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Optimal Formation of Hexyl Laurate by Lipozyme IM-77 in Solvent-free System

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A medium-chain ester, hexyl laurate, with fruity flavor is primarily used in personal care formulations as an important emollient for cosmetic applications. To conform to the "natural" interests of consumers, the ability of immobilized lipase from *Rhizomucor miehei* (Lipozyme IM-77) to catalyze the direct esterification of hexanol and lauric acid by using a solvent-free system was investigated in this study. Response surface methodology (RSM) and four-factor−five-level central composite rotatable design (CCRD) were employed to evaluate the effects of synthesis parameters, such as reaction time (10−50 min), temperature (45−85 °C), lipase amount (10−30 mg/volume; 0.077−0.231 batch acidolysis units of Novo (BAUN), and pH memory (5−9), on percentage molar conversion of hexyl laurate by lipase-catalyzed direct esterification. Reaction time, temperature, and enzyme amount had significant effects on percent molar conversion. On the basis of ridge maximum analysis, the optimum synthesis conditions for hexyl laurate were a reaction time of 40.6 min, a temperature of 58.2 °C, an enzyme amount of 25.4 mg/volume (0.196 BAUN), and a pH memory of 5.9. The predicted percentage molar conversion of hexyl laurate was 69.7 ± 1.4%.

KEYWORDS: Enzymatic; esterification; hexyl laurate; optimization; RSM; solvent-free system

INTRODUCTION

Hexyl esters with "green note" flavor, derived from medium-chain carboxylic acids such as hexyl laurate, are used as an important emollient material in many cosmetic industrial applications (*1*). Customarily, they are produced by chemical synthesis or extracted from natural sources. However, with the steadily growing "natural" demand, the synthesis of such esters by lipase-catalyzed chemical reactions under mild conditions has been receiving much attention for producing these valuable products. In particular, the development of an optimum enzymatic synthesis procedure to improve the yield conversion of hexyl esters and to reduce the production costs would be more attractive for the manufacturers and consumers.

Initially, the lipase-catalyzed reaction for ester synthesis was reported by Inada et al. (*2*), and some relevant research studies regarding the effect of experimental variables on the thermodynamic parameters and optimization of esterification reactions were successively issued (*3*−*5*). Carvalho et al. (*6*) reported that hexyl acetate was synthesized by the cutinase-catalyzed transesterification reaction of butyl acetate with hexanol in a reversed micelle system. Bourg-Garros et al. (*7*) synthesized (*Z*)-3-hexen-1-yl laurate by direct esterification using lipases *Mucor miehei* (Lipozyme IM) and *Candida antarctica* (Novozym 435) in *n*-hexane and solvent-free system. Previously, we have successfully synthesized hexyl laurate by lipase-catalyzed esterification in *n*-hexane (*8*). However, there are some disadvantages of the utilization of organic solvents, such as toxicity, flammability, or costs of recovery and recycling. Therefore, the solvent-free system, without any organic solvent addition, must be the beneficial choice to ward off any possible harmful effects on the enzyme stability or their thermodynamic equilibrium caused by organic solvents. Previously, we have investigated the optimal lipase-catalyzed conditions for hexyl laurate in *n*-hexane (*8*), whereas there is limited information for the solvent-free system.

The present work focuses on the parameters that affect the ability of lipase from *Rhizomucor miehei* (Lipozyme IM-77) to catalyze the synthesis of hexyl laurate using lauric acid as acyl donor in solvent-free system. Our purpose was to better understand relationships between the factors (reaction time, temperature, enzyme amount, and pH memory) and the response (percent molar conversion) and to determine the optimal synthesis conditions for hexyl laurate without adding any organic

