Applications of Immobilized *Thermomyces lanuginosa* Lipase in Interesterification

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ABSTRACT: The aim of this work was to investigate the catalytic functions of a new immobilized *Thermomyces lanuginosa* lipase in interesterification and to optimize the conditions of interesterification for the production of human milk fat substitutes (HMFS) containing n-3 PUFA by response surface methodology (RSM). *Thermomyces lanuginosa* lipase had an activity similar to that of immobilized *Rhizomucor miehei* lipase (Lipozyme RM IM) in the glycerolysis of sunflower oil, but the former had higher activity at a low reaction temperature (5°C). *Thermomyces lanuginosa* lipase was found to have much lower catalytic activity than Lipozyme RM IM in the acidolysis of sunflower oil with caprylic acid. However, the activity of *T. lanuginosa* lipase was only slightly lower than that of Lipozyme RM IM in the ester–ester exchange between tripalmitin (PPP) and the ethyl esters of EPA and DHA (EE). For this reason, the new immobilized *T. lanuginosa* lipase was used to produce HMFS from PPP by interesterification with EE. The optimization of major parameters was conducted with the assistance of RSM. The optimal conditions generated were a substrate molar ratio of 5 (EE/PPP), a lipase load of 20 wt% (on substrates), and a reaction time of 20 h, with acyl incorporation up to 42%. The model generated significantly represented real relationships between the response (incorporation) and reaction parameters.

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KEY WORDS: Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), human milk fat substitutes, interesterification, *Thermomyces lanuginosa* lipase, tripalmitin.

Interesterification commonly refers to three reactions: acidolysis, alcoholysis, and ester–ester exchange. Chemical interesterification, mainly ester–ester exchange, has been used in commercial production of shortening and margarine fats (1). This process has strict requirements for material oils and is usually carried out above 100°C using an inorganic catalyst with no positional specificity. The use of high temperature can lead to deterioration in the finished product. Another attractive approach is enzyme-catalyzed interesterification. The advantages of the enzymatic reaction lie in its selectivity, mild reaction conditions, few side reactions or by-products, ease of product recovery, easy process control, and minimal waste disposal (2).

Although lipases are designed in nature for the hydrolysis and synthesis of lipids and related molecules, it was discovered in the early 1980s that lipases could catalyze the interesterification of acyl groups in microaqueous systems (3,4). Many varieties of lipases have been investigated for the enzymatic modification of oils and fats. Commercial lipases are available from microbial, plant, and animal sources. Among them, microbial lipases are the most attractive ones, and these have been described extensively (5). Owing to the generally high price for enzymes, the real-market penetration of enzymes in commercial modification has been limited. Thus, inexpensive and nearly commercially developed immobilized *Thermomyces lanuginosa* lipase, named Lipozyme TL IM, has gradually gained attention in lipid research and in industry as well. Lipozyme TL IM is a food-grade granulated silica preparation of a microbial 1,3-specific lipase (EC 3.1.1.3) from *T. lanuginosa* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism (6). In the enzymatic interesterification of a blend of palm stearin and coconut oil (75:25, w/w), the activity of Lipozyme TL IM was similar to that of immobilized *Rhizomucor miehei* lipase (Lipozyme RM IM). The price of the former, on the other hand, is about 10% of the latter. The activity of Lipozyme TL IM showed little change after 10 batches in the 1- and 300-kg scale reactors (7). Lipase-catalyzed interesterification between fish oil and medium-chain TAG proved that Lipozyme TL IM was stable in a 2-wk continuous operation without adjustment of water content (8).

In the present work, the objective was to investigate the catalytic functions of Lipozyme TL IM in different interesterification reactions, as compared to the popular Lipozyme RM IM. It was also targeted to find the optimal interesterification conditions for the production of human milk fat substitutes (HMFS) containing n-3 PUFA, i.e., EPA and DHA, by response surface methodology (RSM).

