 50:50 (A/B), hold 4 min, at a flow rate of 1.8 mL/min; 5:95 (A/B), hold 8.5 min at a flow rate of 2.0 mL/min; return to original conditions. Total run time was 18 min.

Statistical analysis. The data were analyzed by means of the Statistical Analysis System (SAS) (12). Regression analysis with backward elimination was used to obtain a second-order polynomial equation in which the level of significance (P-value) of all coefficients was less than 0.1. The backward option in the SAS regression procedure was employed to eliminate insignificant coefficients in a model. Furthermore, the molar substrate ratio and enzyme amount were kept constant for the optimization study. Only two variables, reaction temperature and reaction time, were kept in the final model, which was used to generate contour plots by using Matlab software (MathWorks, Natick, MA).

RESULTS AND DISCUSSION

The data given in Table 1 show the level of incorporation of capric acid into triolein and the total yield of structured lipid. The total yield is the sum of the triacylglycerols that have one or two capric acid molecules incorporated. Up to 100% total yield of structured lipid was obtained in some cases. The best fitting model was determined by regression and backward elimination. The model coefficients (β) and *P*-values are given in Table 2. All *P*-values of coefficient were less than 0.1, and the coefficient of determination (R^2) of the model was 0.868, which indicates that this model is suitable to represent the real relationships among reaction parameters. Furthermore, the model was changed into nine two-variable second-order polynomial equations by keeping molar substrate ratio and enzyme amount constant. All of these nine models were used to generate contour plots (Fig. 1). Such an application allowed us to compare all of the factors simultaneously.

The most efficient condition for this reaction would use the lowest amount of enzyme to achieve full conversion of the substrate in minimal time at the lowest temperature. Figure 1(f–i) identify reaction conditions under which a 100% yield of the structured lipid product is predicted. However, a reaction condition of 30 h, 25°C, molar substrate ratio 6:1, and 10% enzyme [Fig. 1(g)] is suggested as the optimum condition because Figure 1(f,h,i) used more enzyme to achieve the same yield. Even though a lower molar substrate ratio (4:1) was suggested in Figure 1(f), compared to a ratio of 6:1 in Figure 1(g), to obtain a 100% yield of structured lipid, we feel that Figure 1(g) should be employed because it required only 10% enzyme compared to 30% [Fig. 1(f)]. Enzyme is more expensive than substrates. Also, Figure 1(f) required a high temperature of 60°C to achieve 100% yield, whereas Figure 1(g) required only 25°C. Therefore, it will be prudent and more economical to use the conditions of Figure 1(g) for the acidolysis reaction. In other words, high molar substrate ratio (6:1) combined with low enzyme amount (10%, w/w of Trisun 90) at 25°C and 30 h gave the ideal synthesis condition. In addition, the contour plots also can indicate the desirable combination of variables that can be selected by the manufacturer of structured lipids, based on the conditions available, including economic considerations. Because there were several combinations that reach the maximum yield (100%), it allows one to decide which factor is most important. For instance, if it were necessary to complete the synthesis within 12 h without concern for cost, the time factor should be considered first, and then the other factors can be maximized. Therefore, a 12-h synthesis at 65°C at molar substrate ratio of 6:1 and enzyme amount of 20% could be used to obtain a 100% yield, as predicted in Figure 1(h). This flexibility allows the manufacturer to evaluate the most important factor and select suitable synthesis conditions from the contour plots.

Adequacy of the model was examined by comparing experimental data in experiment 6 with the predicted value and then by performing extra independent experiments at the optimal synthetic conditions. The predicted value was obtained by substituting the predicted model variables with the experimental synthetic conditions. Verification results (Table 3) reveal that the experimental results of experiment 6 and the predicted value were close. The results of additional experiments at the optimum condition (30 h, 25°C, molar substrate ratio 6:1, and enzyme amount 10%) were compared with predicted values. The coefficient of determination (R^2 = 0.868) of the model indicated some difference (2.48%) between predicted and experimental values, but it was an acceptable model as determined by chi-square test (*P*-value = 0.910; degree of freedom = 2) (13). Thus, we have demonstrated that optimum synthesis of structured lipid can be successfully predicted by the contour plots.

TABLE 2
Regression Coefficients and *P*-Values of the Second-Order Polynomials After Backward Elimination

Variables[a]	Coefficient (β)[b]	*P*-Value[c]
Intercept	−15.546	0.2530
x_1	0.800	0.0018
x_2	3.762	0.0001
x_3	3.701	0.0001
x_4	1.610	0.0002
x_2^2	−0.024	0.0497
x_1x_2	−0.026	0.0089
x_2x_4	−0.051	0.0015

[a]See Table 1 for description of abbreviations.
[b]Coefficient = values before the variables in the regression model.
[c]*P*-value = level of significance.

