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RESEARCH ARTICLE

Optimal Production and Structural Characterization of Erythorbyl Laurate Obtained through Lipase-catalyzed Esterification

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Abstract Erythorbic acid, a stereoisomer of L-ascorbic acid, has been extensively used as an antioxidant but cannot be applied to lipid-based foods due to its poor lipophilicity. For this reason, synthesis of erythorbyl laurate (6-*O*-lauroyl-erythorbate) was achieved in acetonitrile using an immobilized lipase from *Candida antarctica* as a biocatalyst to increase its lipophilicity. Response surface methodology was used to optimize the erythorbyl laurate synthesis conditions in terms of enzyme content (1,000-5,000 propyl laurate unit, PLU), molar ratio of lauric acid to erythorbic acid (5-25), and reaction temperature (25-65°C). The central composite experimental results showed the conditions for maximum molar conversion yield were as follows: enzyme content, 2,994 PLU; lauric acid to erythorbic acid molar ratio, 24.23; and reaction temperature, 53.03°C. The maximum molar conversion yield reached 77.81%, which was in agreement with the predicted value (76.92%). The erythorbyl laurate was purified and identified by Fourier transform-infrared spectroscopy (FT-IR). This research could help to develop an economical method of synthesizing erythorbyl laurate for use as a novel food-grade emulsifier with antioxidative activity.

Keywords: erythorbyl laurate, esterification, immobilized lipase, response surface methodology, antioxidant

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Introduction

Food antioxidants retard oxidative rancidity caused by atmospheric oxidation and thus prevent oils, fats, and fat-soluble components, such as oil-soluble vitamins or carotenoids, from undergoing oxidative deterioration. Many compounds have been used as food antioxidants, including mainly artificial phenolic substances that terminate free-radical reaction chains in lipid oxidation (1,2). Some of the most widely used synthetic phenolic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and esters of gallic acid. Because some synthetic antioxidants, such as BHA and BHT, may possibly degrade into harmful components under physiological conditions (3), there is great interest in naturally occurring antioxidants, which are presumed safe (4). Two such naturally occurring substances with a high reducing ability that are widely used in the food and cosmetic industries as oxygen scavengers are L-ascorbic acid (vitamin C) and erythorbic acid. These react with oxygen and can thus remove it from a closed system (5,6). However, L-ascorbic acid and erythorbic acid have limited solubility in lipid-based foods. Fatty esters of L-ascorbic acid have been synthesized to overcome this problem (7-12).

Erythorbic acid is widely used as an antioxidant in processed foods like an ascorbic acid and it was used as a food preservative. Erythorbic acid should be available at a relatively low cost because calcium 2-ketogluconate is readily converted to erythorbic acid during the fermentation of glucose (13). Furthermore, it is a potent enhancer of nonheme-iron absorption (14). The previous study reported by our group demonstrated that the synthesis of erythorbyl laurate was possible using the immobilized lipase in acetonitrile and that the potential application of immobilized lipase would be useful to produce the multifunctional food ingredient as an antioxidant and surfactant (15). There are

no studies of the synthesis of erythorbyl esters using the immobilized lipase, and their synthesis control and optimization. Therefore, the present study aimed to better understand the effect of reaction variables on the synthesis of erythorbyl laurate by an immobilized lipase in acetonitrile and to determine the optimum conditions for synthesis of erythorbyl laurate using response surface methodology (RSM).

Materials and Methods

Materials Novozym® 435 with a reported catalytic activity of 10,000 PLU/g (from *Candida antarctica* lipase immobilized onto macroporous acrylic resin) was purchased from Novozymes (Bagsvaerd, Denmark). According to the supplier, a unit (PLU) was defined as the amount of enzyme that synthesizes 1 μmol of propyl laurate/min at 60°C. Erythorbic acid (99%) was purchased from Fluka (Buchs, Switzerland), and lauric acid (99%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (J.T. Backer Co., Phillipsburg, NJ, USA) was dehydrated using a 4 Å molecular sieve (8-12 mesh, Sigma-Aldrich) prior to use as a reaction medium. All other chemicals were of analytical reagent grade.