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Table 1. Central Composite Rotatable Second-Order Design, Experimental Data, and Predicted Values for Four-Factor–Five-Level Response Surface Analysis

treatment[a]	time (min), x_1	temperature (°C), x_2	lipase amount (mg), x_3	pH memory, x_4	yield (% molar conversion), Y
1	–1 (20)[b]	–1 (55)	–1 (15)	1 (8)	54.6 ± 2.8
2	–1 (20)	–1 (55)	1 (25)	–1 (6)	65.0 ± 3.1
3	–1 (20)	1 (75)	–1 (15)	–1 (6)	45.1 ± 1.4
4	–1 (20)	1 (75)	1 (25)	1 (8)	53.2 ± 1.8
5	1 (40)	–1 (55)	–1 (15)	–1 (6)	66.7 ± 3.5
6	1 (40)	–1 (55)	1 (25)	1 (8)	60.4 ± 4.3
7	1 (40)	1 (75)	–1 (15)	1 (8)	52.2 ± 0.05
8	1 (40)	1 (75)	1 (25)	–1 (6)	59.0 ± 2.6
9	0 (30)	0 (65)	0 (20)	0 (7)	61.0 ± 2.6
10	–1 (20)	–1 (55)	–1 (15)	–1 (6)	57.5 ± 0.5
11	–1 (20)	–1 (55)	1 (25)	1 (8)	58.5 ± 2.2
12	–1 (20)	1 (75)	–1 (15)	1 (8)	46.0 ± 1.7
13	–1 (20)	1 (75)	1 (25)	–1 (6)	45.7 ± 9.4
14	1 (40)	–1 (55)	–1 (15)	1 (8)	60.3 ± 2.2
15	1 (40)	–1 (55)	1 (25)	–1 (6)	71.2 ± 2.4
16	1 (40)	1 (75)	–1 (15)	–1 (6)	52.6 ± 0.3
17	1 (40)	1 (75)	1 (25)	1 (8)	63.1 ± 0.1
18	0 (30)	0 (65)	0 (20)	0 (7)	61.4 ± 1.1
19	–2 (10)	0 (65)	0 (20)	0 (7)	49.4 ± 0.8
20	2 (50)	0 (65)	0 (20)	0 (7)	63.4 ± 1.0
21	0 (30)	–2 (45)	0 (20)	0 (7)	63.4 ± 4.0
22	0 (30)	2 (85)	0 (20)	0 (7)	39.0 ± 4.5
23	0 (30)	0 (65)	–2 (10)	0 (7)	51.5 ± 0.4
24	0 (30)	0 (65)	2 (30)	0 (7)	67.8 ± 1.4
25	0 (30)	0 (65)	0 (20)	–2 (5)	61.6 ± 0.2
26	0 (30)	0 (65)	0 (20)	2 (9)	61.0 ± 1.1
27	0 (30)	0 (65)	0 (20)	0 (7)	60.8 ± 0.01

[a] Treatments were run in a totally random order. [b] Numbers in parentheses represent actual experimental amounts.

solvents by using central composite rotatable design (CCRD) and response surface methodology (RSM).

MATERIALS AND METHODS

Reagents. Immobilized lipase [triacylglycerol hydrolase, EC 3.1.1.3; Lipozyme IM-77, the unit of enzyme activity was defined as 7.7 batch acidolysis units of Novo (BAUN)/g (water 5.4% w/w)] from *R. miehei* supported on macroporous weak anionic resin beads was purchased from Novo Nordisk Bioindustrials, Inc. (Bagsvaerd, Denmark). Hexanol (98% pure), lauric acid (99% pure), and glyceryl tributyrate (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO), and molecular sieve 4 Å was purchased from Davison Chemical (Baltimore, MD). All other chemicals were of analytical reagent grade.

Esterification. Lipozyme IM-77 was employed as a biocatalyst to perform the direct esterification of hexanol by lauric acid:

$$CH_3(CH_2)_5OH + CH_3(CH_2)_{10}COOH \rightleftharpoons CH_3(CH_2)_5OCO(CH_2)_{10}CH_3 + H_2O \quad (I)$$

The water content of Lipozyme IM-77 was measured by Karl Fischer titrator (Mettler-Toledo, DL31), and the 5.1% initial water content of Lipozyme IM-77 was manipulated in this study. Molecular sieve 4 Å (10% w/w of substrate) was used to remove H_2O of all chemicals for at least 24 h before the reaction was begun. Lauric acid (0.3 mmol) was mixed with hexanol (3 mmol), followed by the addition of different amounts of lipase to allow the direct esterification reaction, accompanying the H_2O byproduct formation, to proceed in an orbital shaking water bath (200 rpm) at different reaction temperatures and reaction times, as shown in **Table 1**.

