Journal of Molecular Catalysis B: Enzymatic 56 (2009) 7–12

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Optimized synthesis of lipase-catalyzed L-ascorbyl laurate by Novozym® 435

S.-W. Chang [a], C.-J. Yang [a], F.-Y. Chen [a], C.C. Akoh [b], C.-J. Shieh [a,c,*]

[a] Department of Bioindustry Technology, Dayeh University, 112 Shan-Jiau Road, Da-Tsuen, Chang-Hua 515, Taiwan
[b] Department of Food Science and Technology, University of Georgia, Athens, GA 30602, USA
[c] Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan

ARTICLE INFO

Article history:
Received 31 October 2007
Received in revised form 7 April 2008
Accepted 7 April 2008
Available online 16 April 2008

Keywords:
Antioxidant
Ascorbyl esters
Lipase
Optimization
Response surface methodology

ABSTRACT

L-Ascorbyl laurate is a fatty acid derivative of L-ascorbic acid which can be widely used as a natural antioxidant in both lipid containing food and cosmetic applications. To avoid any possible harmful effects from chemically synthesized product, the enzymatic synthesis appears to be the best way to satisfy the consumer demand for natural antioxidants. The ability of immobilized lipase from *Candida antarctica* (Novozym® 435) to catalyze the direct esterification between L-ascorbic acid and lauric acid was investigated. Response surface methodology (RSM) and 5-level-4-factor central composite rotatable design (CCRD) were employed to evaluate the effects of synthesis parameters, such as reaction time (2–10 h), temperature (25–65 °C), enzyme amount (10–50% w/w of L-ascorbic acid), and substrate molar ratio of L-ascorbic acid to lauric acid (1:1–1:5) on percentage molar conversion to L-ascorbyl laurate. Based on the analysis result of ridge max, the optimal enzymatic synthesis conditions were predicted as follows: reaction time 6.7 h, temperature 30.6 °C, enzyme amount 34.5%, substrate molar ratio 1:4.3; and the optimal actual yield was 93.2%.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Polyphenols are useful antioxidants that can protect cells or DNA from damage by free radicals and are widely used by the food and cosmetic industries. Among the available antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are the most common polyphenols used. However, they might possibly generate toxic or carcinogenic components as they degrade during storage [1]. L-Ascorbic acid (vitamin C), a natural hydrophilic antioxidant, has been used but limited in its application in hydrophobic foods and cosmetics [2]. To overcome solubility problem, a fatty acid derivative of L-ascorbic acid, such as L-ascorbyl laurate which possesses similar antioxidant function as L-ascorbic acid was used to prevent the enzymatic browning of apple juices or mixed with other fatty acid esters of ascorbic acid for treating and preventing sunburn damage to the skin [3,4]. Traditionally, such polyphenol compounds could be synthesized by chemical methods. Due to the steady growing demand for natural materials, the biosynthesis of such esters by lipase-catalyzed reactions, under mild conditions, has become a current commercial interest. An optimized enzymatic synthesis of L-ascorbyl esters with improved yield at reduced cost in the most favorable conditions would be more appealing to the consumer and of benefit to the manufacturers.

The importance of the enzymatic synthesis, catalyzed by lipases, to produce L-ascorbyl esters via esterification in water-miscible organic solvents has been emphasized in several works [1,5,6]. The lipase-catalyzed esterification reactions were reviewed, including the parameters affecting the lipase activities on esterification reactions such as reaction time, synthesis temperature, added water, water activity, pH memory, and acyl donors. [7]. Response surface methodology (RSM) and central composite rotatable design (CCRD) are useful statistical techniques for complex processes investigation and have been successfully applied to optimizing ester production by lipase [8].