Erythorbyl laurate synthesis Erythorbyl laurate synthesis was performed in screw-capped glass vials. Erythorbic acid (0.12 mmol) and the indicated molar ratios of lauric acid were added to 20 mL acetonitrile (Table 1). The vial headspace was then filled with nitrogen gas, and vials were pre-incubated at 50°C in an orbital shaking water bath at 200 rpm for 30 min. The reaction was initiated by adding the desired amount of enzyme and carried out at pre-determined temperatures in a water bath at 200 rpm for 6 h.

Quantitative analysis of esterification product Esterification products were periodically analyzed using an HPLC instrument (LC-2002; Jasco, Tokyo, Japan) equipped with a Spherisorb-ODS column (5 μm, 100 Å, i.d. 4.6× 250 mm, Waters, Milford, MA, USA), a refractive index (RI) detector (RI-2031; Jasco), and a UV detector (UV-2075; Jasco). The mobile phase was acetonitrile/water/acetic acid (90:5:5, v/v/v) at 1.0 mL/min flow rate for 10 min. Samples collected at various time intervals were filtered through a 0.45-μm membrane filter, and 20 μL of each was analyzed. Lauric acid was detected using an RI detector, and erythorbic acid and erythorbyl laurate were detected using a UV detector at 256 nm. The retention times were 2.51±0.01, 3.39±0.03, and 4.63±0.03 min for erythorbic acid, erythorbyl laurate, and lauric acid, respectively. The molar conversion yield (%) was calculated by calibration curves prepared using standard erythorbic acid, lauric acid, and erythorbyl laurate solution as defined by the following equation:

$$\text{Molar conversion (\%)} = \frac{C_{erythorbyl\ laurate}}{C_{erythorbic\ acid} + C_{erythorbyl\ laurate}} \times 100$$

where, $C_{erythorbyl\ laurate}$, mol (molar concentration) of erythorbyl laurate; $C_{erythorbic\ acid}$, mol (molar concentration) of erythorbic acid.

All data were the means of triplicate samples and were reproducible within ±10%.

Experimental design and statistical analysis A central composite experimental design with 3 independent variables and 5 levels was employed to optimize the synthesis of erythorbyl laurate. The 20 experiments consisted of 2^3 factorial points, 6 axial points (α=2), and the center point, which was replicated 6 times. To avoid bias, the 20 experiments were performed in a random order (Table 2). Enzyme content (X_1), molar ratio of lauric acid to erythorbic acid ([lauric acid]/[erythorbic acid], X_2), and reaction temperature (X_3) were chosen as independent variables in this design. X_1 (1,000 to 5,000 PLU), X_2 (5 to 25), and X_3 (25 to 65°C) were determined as critical levels having a significant effect on the synthesis of erythorbyl laurate. The quadratic polynomial regression model was assumed for predicting the response (Y, molar conversion yield) using the following Eq. 1:

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

where, Y is response (molar conversion yield, %), and β_0, β_i, β_{ii}, and β_{ij} are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively. X_i and X_j are independent variables. The experimental data were analyzed using the statistical software package Design-

Table 1. Variables and their levels for central composite design experiment

Variables	Coded X_i	Coded levels					X
		-2	-1	0	1	2	
Amount of enzyme (PLU)	X_1	1,000	2,000	3,000	4,000	5,000	1,000
Substrate molar ratio	X_2	5	10	15	20	25	5
Reaction temperature (°C)	X_3	25	35	45	55	65	10