TABLE 3
Verification of Predicted and Experimental Percent Yields[a]

	Predicted from model (%)	Experimental (%)
Experiment 6	95.29	95.81
Additional experiments at optimum condition	99.42	96.94

[a]Values are total yields of structured lipid (combined monocaproolein and dicaproolein).

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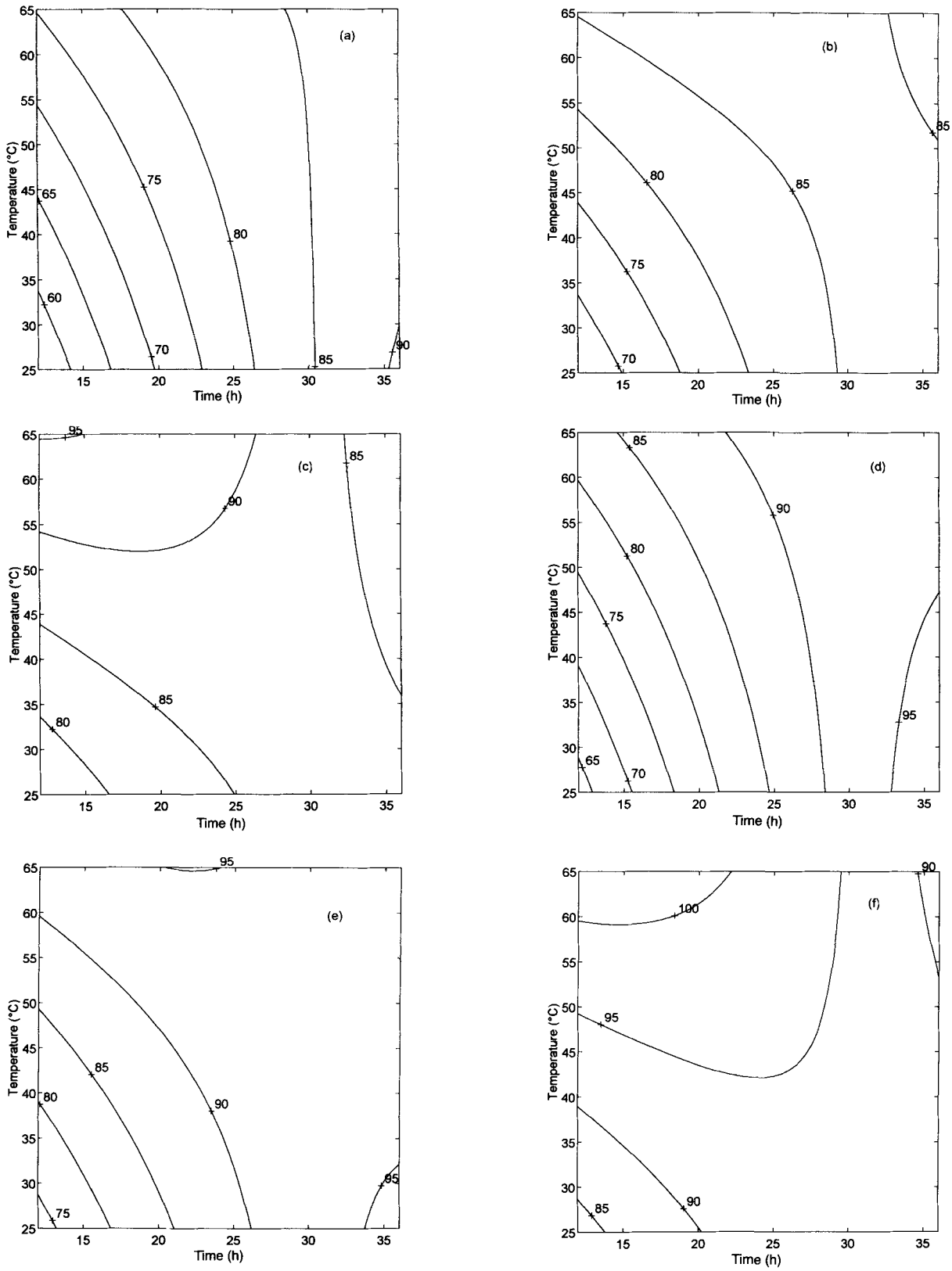

FIG. 1(a–f). Contour plots of total yield of structured lipid (combined monocaproolein and dicaproolein). Molar substrate ratio represents capric acid (Sigma Chemical Co., St. Louis, MO) to Trisun 90 (90% triolein) (SVO Enterprises, Eastlake, OH). Enzyme amount is the wt% of Trisun 90. The numbers inside the contour plots indicate percentage yield at given reaction condition. (a) Molar substrate ratio = 2:1, enzyme amount = 10%; (b) molar substrate ratio = 2:1, enzyme amount = 20%; (c) molar substrate ratio = 2:1, enzyme amount = 30%; (d) molar substrate ratio = 4:1, enzyme amount = 10%; (e) molar substrate ratio = 4:1, enzyme amount = 20%; and (f) molar substrate ratio = 4:1, enzyme amount = 30%.