MATERIALS AND METHODS

Materials. Tripalmitin (PPP) was purchased from Sigma (St. Louis, MO) (purity >95%). Caprylic acid was obtained from Riedel-de Haën (Seelze, Germany). EPAX 6000 ethyl ester (EE) was obtained from Pronova Biocare with contents of EPA >33% and DHA >22% (Sandefjord, Norway). Sunflower oil was obtained from a local supermarket and had an FFA content of 0.08%, a PV of 0.4 meq/kg, and a water content of 0.07%. Lipozyme TL IM, a silica granulated *T. lanuginosa* lipase, and Lipozyme RM IM, an immobilized lipase from *R. miehei*, were

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supplied by Novozymes A/S (Bagsvaerd, Denmark). Both lipases are *sn*-1,3 specific. The water contents of Lipozyme TL IM and Lipozyme RM IM were 6.0 and 3.0%, respectively. FAME and TAG standards were purchased from Sigma. All other reagents and solvents were of analytical grade.

Acidolysis of sunflower oil with caprylic acid in a 50-mL flask. Substrates were preheated in a water bath to 60°C. Mixed substrates (20 g) with a substrate weight ratio of 1:1 (sunflower oil/caprylic acid) were used. A magnetic bar was added for stirring (300 rpm). The reaction was started by adding lipase (10 wt% based on the total substrates), and the reaction was carried out in a closed system. During the reaction, samples were withdrawn from the mixture at 0.5, 1, 2, 3, 4, and 5 h.

Glycerolysis of sunflower oil. Ten grams of sunflower oil, 0.6 g glycerol, and 2.0 g Lipozyme RM IM or Lipozyme TL IM were reacted in a 50-mL flask with 300 rpm magnetic stirring. The reaction temperature was first set at 40°C (17 h), then at 5°C for another 24 h. Before analysis, the samples from the reaction system were filtered, dissolved in hexane, washed with water, and dried with anhydrous sodium sulfate.

Interesterification of PPP with EPAX 6000 EE. Interesterification of 10 g of PPP and EPAX 6000 EE with Lipozyme RM IM or Lipozyme TL IM was carried out in a 50-mL flask. The reaction temperature was 60°C, with 250 rpm stirring. Other conditions were as indicated in Table 1. Acyl incorporation was defined as 2/3[EPE] + 1/3[EPP], where [EFE] and [EPP] are percentages of EPE and EPP in the TAG products analyzed by HPLC (E = EPA and DHA, and P = palmitic acid).

Analysis by HPLC. TAG compositions in the interesterification samples were determined by HPLC. The HPLC system was a JASCO high-performance liquid chromatograph (Tokyo, Japan) equipped with two PU-980 pumps, an HG-980-30 solvent mixing module, an AS-950 autosampler, a UV-970 UV/vis detector, and a Sedex 55 ELSD (Sedere, Alfortville, France). The ELSD was operated at a temperature of 40°C with air as the nebulizing gas at 2.2 bar. The column used was SUPELCOSIL LC-18 (250 × 4.6 mm; Supelco Inc., Bellefonte, PA) with a particle size of 5 μm. The mobile phase was a binary mixture of acetonitrile and isopropanol/hexane (2:1, vol/vol), and the flow rate was 1 mL/min at ambient temperature. The samples were dissolved in chloroform (10 mg/mL) and 10 μL was injected. TAG peaks were identified by TAG standards and theoretical carbon number.

Methylation and GC analysis. FAME were prepared through saponification of TAG and esterification with methanol in the presence of boron trifluoride (9). The FAME were analyzed by GLC with an HP 6890 series gas chromatograph (Hewlett-Packard, Waldbronn, Germany) and a fused-silica capillary column (SUPELCOWAX 10, 60 m × 0.25 mm i.d., 0.25 mm film thickness; Supelco Inc.). The carrier gas was helium with a flow rate of 40 mL/min. The injector was used in split mode with a ratio of 1:20. The oven temperature was programmed from 70 to 160°C at a rate of 15°C/min, increased to 180°C at a rate of 1°C/min, further to 185°C at a rate of 0.5°C/min, and finally to 200°C at a rate of 20°C/min and held for 10 min. The injector and detector temperatures were 250 and 260°C, respectively. FAME were identified by comparing their retention times with authentic standards (Sigma Chemical, St. Louis, MO).