Determination of Hexyl Laurate. Immobilized Lipozyme IM-77 and any residual water were removed by passing reaction media through an anhydrous sodium sulfate column. Then, hexyl laurate formation was determined by injecting a 1 μL aliquot in a splitless mode into a gas chromatograph (GC) (Hewlett-Packard 6890, Avondale, PA) equipped with a flame ionization detector (FID) and a DB-5 fused-silica capillary column (30 m × 0.32 mm i.d.; film thickness = 1 μm; J&W Scientific, Folsom, CA). The reactant was mixed with 43.6 μL of 1.5 mmol of tributyrin, which was employed as an internal standard. Injector and FID temperatures were set at 280 and 280 °C, respectively. Oven temperature was maintained at 180 °C for 8.5 min. Nitrogen was used as carrier gas at the flow rate of 40 cm^3/s. The percentage yield (molar conversion) was defined as (mmol of hexyl laurate/mmol of initial hexanol) × 100% and was estimated using peak area integrated by on-line software Hewlett-Packard 3365 series II ChemStation.

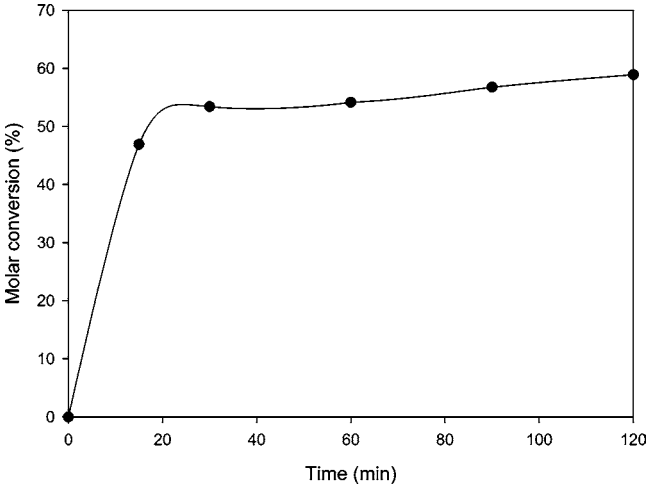

Figure 1. Time course of the direct esterification of hexanol with lauric acid by Lipozyme IM-77. The reaction was carried out at 45 °C containing 0.1 M lauric acid and 15.3 mg of Lipozyme IM-77 in solvent-free system. The activity of 10 mg of Lipozyme IM-77 is 0.077 BAUN.

Experimental Design and Statistical Analysis. A four-factor−five-level CCRD was employed in this study, requiring 27 experiments (*9*). To avoid bias, 27 runs were performed in a totally random order. The CCRD consists of 16 factorial points, 8 axial points (2 axial points on the axis of each design variable at a distance of 2 from the design center), and 3 center points. The parameters and their levels selected for the study of hexyl laurate synthesis were as follows: reaction time, 10−50 min; temperature, 45−85 °C; lipase amount, 10−30 mg (0.077−0.231 BAUN); pH memory, 5−9. **Table 1** shows the independent factors (x_i), levels, and experimental design in terms of coded and uncoded. The experimental data were analyzed by the response surface regression (RSREG) procedure to fit the following second-order polynomial equation (*10*)

$$Y = \beta_{k0} + \sum_{i=1}^{4}\beta_{ki}x_i + \sum_{i=1}^{4}\beta_{kii}x_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{kij}x_ix_j \quad (II)$$

where Y is response (% molar conversion), β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients, and x_i are the uncoded independent variables. The RIDGE MAX option was used to compute the estimated ridge of maximum response for increasing radii from the center of the original design.