Table 2. Experimental results based on central composite design

Run no.	X_1[1)]	X_2	X_3	Molar conversion yield (%)	
				Observed	Predicted
1	1,000 (-1)	10 (-1)	35 (-1)	46.93±0.99	44.03
2	4,000 (1)	10 (-1)	35 (-1)	44.62±1.24	42.87
3	2,000 (-1)	20 (1)	35 (-1)	61.09±1.65	58.47
4	4,000 (1)	20 (1)	35 (-1)	60.94±0.47	58.01
5	2,000 (-1)	10 (-1)	55 (1)	53.28±1.23	55.55
6	4,000 (1)	10 (-1)	55 (1)	52.55±0.36	54.51
7	2,000 (-1)	20 (1)	55 (1)	71.81±2.16	72.92
8	4,000 (1)	20 (1)	55 (1)	70.34±1.27	72.58
9	1,000 (-2)	15 (0)	45 (0)	58.12±1.62	58.87
10	5,000 (2)	15 (0)	45 (0)	57.46±1.26	57.37
11	3,000 (0)	5 (-2)	45 (0)	41.23±0.97	41.11
12	3,000 (0)	25 (2)	45 (0)	72.84±0.52	73.61
13	3,000 (0)	15 (0)	25 (-2)	29.39±0.42	34.15
14	3,000 (0)	15 (0)	65 (2)	64.36±2.27	60.24
15	3,000 (0)	15 (0)	45 (0)	68.22±3.37	66.80
16	3,000 (0)	15 (0)	45 (0)	67.44±1.41	66.80
17	3,000 (0)	15 (0)	45 (0)	64.97±3.22	66.80
18	3,000 (0)	15 (0)	45 (0)	65.52±0.92	66.80
19	3,000 (0)	15 (0)	45 (0)	66.87±0.77	66.80
20	3,000 (0)	15 (0)	45 (0)	67.14±1.95	66.80

[1)]For the identification of X_1, X_2, and X_3, refer to Table 1.

Expert® 8 (Stat-Ease, Minneapolis, MN, USA) for regression analysis and to evaluate the statistical significance of the equation. The quality of fit of the polynomial model equation was expressed by the coefficient of determination (R^2).

Product identification The isolated erythorbyl laurate was identified by Fourier transform infrared spectroscopy (FT-IR). FT-IR spectra were obtained using a Nicolet 6,700 FT-IR spectrophotometer (Thermo Scientific, Waltham, MA, USA) equipped with an attenuated total reflectance accessory. The spectra were recorded in transmission mode from 650 to 4,000/cm at a resolution of 8/cm at 25ºC.

Results and Discussion

Identification of the reaction product The FT-IR spectra of erythorbic acid (A), lauric acid (B), and erythorbyl laurate (C) are shown in Fig. 1. FT-IR spectra of erythorbyl laurate revealed a relatively strong peak at 1,739.4/cm against erythorbic or lauric acids, indicating the newly introduced CO-O ester bond in erythorbyl laurate (16). The spectra of erythorbyl laurate showed a broad peak in the range 3,000-3,500/cm, representing the OH group of the erythorbyl moiety of erythorbyl laurate. Stretch vibration of the CH bonds of the laurate moiety of erythorbyl laurate lead to high absorptions at 2,954.2, 2,917.6, and 2,851.3 1/cm. This result indicated that synthesis of erythorbyl laurate ester was catalyzed by lipase in organic media.

Analysis of central composite experiment design for the synthesis of erythorbyl laurate The synthesis conditions including the amount of enzyme, molar ratio of lauric acid to erythorbic acid, and reaction temperature were optimized independently to maximize erythorbyl laurate production. The central composite design is useful in determining the optimal values of independent variables and of any interaction effects thereof (17,18). The regression equation obtained after an analysis of variance (ANOVA) (Table 3) gives the molar conversion yield as a function of the levels of the 3 independent variables. The observed and predicted molar conversion yield values of the 20 experiments carried out are shown in Table 2. These were fitted into a quadratic polynomial Eq. 2, as follows:

$$Y = -113.92193 + 0.011975X_1 + 3.69344X_2 + 4.83346X_3 - 0.00000217028X_1^2 - 0.094376X_2^2 - 0.049002X_3^2 + 0.0000351305X_1X_2 + 0.00000321625X_1X_3 + 0.014619X_2X_3 \quad (2)$$

where, Y is the response variable (molar conversion yield, %), and X_1, X_2, and X_3 are the amount of enzyme, molar ratio of lauric acid to erythorbic acid, and reaction temperature, respectively. Figure 2 shows the observed and predicted molar conversion yield values determined by the model Eq. 2.