Analysis of FFA, MAG, DAG, and TAG content during glycerolysis. Samples were developed by TLC (impregnated with 3% boric acid, Kieselgel G; Merck Co., Darmstadt, Germany) with chloroform/acetone (90:10, vol/vol) as the developing solvent. The band containing acylglycerols or FFA was scraped off separately, mixed with relative weighed standards (15:0 TAG, DAG, MAG, and FFA), and extracted with hexane. The solvent was removed under a stream of N_2. The residuals were used for the methylation and analysis of FA compositions. Results were

TABLE 1
Set Factor Levels and Observed Responses in Response Surface Methodology Experiments[a]

	Factors			
No.	Time	Substrate ratio	Enzyme load	Incorporation
1	10	4	10	24.74
2	20	4	10	28.09
3	10	6	10	33.56
4	20	6	10	37.75
6	20	4	20	36.11
7	10	6	20	40.94
8	20	6	20	44.21
9	5	5	15	29.38
10	25	5	15	37.71
11	15	3	15	27.51
12	15	7	15	41.27
13	15	5	5	21.25
14	15	5	25	40.23
15	15	5	15	35.78
16	15	5	15	36.19
17	15	5	15	38.50

[a]Time = reaction time (h); substrate ratio = ethyl esters of EPA and DHA/tripalmitin (PPP) (mol/mol); enzyme load = dosage of immobilized *Thermomyces lanuginosa* (Lipozyme TL IM; Novozymes, Bagsvaerd, Denmark) (wt% on total substrates); and incorporation = percentages of EPA and DHA in TAG products (% area).

calculated according to the FA compositions of TAG, DAG, MAG, FFA, and their standards and were expressed as weight percentages.

Experimental design and statistical analysis. RSM enables the evaluation of effects of multiple parameters, alone or in combination, on response variables. The software Modde 6.0 (Umetri, Umeå, Sweden) was used to assist in study design, statistical analysis, and reaction optimization. A three-factor fractional factorial design with three central points was adopted to optimize the interesterification of PPP with EE. Lipozyme TL IM was more active in the temperature range 55–70°C, and water had less effect in the reaction (7). Therefore, the three factors chosen for the optimization were substrate molar ratio (EE/TAG, S_r), enzyme load (E_l), and reaction time (T_i). The variables and their levels are presented in Table 1. The quadratic response surface model was fitted by using the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad [1]$$

where Y = the response (incorporation of EPA and DHA), β_0 = the intercept, β_i = first-order model coefficients, β_{ii} = quadratic coefficients for the ith variable, β_{ij} = interaction coefficients for the interaction of variables i and j, and X_i = independent variables. Second-order coefficients were generated by regression analysis with backward elimination. Responses were first fitted to the factors by multiple regression. The fit of the model was then evaluated by the coefficients of determination (R^2 and Q^2) and ANOVA. Insignificant coefficients were eliminated, and the model was finally refined.