RESULTS AND DISCUSSION

Effect of Reaction Time. Figure 1 shows the time course for the direct esterification of hexanol with lauric acid by Lipozyme IM-77 at 45 °C in a solvent-free system. The percent molar conversion of hexyl laurate increased >50% after 20 min, which showed higher molar conversion than that (~38% at 20 min) obtained by Chang et al. (*8*). Therefore, the range of reaction time from 10 to 50 min was chosen extremely precisely in the study of CCRD, and the optimal synthesis condition can be found inside the experimental region through the analyses

Table 2. Analysis of Variance for Synthetic Variables Pertaining to Response Percent Molar Conversion

source	degrees of freedom	sum of squares	prob > *F*
model	14	1242.94	<0.0001
linear	4	1056.43	<0.0001
quadratic	4	140.38	0.0009
cross product	6	46.12	0.1199
total error	12	42.59	
R^2 [a]	0.967		

[a] Coefficient of determination.

of statistics and contour plots. However, as the reaction time exceeded 20 min, the molar conversion of hexyl laurate was only slightly increased (**Figure 1**), which differs from the behavior of the *n*-hexane system (*8*). Therefore, the pH memory of the enzyme was further controlled in this reaction system to let us understand if the pH factor is important for enhancing enzymatic production of hexyl laurate or not.

Model Fitting. The major objective of this paper was the development and evaluation of a statistical approach to better understand the relationship between the variables of a lipase-catalyzed direct esterification reaction in a solvent-free system. In particular, the process can be optimized and allowed us to obtain economically a high-quality useful material with lowest costs such as work, money, and time saving for the scaling-up procedure. Compared with one-factor-at-a-time design, which has been adopted most often in the literature, the combination of RSM and four-factor−five-level CCRD employed in this study was more efficient in reducing the experimental runs and times for investigating the optimized enzymatic synthesis of hexyl laurate.

The RSREG procedure was employed to fit the second-order polynomial eq II to the experimental data−percent molar conversions (**Table 1**). Among the various treatments, the highest molar conversion (71.2%) was treatment 15 [40 min, 55 °C, 25 mg (0.193 BAUN) lipase amount, and pH memory 6.0], and the lowest conversion (38.9%) was treatment 22 [30 min, 85 °C, 20 mg (0.154 BAUN) lipase amount, and pH memory 7.0]. In addition, the lowest conversion (38.9%) of hexyl laurate might be because the protein conformation of Lipozyme IM-77 changed or denatured as the reaction temperature stayed at 85 °C (treatment 22). From the SAS (Strategic Applications System) output of RSREG, the second-order polynomial eq **III** is

$$Y = 36.338 + 1.008x_1 + 1.290x_2 - 445.099x_3 - 6.261x_4 - 0.011x_1^2 + 0.003x_2x_1 - 0.023x_2^2 + 5.723x_3x_1 + 23.477x_3x_2 - 17489x_3^2 - 0.049x_4x_1 + 0.104x_4x_2 + 12.781x4x_3 + 0.008x_4^2 \quad \text{(III)}$$

Analysis of variance (**Table 2**) indicated that the second-order polynomial model was highly significant and adequate to represent the actual relationship between the response (percent molar conversion) and the significant variables with very small *p* value (0.0001) and a satisfactory coefficient of determination ($R^2 = 0.967$). Furthermore, the overall effect of the four synthesis variables on the percent molar conversion of hexyl laurate in a solvent-free system was further analyzed by a joint test (**Table 3**). The results revealed that the reaction time (x_1), temperature (x_2), and lipase amount (x_3) were the important parameters and exerted a statistically significant overall effect ($p < 0.0001$) on the response molar conversion of hexyl laurate in a solvent-free system.

Table 3. Analysis of Variance for Joint Test

factor	degrees of freedom	sum of squares	prob > *F* [a]
time (x_1)	5	300.51	<0.0001
temperature (x_2)	5	689.52	<0.0001
lipase amount (x_3)	5	275.20	<0.0001
pH memory (x_4)	5	29.87	0.2129[b]

[a] prob > *F* = level of significance. [b] Not significant at $p > 0.1$.