Humeau et al. [1] synthesized ascorbyl palmitate through transesterification by using an immobilized lipase (Novozym® 435) from *Candida antarctica* in nonaqueous environment containing a 1:5 initial molar ratio of ascorbic acid to acyl donor. When palmitic acid methyl ester was used as acyl donor, 68% of ascorbic acid was converted to the ascorbyl ester compared to 56% with palmitic acid. Humeau et al. [1] also reported the enzymatic synthesis of fatty acid ascorbyl esters by lipase B (*C. antarctica*) in 2-methyl-2-butanol. They utilized palmitic acid methyl ester, EPA ethyl ester and DHA ethyl ester as acyl donors to react with L-ascorbic acid in the transesterification reactions. When methyl palmitate/ascorbic acid molar

* Corresponding author at: Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan. Tel.: +886 4 2284 0452x5121; fax: +886 4 2285 2609.
E-mail address: cjshieh@nchu.edu.tw (C.-J. Shieh).

1381-1177/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2008.04.001

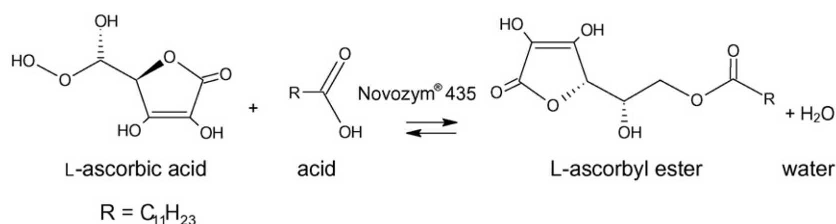

Fig. 1. Biosynthesis of L-ascorbyl laurate by Novozym® 435 in acetonitrile system.

ratio (R) increased to 9, the yield was 19 g/L of ascorbyl palmitate ester after 5 h of reaction. Yan et al. [6] investigated the enzymatic synthesis of vitamin C fatty acid esters in *t*-butanol and obtained the optimal conditions as follows: 50 °C reaction temperature, vitamin C/fatty acid ester molar ratio of 1:2, 50 mg immobilized *C. antarctica* lipase B, 48 h reaction time, and the molar conversion/yield of various ascorbyl fatty acid esters (C_8, C_{10}, C_{12}, C_{16}) were 65, 87, 74, and 91%, respectively. Tang et al. [9] reported the kinetics of the synthesis of L-ascorbyl laurate and L-ascorbyl palmitate catalyzed by immobilized lipase with L-ascorbic acid and an acyl donor (i.e., lauric acid, palmitic acid and methyl or ethyl esters) in 2-methyl-2-butanol. The optimum reaction condition was suggested as follows: shaking speed 200 rpm, temperature 55 °C, and 17–20% enzyme (w/w of substrate).

The present work focused on the reaction parameters that affect the synthesis of L-ascorbyl laurate catalyzed by immobilized lipase from *C. antarctica* (Novozym® 435) in acetonitrile system (Fig. 1). Our objectives were to better understand the relationships between various reaction variables (i.e., time, temperature, enzyme amount, and substrate molar ratio) and the response (molar conversion); and to obtain the optimum conditions for L-ascorbyl laurate synthesis by using central composite rotatable design and response surface methodology.

2. Materials and methods

2.1. Materials

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym® 435) from *C. antarctica* was a gift from Novozymes (Bagsvaerd, Denmark), whose catalytic activity was 7000 PLU/g (propyl laurate units per gram) with 1–2 wt% water content. L-Ascorbic acid (99% pure) and lauric acid (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Molecular sieve 4 Å was purchased from Davison Chemical (Baltimore, MD, USA) and acetonitrile was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical reagent grade.

2.2. Methods

2.2.1. Experimental design

A 5-level-4-factor CCRD was employed in this study, requiring 27 experiments [10]. The fractional factorial design consisted of 16 factorial points, 8 axial points (two axial points on the axis of each design variable at a distance of 2 from the design center), and 3 center points. The variables and their levels selected for the study of L-ascorbyl laurate synthesis were: reaction time (2–10 h); tem-

Table 1
Central composite rotatable second-order design and experimental data for 5-level-4-factor response surface analysis