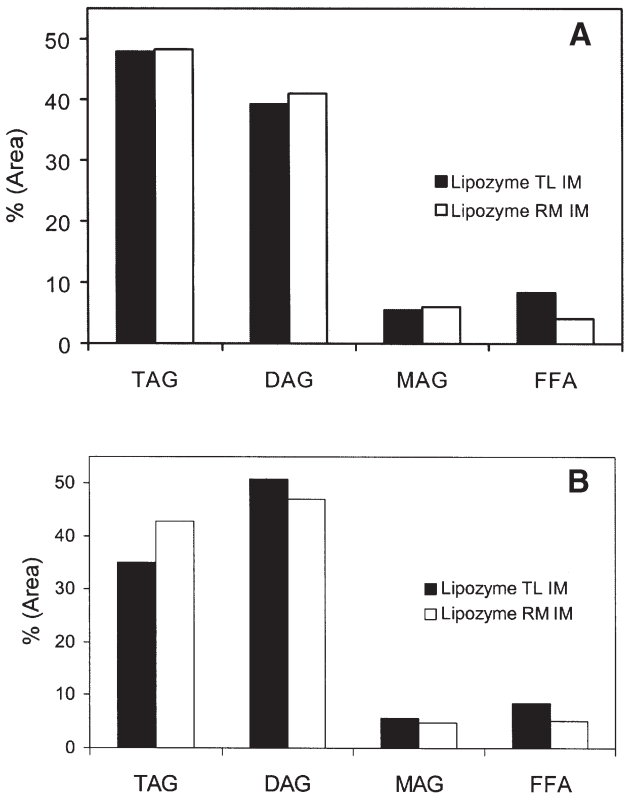

FIG. 1. Comparison of immobilized *Thermomyces lanuginosa* lipase (Lipozyme TL IM) with immobilized *Rhizomucor miehei* lipase (Lipozyme RM IM) (Novozymes A/S Bagsvaerd, Denmark) during the glycerolysis of high-oleic sunflower oil. Conditions: 20% enzyme load (based on total substrates), oil/glycerol 1:1 (mol/mol): (A) 40°C, 17 h, and 300 rpm stirring; (B): 40°C, 17 h, and 300 rpm stirring, then decreasing to 5°C for 24 h with unstable stirring (200 rpm).

RESULTS AND DISCUSSION

Glycerolysis. Lipase-catalyzed glycerolysis can be used for the production of MAG and DAG with possible quality improvements, especially for those containing PUFA. Glycerolysis is strongly influenced by lipase type and reaction temperature (10). During glycerolysis of sunflower oil in a solvent-free system at 40°C, Lipozyme TL IM had the same activity as Lipozyme RM IM (Fig. 1A). However, initiating the reaction at 40°C and then reducing the temperature for 5°C led to different results (Fig. 1B). Lowering the reaction temperature favored glycerolysis catalyzed by Lipozyme TL IM, as seen by a reduction in TAG content and an increase in DAG content. This indicated that Lipozyme TL IM could be more active than Lipozyme RM IM at low temperatures, at least in the present system.

Acidolysis. Lipozyme TL IM had much lower activity than Lipozyme RM IM in the enzymatic acidolysis of sunflower oil with caprylic acid (Fig. 2). Although the reaction is commonly used for the production of structured lipids (5), the reason for the low activity of the former lipase is not clear and needs to be explored. For some lipases, it has been reported that a high concentration of free acids as acyl donors could decrease lipase activity in the acidolysis reaction (11,12). It is known that the active sites of lipases subjected to structural studies to date are covered by a helical surface loop or lid that makes the active site inaccessible to substrate. The surface activation between lipids and water can open the lid (13,14). In the acidolysis reaction, the opening of the lid may be hindered by the presence of a large amount of FFA. This could explain the lower catalytic activity in the acidolysis reaction for some lipases including Lipozyme TL IM. However, this either did not affect Lipozyme RM IM or the extent of influence was lower. In a recent study, Kim *et al.* (15) reported that acyl incorporation in the lipase-catalyzed acidolysis of perilla oil with caprylic acid showed no difference for Lipozyme RM IM and Lipozyme TL IM in reactions at the milligram scale and with additional water. We could not repeat this conclusion in our study, especially in our large-scale studies.