Mutual Effect of Parameters. Reaction time and temperature were investigated in the range of 10−50 min and 45−85 °C, respectively. **Figure 2A** represents the effect of various reaction times and temperatures on esterification with 20 mg (0.154 BAUN) of lipase and a pH memory of 7.0. Under the appropriate reaction temperature (55 °C) and moderate reaction time (45 min) environment, the maximum percent molar conversion (65.5%) of hexyl laurate was obtained. However, the lowest reaction time and highest temperature drastically decreased the molar conversion to 30%, which might be because of the occurrence of protein denaturation at high reaction temperature. Although the molar conversion of hexyl laurate of a solvent-free system is lower than that of a *n*-hexane system, the lipase amount required in a solvent-free system is much less than that in a *n*-hexane system (*8*). This indicates that the production cost can be significantly reduced by the lower lipase amount and reaction time required in a solvent-free system rather than in a *n*-hexane system (*8*).

Figure 2B shows the effect of lipase amount, reaction temperature, and their mutual interaction on hexyl laurate synthesis at 30 min and a pH memory of 7.0. At any given lipase amount ranging from 10 to 30 mg (0.077−0.231 BAUN), there is no significant effect on the molar conversion of hexyl laurate, whereas the molar conversion was significantly decreased as a reaction temperature >70 °C. That means a reaction temperature (60 °C), higher than that used in the *n*-hexane system (*8*), might lead the catalytic ability of Lipozyme-IM77 to be more active to give maximum molar conversion of hexyl laurate (66%) without the addition any organic solvents.

The effect of varying the lipase amount and pH memory on esterification at constant reaction time (30 min) and reaction temperature (65 °C) is shown in **Figure 2C**. At any given pH memory (5−9), an increasing lipase amount tends toward higher yields. At the reaction conditions of lowest pH memory (5.0) and highest lipase amount (30 mg; 0.231 BAUN), the maximal yield (66.7%) will be reached. However, increasing pH memory resulted in only slightly decreased esterification efficiency at any given lipase amount. That means the Lipozyme IM-77 possesses high stability over a broad pH range, in either acidic or alkaline environment. Regardless of how the pH memory affects the solvent-free system, the molar conversion of hexyl laurate was significantly affected by lipase amount, which is consistent with results obtained with the *n*-hexane system (*8*).

The relationships between reaction factors and response can be better understood by examining the planned series of contour plots (**Figure 3**) generated from the predicted model (eq III) by holding constant the lipase amount (10, 20, 30 mg; 0.077, 0.154, 0.231 BAUN) and pH memory (5.0, 7.0, 9.0). Panels **A**−**C** of **Figure 3** represent the same pH memory (5.0); and panels **A**, **D**, and **G** represent the same lipase amount (10 mg; 0.077 BAUN). Such an application could be adopted to study the synthesis variables simultaneously in a five-dimensional space. Reaction time (x_1), temperature (x_2), and lipase amount (x_3) were important variables between hexyl laurate synthesis periods and were considered as indicators of effectiveness and