Treatment # [a]	Time (h) x_1	Temperature (°C) x_2	Enzyme amount (% w/w of L-ascorbic acid) x_3	Substrate molar ratio (L-ascorbic acid:lauric acid) x_4	Yield (%) Y
1	−1(4) [b]	−1(35)	−1(20)	1(1:4)	80.80
2	−1(4)	−1(35)	1(40)	−1(1:2)	72.49
3	−1(4)	1(55)	−1(20)	−1(1:2)	49.53
4	−1(4)	1(55)	1(40)	1(1:4)	77.37
5	1(8)	−1(35)	−1(20)	−1(1:2)	65.84
6	1(8)	−1(35)	1(40)	1(1:4)	91.57
7	1(8)	1(55)	−1(20)	1(1:4)	73.08
8	1(8)	1(55)	1(40)	−1(1:2)	65.98
9	0(6)	0(45)	0(30)	0(1:3)	68.54
10	−1(4)	−1(35)	−1(20)	−1(1:2)	43.49
11	−1(4)	−1(35)	1(40)	1(1:4)	87.21
12	−1(4)	1(55)	−1(20)	1(1:4)	52.06
13	−1(4)	1(55)	1(40)	−1(1:2)	58.77
14	1(8)	−1(35)	−1(20)	1(1:4)	79.80
15	1(8)	−1(35)	1(40)	−1(1:2)	74.47
16	1(8)	1(55)	−1(20)	−1(1:2)	40.16
17	1(8)	1(55)	1(40)	1(1:4)	65.07
18	0(6)	0(45)	0(30)	0(1:3)	66.95
19	−2(2)	0(45)	0(30)	0(1:3)	57.62
20	2(10)	0(45)	0(30)	0(1:3)	69.58
21	0(6)	−2(25)	0(30)	0(1:3)	73.26
22	0(6)	2(65)	0(30)	0(1:3)	62.47
23	0(6)	0(45)	−2(10)	0(1:3)	54.27
24	0(6)	0(45)	2(50)	0(1:3)	75.70
25	0(6)	0(45)	0(30)	−2(1:1)	40.08
26	0(6)	0(45)	0(30)	2(1:5)	85.02
27	0(6)	0(45)	0(30)	0(1:3)	68.90

[a] The treatments were run in a random order.
[b] Numbers in parenthesis represent actual experimental amounts.

perature (25–65 °C); enzyme amount (10–50% w/w of L-ascorbic acid); and substrate molar ratio (1:1–1:5; L-ascorbic acid: lauric acid). Table 1 shows the independent factors (x_i), levels and experimental design in terms of coded and uncoded variables. To avoid bias, 27 runs were performed in a totally random order.

2.2.2. Enzymatic synthesis and analysis

All materials were dehydrated by molecular sieve 4 Å (10% w/w) for 24 h before use. L-Ascorbic acid (30 mM) and different molar ratios of lauric acid were added into 3 mL acetonitrile, followed by different amounts of enzyme (10–50% w/w of L-ascorbic acid). The mixtures containing L-ascorbic acid, lauric acid and Novozym® 435 were stirred in an orbital shaking water bath (180 rpm) at different reaction temperatures and reaction times as shown in Table 1. The solid enzyme and any possible residual water were removed in a column packed with anhydrous sodium sulfate. All samples were analyzed with a Hewlett Packard 1100 HPLC (Avondale, PA, USA) equipped with a Spherisorb ODS-2 column (5 μm, 250 mm × 4.6 mm) (Macherey-Nagel, Duren, Germany) and a UV detector at 280 nm. The mobile phase was acetonitrile/water (30/70) at 1.0 mL/min flow rate for 12 min. The relative percentage yield (molar conversion) was defined as: L-ascorbyl laurate/(L-ascorbic acid + L-ascorbyl laurate) × 100% and was estimated using peak area integrated by on-line software, Hewlett Packard 3365 Series II ChemStation (Avondale, PA).

2.2.3. Purification of product

The purification procedure for the reaction product and ^{1}H NMR analysis method were all according to the previous report [11]. Our purified product was identified as 6-O-lauroyl L-ascorbic acid (Fig. 1) which was consistent with that obtained by Watanabe et al. [11].