Ester–TAG exchange. With reference to the lower activity of Lipozyme TL IM in acidolysis (Fig. 2) but similar activity in interesterification between the two oils (7), we decided to compare the activity of the two immobilized lipases in the reaction between TAG and FA EE. As indicated in Figure 3, the activity of Lipozyme TL IM was only slightly lower than that of Lipozyme RM IM in the ester–TAG exchange of PPP with the EE of EPA and DHA. This phenomenon was very different from acidolysis, in which the activities of the two lipases were

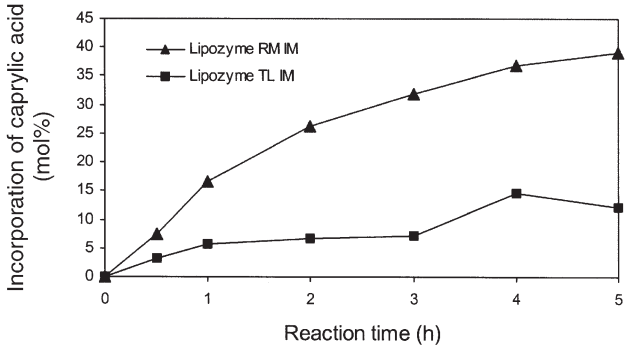

FIG. 2. Lipase-catalyzed acidolysis of sunflower oil with caprylic acid. Reaction conditions: temperature, 60°C; stirring, 300 rpm; lipase dosage, 10 wt% (based on total substrates); and substrate weight ratio, 1:1. For abbreviations see Figure 1.

substantially different (Fig. 2). In the ester–TAG exchange, Lipozyme RM IM reached equilibrium faster than Lipozyme TL IM. Lipozyme RM IM also had faster acyl incorporation and promoted more formation of products (EPP and EPE) at the beginning of the reaction (Figs. 4A and 4B). Considering the dramatic difference in the cost of the two lipases and the large difference in their densities, we chose Lipozyme TL IM as the catalyst for further optimization of the ester–TAG interesterification to produce HMFS containing EPA and DHA.

HMFS. As TAG represent a major nutrient source in infancy, their structure is important for infants' nutrition. Previous reports (16,17) provided convincing evidence that the high absorption efficiency of human milk fat was the result of the specific positioning of saturated FA at the 2-position and unsaturated FA at the 1,3-positions in the TAG moiety. Moreover, PUFA are very important nutrients for infants' growth and the maturation of the infant brain, nervous system, and visual process (18). EPA (20:5n-3), like arachidonic acid (AA), serves as the precursor for eicosanoids, which regulate numerous cell and organ functions. DHA (22:6n-3) is the most abundant PUFA contained in the human brain and retina. Recent studies support the conditionally essential nature of EPA and DHA in humans, particularly in early life (19,20). Therefore, suggestions have been made that TAG containing EPA and DHA should be added to infant formula. 1,3-Eicosapentaenoyl-2-palmitoyl glycerol and 1,3-docosahexaenoyl-2-palmitoyl glycerol are considered ingredients of infant formula. These structured lipids would be produced most effectively by *sn*-1,3-specific lipase-catalyzed

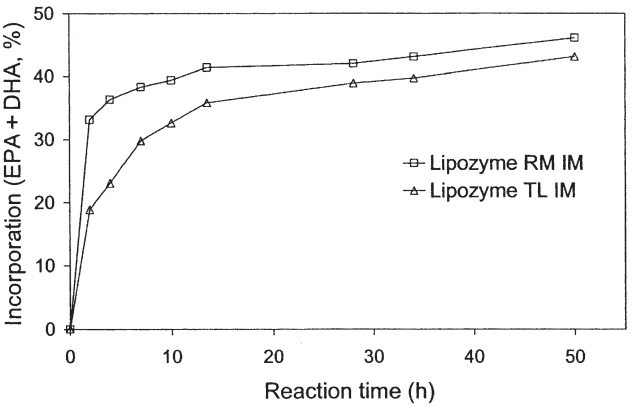

FIG. 3. Time course of EPA/DHA incorporation during the interesterification between tripalmitin (PPP) and the ethyl esters of EPA and DHA (EE). Conditions: 60°C, 10 wt% enzyme load (based on total substrates), PPP/EE 1:6 (mol/mol), 250 rpm stirring, and no water addition. For other abbreviations see Figure 1.