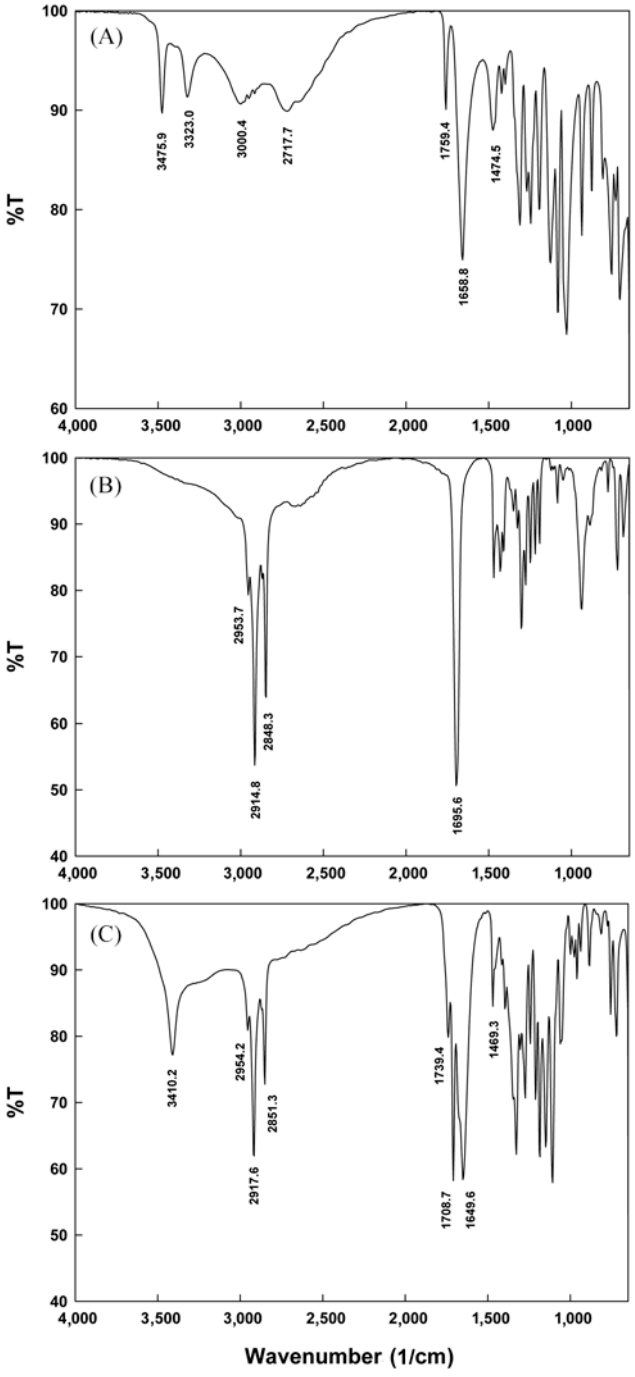

Fig. 1. FT-IR spectra of erythorbic acid (A), lauric acid (B), and erythorbyl laurate (C).

The statistical significance of Eq. 2 was determined using an ANOVA (Table 3). The effects of independent variables as linear, quadratic, or their interaction on response were tested for adequacy. The R^2 value, the coefficient of multiple determination of the polynomial model, was 0.9639, indicating that the fitted model could explain 96.39% of the variability in the response. A precision ratio of 18.554 indicates an adequate signal. A ratio greater than 4 is desirable. The relatively low coefficient of variation value (CV=5.08%) indicated good precision and reliability. In Table 3, the *p*-value (<0.0001) implies that the quadratic model was highly significant ($p<0.01$). Meanwhile the lack-of-fit *p*-value of 0.0097 indicated that the lack of fit was significant. Enzyme level had no significant linear or quadratic effects ($p>0.05$; Table 3). The molar ratio of lauric acid to erythorbic acid had a highly significant linear effect ($p<0.01$) but no significant quadratic effect ($p>0.05$). The reaction temperature also had a highly significant linear effect ($p<0.01$) but no significant quadratic effect ($p>0.05$). However, the interaction effects of the independent variables were found to be very significant (*p*-values: X_1X_2 =0.0047, X_1X_3=0.0028, and X_2X_3<0.0001).