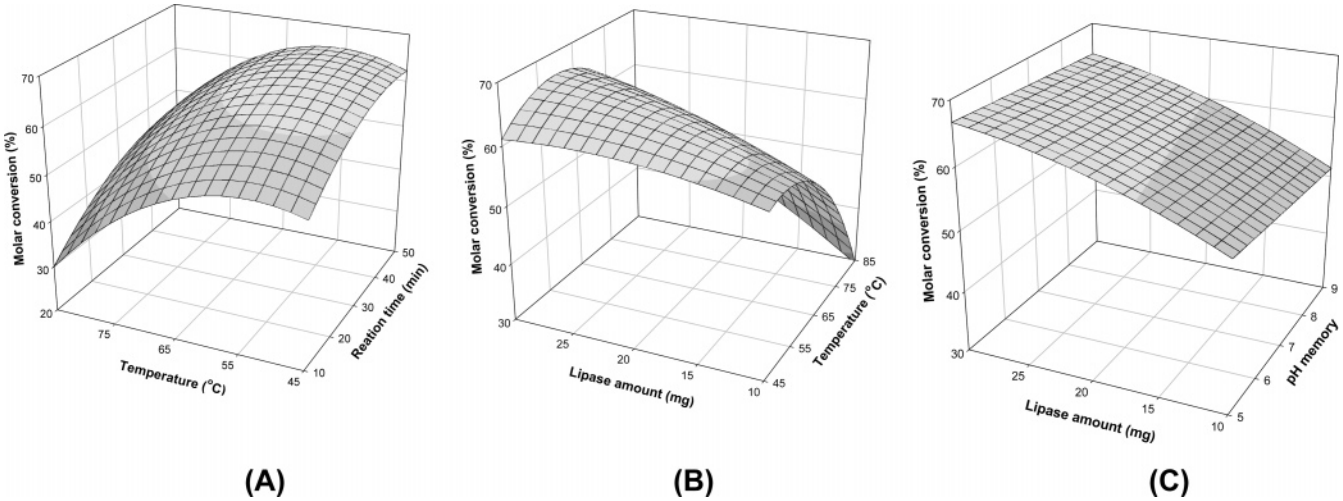

Figure 2. Response surface plot showing the effect of (**A**) reaction temperature (45–80 °C), reaction time (10–50 min), and their mutual interaction on hexyl laurate synthesis, (**B**) the effect of lipase amount (10–30 mg), reaction temperature (45–85 °C), and their mutual interaction on hexyl laurate synthesis, and (**C**) the effect of pH memory (5.0–9.0), lipase amount (10–30 mg), and their mutual interaction on hexyl laurate synthesis. All other reaction parameters are constant at 0 levels.

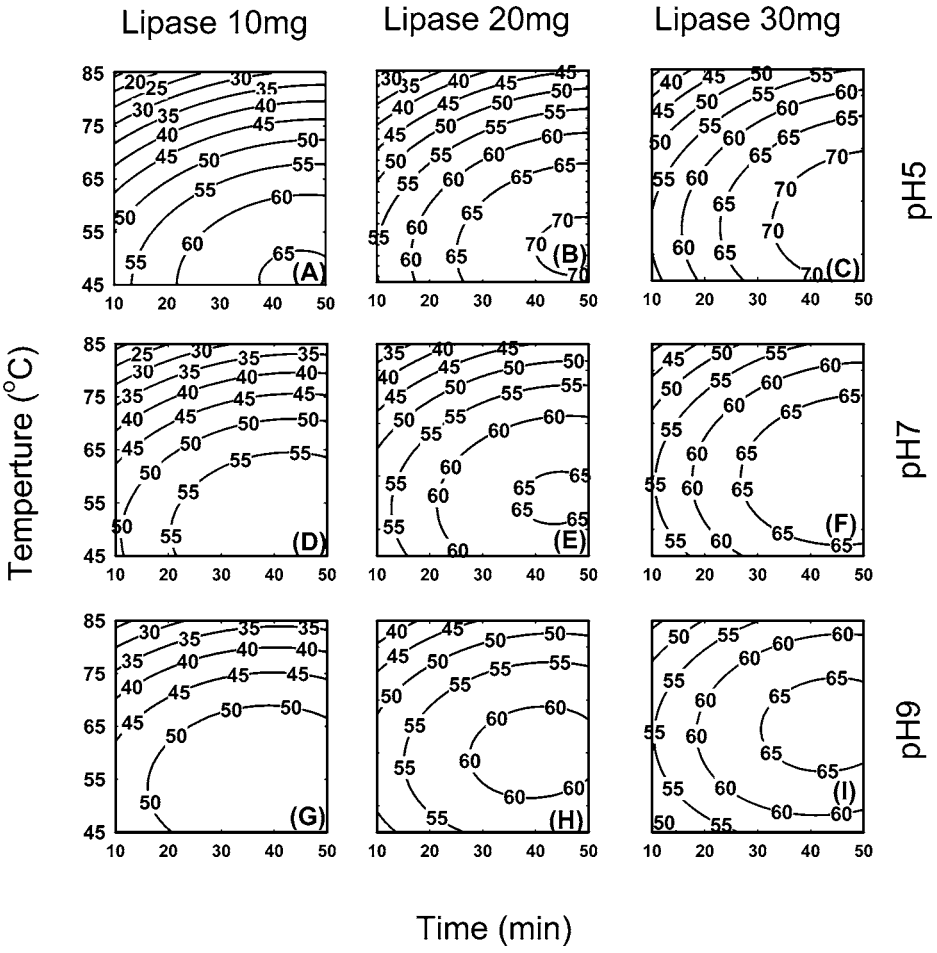

Figure 3. Contour plots of percent molar conversion of hexyl laurate in solvent-free system (numbers inside the contour plots indicate molar conversions at given reaction conditions).