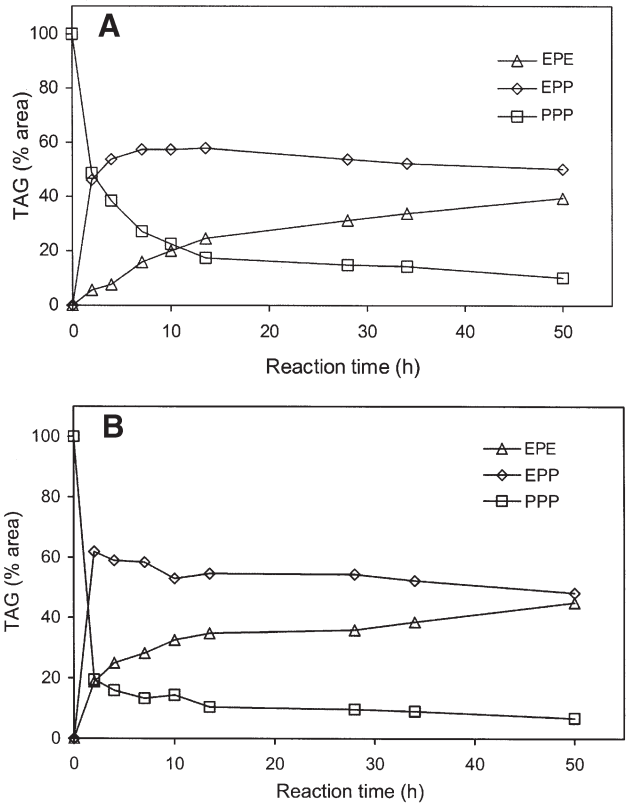

FIG. 4. Time course of TAG composition during the interesterification between PPP and the EE. Conditions: 60°C, 10 wt% enzyme load (based on total substrates), PPP/EE 1:6 (mol/mol), and 250 rpm stirring. (A) Lipozyme TL IM, and (B) Lipozyme RM IM. E = EPA + DHA; P = palmitic acid; for other abbreviations see Figures 1 and 3.

TABLE 2
Regression Coefficients and Significance (*P*) Values of the Second-Order Polynomials After Backward Elimination[a]

	Acyl incorporation	
Variables	Coefficient	*P*
Constant	37.08	1.02e-09
T_i	2.01	1.39e-03
S_r	3.98	2.01e-05
E_1	4.16	1.51e-05
$T_i{}^*T_i$	–0.79	0.064
$E_1{}^*E_1$	–1.49	4.25e-03

[a]n = 17, df = 7, Q^2 = 0.82, and R^2 = 0.97. Abbreviations: T_i, reaction time (h); S_r, substrate molar ratio [ethyl esters of EPA and DHA (EE)/TAG]; E_1, enzyme load (wt% on total substrates).

TABLE 3
ANOVA Results[a]

	df	SS	MS (variance)	*F*	*P*	SD
Total	17	20761.01	1221.23			
Constant	1	20105.43	20105.43			
Total corrected	16	655.59	40.97			6.40
Regression	9	638.19	70.91	28.52	0.000105	8.42
Residual	7	17.40	2.49			1.58
Lack of fit (model error)	5	13.10	2.62	1.23	0.51	1.62
Pure error (replicate error)	2	4.30	2.15			1.47

[a]SS, sum of squares; MS, mean squares; *P*, probability.

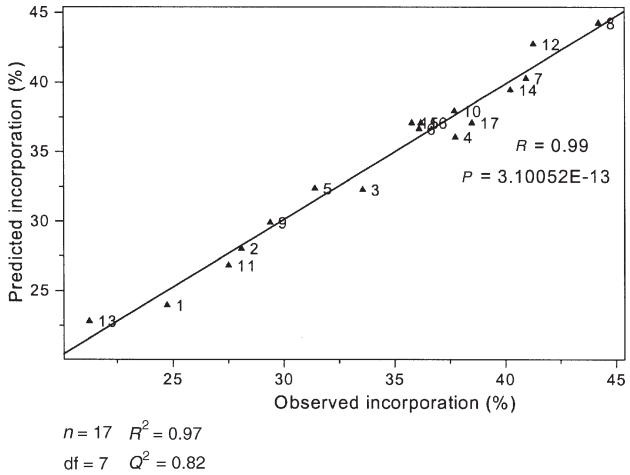

FIG. 5. Linear relationship between the observed incorporation and the predicted response for the reaction between PPP and EE with the catalysis of Lipozyme TL IM. For abbreviations see Figure 3.

interesterification (21,22). For these reasons, we chose the reaction between PPP and EE for optimization to produce PUFA–palmitic–PUFA-type HMFS as fat ingredients for infant formula with a much cheaper commercial lipase preparation.