Effects of independent variables on the synthesis of erythorbyl laurate The response surface and contour plots in Fig. 3-5 show the main, interaction, and quadratic effects of 2 independent variables on molar conversion yield. Figure 3 shows the effect of enzyme content (1,000-5,000 PLU) and the molar ratio of lauric acid to erythorbic acid (5-25) on the synthesis of erythorbyl laurate at a reaction temperature of 45°C. Molar conversion was enhanced by increasing the molar ratio of lauric acid to erythorbic acid. At a fixed molar ratio of lauric acid to erythorbic acid, varying enzyme content had a little effect on the molar conversion yield. Figure 3 shows that a high molar conversion yield (over 60%) could be obtained using a high molar ratio of lauric acid to erythorbic acid (over 20) regardless of the amount of enzyme used. These data indicate that the substrate molar ratio was the most important variable. Figure 4 shows the effect of the enzyme content (1,000-5,000 PLU) and reaction temperature (25-65°C) on the synthesis of erythorbyl laurate at a molar ratio of lauric acid to erythorbic acid of 15. At a fixed enzyme content, the molar conversion yield increased rapidly when the temperature reached approximately 50°C, and it then leveled off. At a fixed temperature, the molar conversion yield was varied slightly with increasing enzyme content, especially when the temperature exceeded 50°C. This result indicates that the reaction temperature had the greatest effect on the molar conversion yield. The effect of the substrate ratio on the molar conversion yield at varying temperatures and at an enzyme content of 3,000 PLU is shown in Fig. 5. The increase in both the reaction temperature and the molar ratio of lauric acid to erythorbic acid elevated the molar conversion yield. However, increasing the reaction temperature and molar ratio of lauric acid to erythorbic acid above their optimum values (approximately 50°C and 20, respectively) did not further increase the molar conversion yield.

Optimization of the synthesis of erythorbyl laurate and verification of model The conditions that resulted in the

Table 3. Analysis of variance of the response surface quadratic model

Source[1)]	Sum of square	Degree of freedom	Mean square	*F* value	*p*-value
Model	2,414.55	9	268.28	29.66	<0.0001
X_1	2.24	1	2.24	0.25	0.6292
X_2	1,056.75	1	1,056.75	116.81	<0.0001
X_3	680.62	1	680.62	75.23	<0.0001
X_1^2	0.25	1	0.25	0.027	0.8721
X_2^2	0.008275	1	0.008275	0.0009147	0.9765
X_3^2	4.27	1	4.27	0.47	0.5075
X_1X_2	118.43	1	118,43	13.09	0.0047
X_1X_3	139.97	1	118.43	15.47	0.0028
X_2X_3	603.73	1	139.97	66.73	<0.0001
Residual	90.47	10	603.73		
Lack of fit	83.02	5	9.05	11.14	0.0097
Pure error	7.45	5	16.60		
Corrected total	2,505.02	19	1.49		

[1)]For the identification of X_1, X_2, and X_3, refer to Table 1; R^2=0.9639; adj R^2=0.913; CV=5.08%; adequate precision ratio=18.554

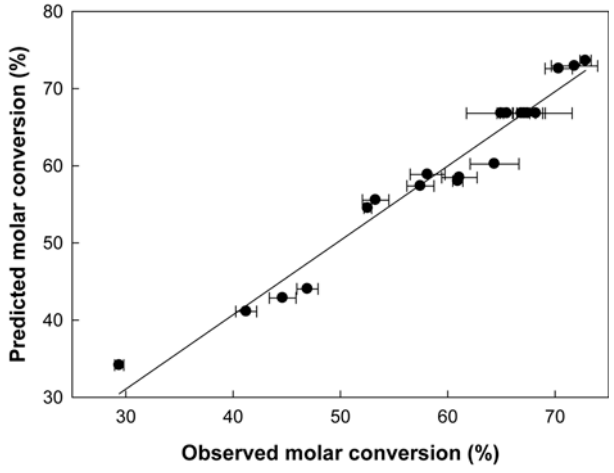

Fig. 2. Plot of predicted and observed molar conversion yield (%).