economical performance. In general, all nine contour plots in **Figure 3** exhibited similar behavior in that predicted molar conversion increased with reaction time. Dissimilarly, the reaction temperature showed a more evident effect on the solvent-free system than on the *n*-hexane system (*8*). Otherwise, the highest lipase amount (30 mg; 0.231 BAUN) gave higher percent molar conversion in a shorter reaction time than that observed with a lower lipase amount (10 or 20 mg; 0.077 or 0.154 BAUN). That means the optimal reaction condition, which represented higher predicted molar conversion than the others

Table 4. Estimated Ridge of Maximum Response for Variable Percent Molar Conversion

coded radius	estimated response (% conversion)	standard error	x_1 (min)	x_2 (°C)	x_3 (mg)	x_4 (pH memory)
0	61.06	1.04	30.00	65.00	20.00	7.00
0.2	63.43	1.01	32.19	62.51	21.06	6.93
0.4	65.34	0.94	34.56	60.78	22.25	6.77
0.6	66.95	0.90	36.83	59.67	23.44	6.53
0.8	68.37	1.02	38.86	58.88	24.51	6.23
1.0	69.68	1.44	40.64	58.20	25.37	5.88

in **Figure 3**, for enzymatic synthesis of hexyl laurate in a solvent-free system was suggested as the combination of 30 mg of lipase (0.231 BAUN) and a pH memory of 5.0 (**Figure 3C**).

Attaining Optimum Conditions. The optimum point was determined by ridge maximum analysis (*10*). The method of ridge analysis computes the estimated ridge of maximum response for increasing radii from the center of the original design. The ridge maximum analysis (**Table 4**) indicated that maximum molar conversion was 69.7 ± 1.4% at 40.6 min, 58.2 °C, 25.4 mg (0.196 BAUN) of lipase, and pH memory of 5.9. In comparison with our previous work, the Lipozyme IM-77 represented a higher molar conversion (96.2%) for hexyl butyrate than that observed by hexyl laurate (69.7%) as the reaction condition was constant at 50 °C, 8.3 h, 42.7% (0.1 BAUN) enzyme amount, 12.6% water content, and 1.8:1 (tributyrin/hexanol) substrate molar ratio (*11*). Although the optimized enzyme amount and reaction temperature were fairly similar between hexyl butyrate and hexyl laurate cases, the reaction time required by hexyl butyrate synthesis was much longer (≈12.3-fold) than that required by hexyl laurate, indicating the Lipozyme IM-77 possessed higher catalytic efficiency toward medium-chain fatty acid than that short-chain one (*11*).

Model Verification. The validity of the predicted model was examined by experiments at the suggested optimum synthesis conditions and three center points of CCRD (treatments 9, 18, and 27). The predicted value was 69.7% obtained by ridge maximum analysis, and the actual value was 70.2 ± 2.6%. A chi-square test (p value = 0.961, degrees of freedom = 5) indicated that observed values were significantly the same as the predicted values and that the generated model adequately predicted the percent molar conversion (*12*). With only one-fifth of the lipase amount required by the *n*-hexane system (*8*), the optimal molar conversion of hexyl laurate resulted in 20% variation, which demonstrated that the solvent-free system is an alternative choice for further economical production applications. As a continuous solvent-free process, the conversion (65%) of butyl ester (*13*) was similar to the optimal hexyl ester conversion (70.2%) obtained in this study, which established the practicality of our predictive model. Thus, the optimization of lipase-catalyzed synthesis of hexyl laurate in a solvent-free system was successfully developed by CCRD and RSM.

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