Model fitting of optimization. The parameter setting generated by RSM in Table 1 and the responses from experiments were fitted with the assistance of Modde 6.0 software. Multiple regression and backward elimination analyses were used to determine the best-fitting quadratic model. The model coefficients and *P*-values for the response (acyl incorporation) were calculated statistically and are given in Table 2. All *P*-values of the coefficients were below 0.1 after the model was refined. The coefficients of determination of the model (R^2) and the predictive power of the model (Q^2) were 0.97 and 0.82, respectively. The R^2 and Q^2 values indicated that the model generated was satisfactory, with acceptable predictive power. Considering the results in Table 3, there was no lack of fit in the model and all results obtained were satisfactory. Predicted acyl incorporation correlated well in linearity with the observed results (Fig. 5, $R^2 = 0.98$). This also demonstrated that the model generally represented real relationships between the response (incorporation) and reaction parameters.

Optimization. The purpose of optimization is to identify the significance of the related parameters, to evaluate their interactions, to generate optimal conditions, and to predict responses in certain conditions. From Table 2 and Figure 6, one can see that all three parameters had positive effects on the incorporation of EPA and DHA, with enzyme load being the most significant and reaction time being the least significant. There were no interactions between parameters, but the second-order effects remained significant. Enzyme load and substrate ratios (EE/PPP) were the main factors affecting acyl incorporation

TABLE 4
Optimal Conditions Generated by Modde 4.0 Software in the Set Parameter Ranges and Targets Through Iterative Calculation[a]

No.	Time	Substrate ratio	Enzyme load	Incorporation	Iteration
1	10.0000	6.0000	14.4635	37.3054	281
2	20.0000	4.4894	19.9946	38.9284	225
3	10.4797	6.0000	14.4869	39.1391	243
4	17.8971	5.9999	19.8767	43.8968	139
5	12.9597	5.9999	17.7330	41.3108	139
6	14.4152	5.9999	18.4685	42.3265	175
7	17.8971	5.9999	19.8767	43.8968	139
8	19.8415	5.2056	19.9598	41.7494	158

[a]Set parameter ranges: time = reaction time (10–20 h), substrate ratio = EE and DHA/PPP (4–6, mol/mol), enzyme load = dosage of Lipozyme TL IM (10–20%, wt% on total substrates), and incorporation = percentages of EPA and DHA in TAG products (% area). Set targets: incorporation of EPA and DHA 43.06–45.35%. For abbreviations see Tables 1 and 2; for supplier of TL IM, see Table 1.