highest molar conversion yield were as follows: an enzyme content of 2,994 PLU, a molar ratio of lauric acid to erythorbic acid of 24.23, and a reaction temperature of 53.03°C. Under these conditions, the molar conversion yield was 77.81%, which was not significantly different (p<0.05) from the predicted value (76.92%) obtained from the quadratic model. This good agreement between the observed and predicted values verified the validity of the model designed in this study.

Conclusively, the lipase-catalyzed synthesis of erythorbyl laurate in acetonitrile was studied using response surface methodology. The data suggested that the molar ratio of lauric acid to erythorbic acid ([lauric acid]/[erythorbic acid]) and temperature had a significant effect on the synthesis of erythorbyl laurate. The maximal molar conversion yield of 77.81% was achieved using an enzyme content of 2,994

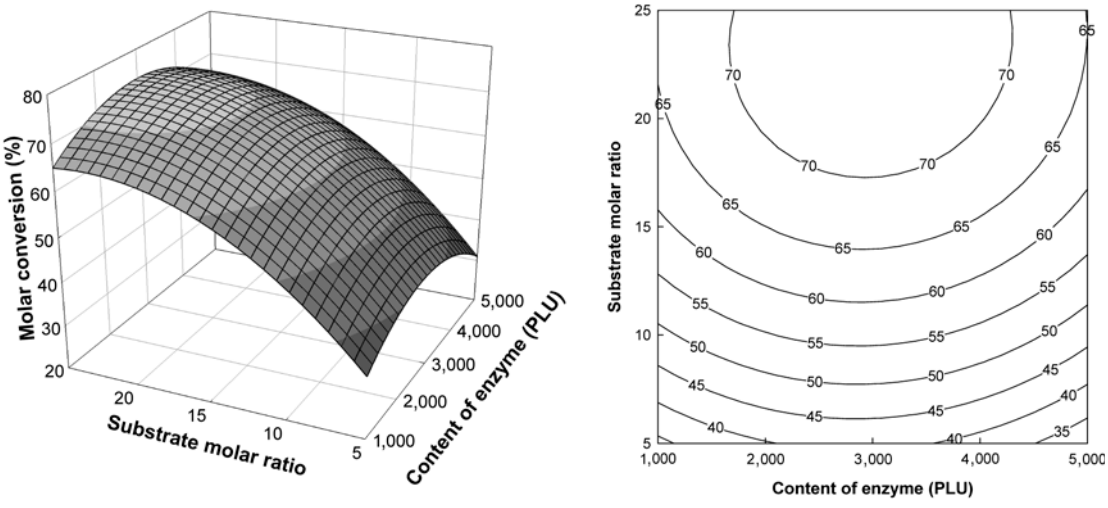

Fig. 3. Response surface and contour plots showing the effect of the content of enzyme and molar ratio of lauric acid to erythorbic acid on the molar conversion yield at a reaction temperature of 45°C.