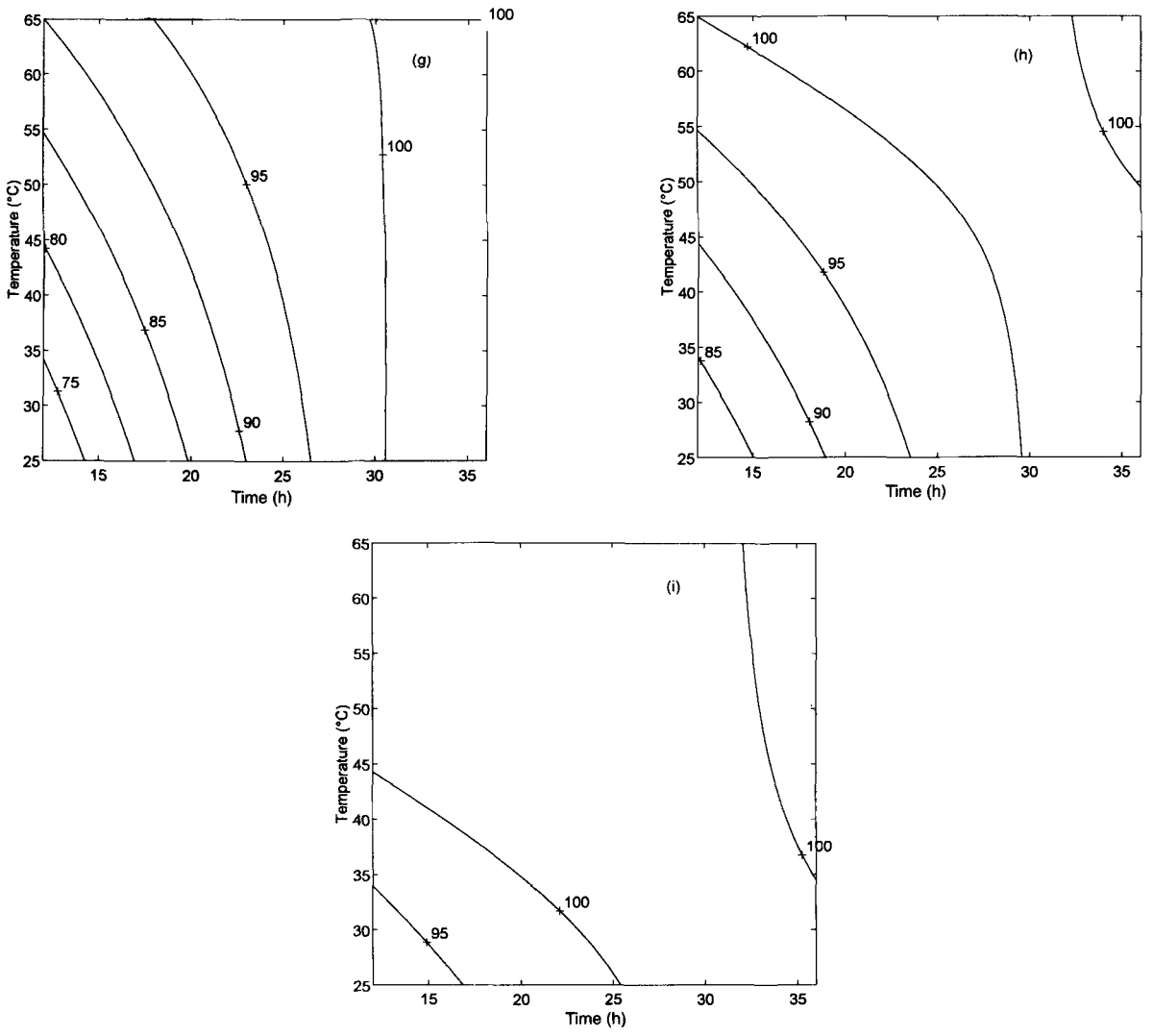

FIG. 1(g–i). (g) Molar substrate ratio = 6:1, enzyme amount = 10%; (h) molar substrate ratio = 6:1, enzyme amount = 20%; and (i) molar substrate ratio = 6:1, enzyme amount = 30%.

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