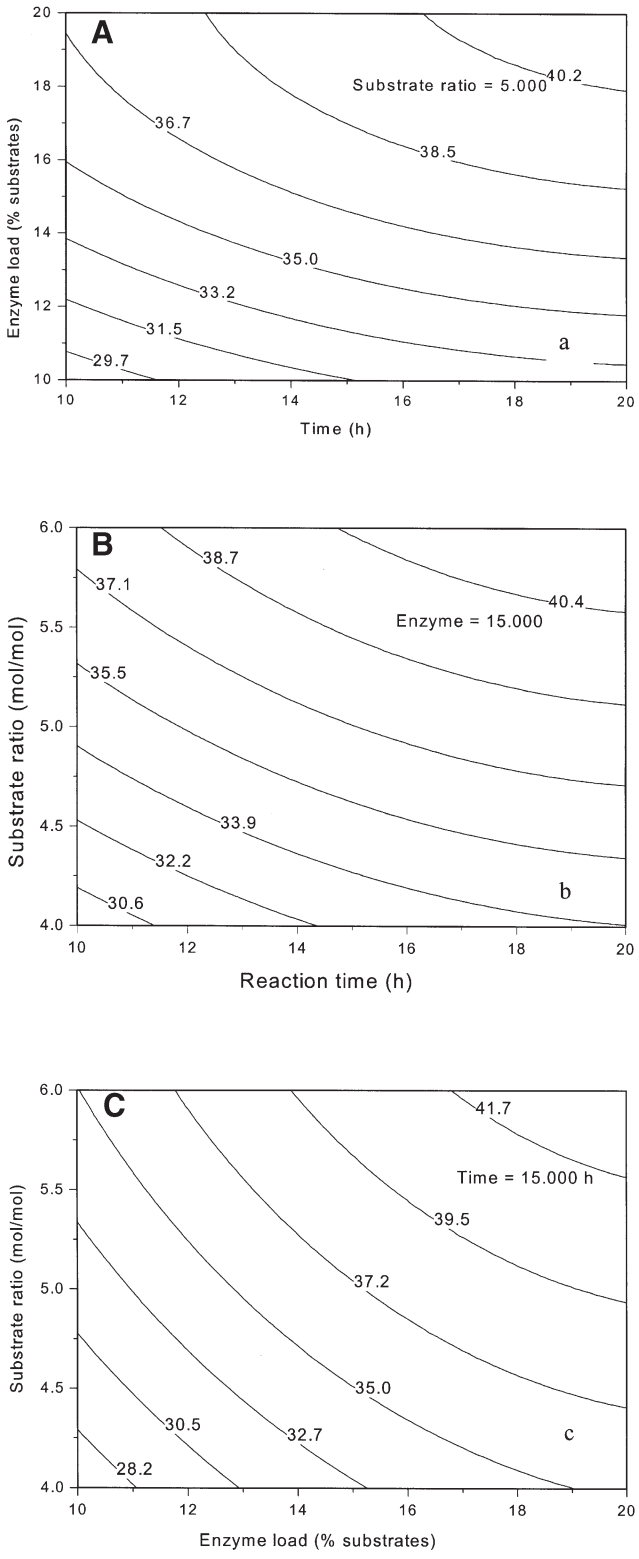

FIG. 6. Contour plots between two parameters for acyl (EPA + DHA) incorporation in the reaction between PPP and EE with the catalysis of Lipozyme TL IM. (A) Enzyme load (wt%, based on all substrates) and reaction time (h), substrate ratio = EE/PPP (mol/mol); (B) substrate molar ratio (EE/PPP) and reaction time, enzyme = enzyme load; (C) substrate molar ratio and enzyme load, time = reaction time (h). For abbreviations see Figure 3.

during the interesterification of PPP with EE. It was not unusual that enzyme load had a strong effect on acyl incorporation in interesterification, but the recommended dosage for Lipozyme TL IM (B1276b-GB, Novozymes 6–10 wt%) was not suitable for the present reaction system. Reaction time had less effect on incorporation than did enzyme load or substrate ratio, probably reflecting the characteristics of the reactions with EPA and DHA or their derivatives.

The use of RSM has merit for evaluating the relationships of parameters and for predicting results and behavior under given reaction conditions. Moreover, optimal parameters can be obtained by iterative calculation with designed responses and targets. The contour plots shown in Figures 6A–6C indicate that enzyme load, substrate ratio, and reaction time all had positive effects on the incorporation of EPA and DHA into PPP, as discussed above. The optimization results generated (Table 4) suggest that an S_r of 6, an enzyme load of 20%, and a 17.9-h reaction would produce the highest incorporation (43.9%). However, a high S_r would add more cost to the downstream purification process. Therefore, we decided the optimal conditions to be an S_r of 5, a Lipozyme TL IM load of 20%, and a 20-h reaction time, at which point acyl incorporation of 42% can be obtained.

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