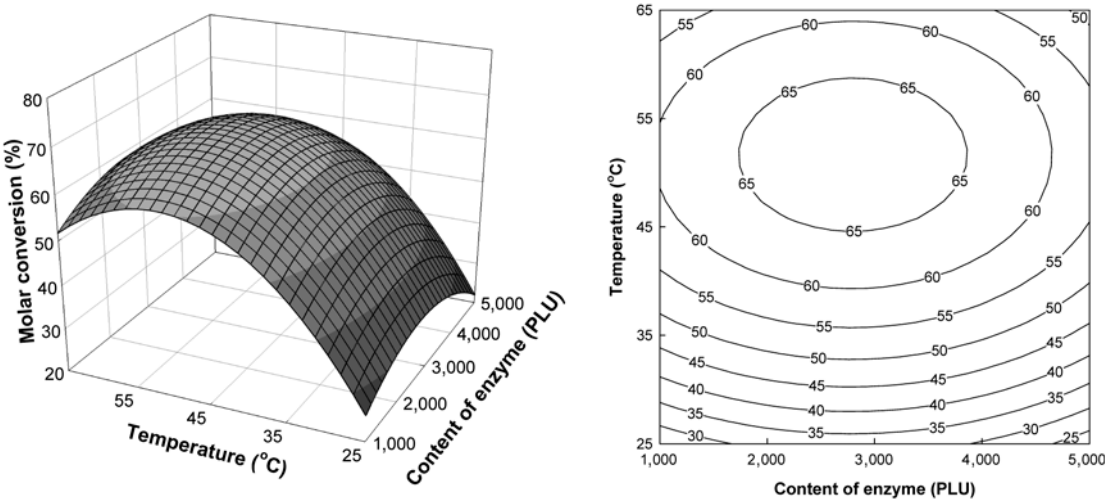

Fig. 4. Response surface and contour plots showing the effect of the content of enzyme and reaction temperature on the molar conversion yield at 15 of molar ratio of lauric acid to erythorbic acid.

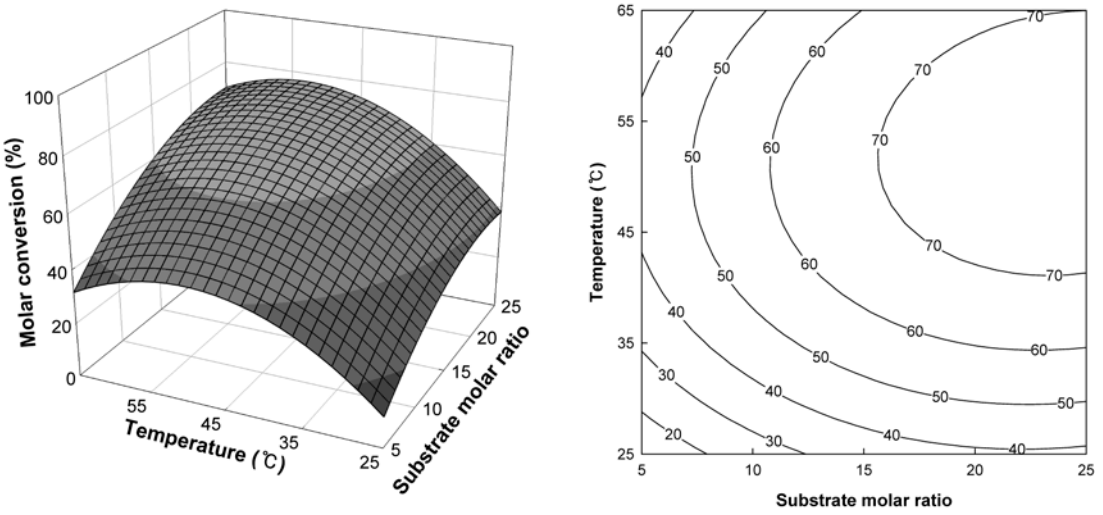

Fig. 5. Response surface and contour plots showing the effect of reaction temperature and molar ratio of lauric acid to erythorbic acid on the molar conversion yield at an enzyme content of 3,000 PLU.

PLU, a molar ratio of lauric acid to erythorbic acid of 24.23, and a reaction temperature of 53.03ºC. These results suggest that the efficient production of erythorbyl laurate could be achieved in a batch-type reaction under optimum conditions. Furthermore, the erythorbyl laurate produced could be used by the food and cosmetic industries as a new kind of emulsifier with antioxidant activity that is able to retard lipid oxidation. For these applications, however, further investigations are required, such as evaluation of the antioxidant properties of erythorbyl laurate, technical developments to facilitate continuous production, and research into additional functionality.

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