2.2.4. Statistical analysis

The experimental data (Table 1) were analyzed by the response surface regression (Proc RSREG) procedure to fit the following second-order polynomial equation [12]:

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

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

3. Results and discussion

3.1. Model fitting

The RSREG procedure for SAS (Statistics Analysis System) was employed to fit the second-order polynomial Eq. (I) to the experimental data percent molar conversions (Table 1). Among the various treatments, the highest molar conversion (91.6%) was treatment #6 (8 h, 35 °C, 40% enzyme, substrate molar ratio 1:4), and the smallest conversion (40.1%) was treatment #25 (6 h, 45 °C, 30% enzyme, substrate molar ratio 1:1). From the SAS output of RSREG, the second-order polynomial Eq. (I) was given below:

$$\begin{aligned} Y = & -62.049 + 10.200x_1 + 0.128x_2 + 2.231x_3 + 32.478x_4 \\ & - 0.175x_1x_1 - 0.066x_2x_1 + 0.004x_2x_2 - 0.099x_3x_1 \\ & - 0.002x_3x_2 - 0.004x_3x_3 - 0.315x_4x_1 - 0.187x_4x_2 \\ & - 0.233x_4x_3 - 0.961x_4x_4 \end{aligned} \qquad \text{(II)}$$

Table 2
Results of regression analysis of central composite rotatory design experiment

Parameters	Estimate	Standard error	t value	p value
Intercept	−62.049453	73.453762	−0.84	0.4148
x_1	10.200469	7.489142	1.36	0.1982
x_2	0.127760	1.734729	0.07	0.9425
x_3	2.230510	1.497828	1.49	0.1622
x_4	32.477813	14.978284	2.17	0.0509
$x_1{}^*x_1$	−0.174557	0.399425	−0.44	0.6699
$x_2{}^*x_1$	−0.066031	0.092243	−0.72	0.4878
$x_2{}^*x_2$	0.003680	0.015977	0.23	0.8217
$x_3{}^*x_1$	−0.099219	0.092243	−1.08	0.3032
$x_3{}^*x_2$	−0.002156	0.018449	−0.12	0.9089
$x_3{}^*x_3$	−0.003520	0.015977	−0.22	0.8293
$x_4{}^*x_1$	−0.315313	0.922432	−0.34	0.7384
$x_4{}^*x_2$	−0.187187	0.184486	−1.01	0.3303
$x_4{}^*x_3$	−0.232563	0.184486	−1.26	0.2314
$x_4{}^*x_4$	−0.960729	1.597699	−0.60	0.5588

The analysis of variance (ANOVA) indicated that the second-order polynomial model [Eq. (II)] was statistically significant and adequate to represent the actual relationship between the response (percent molar conversion) and the significant variables, with very small p value ($p < 0.01$) and a satisfactory coefficient of determination ($R^2 = 0.867$). Both t and p values of the variables and their combined items were indicated as Table 2. Furthermore, the overall effect of the four synthesis variables on the molar conversion of L-ascorbyl laurate was further analyzed by a joint test (Table 3). The results revealed that the reaction temperature (x_2), enzyme amount (x_3), and substrate molar ratio (x_4) were the most important factors that exhibited a statistically significant overall effect ($p < 0.05$) on the molar conversion of L-ascorbyl laurate. However, reaction time (x_1) showed a less significant effect ($p > 0.05$) on the enzymatic synthesis of L-ascorbyl laurate.

3.2. Mutual effect of parameters

Due to the conversion rate will be significantly changed as the enzyme concentration or reaction time varied. Enzyme amount and reaction time were investigated in the range of 10–50% and 2–10 h, respectively. Fig. 2 shows the effect of enzyme amount, reaction time, and their mutual interaction on L-ascorbyl laurate synthesis at 45 °C and 1:2 substrate molar ratio. As the enzyme amount and reaction time decreased, the molar conversion of L-ascorbyl laurate was reduced, whereas the effect of reaction time was lower than the effect of enzyme amount. This result was consistent with that obtained from Table 2, which indicated that reaction time (x_1) do not have significant effect ($p > 0.05$) on the biosynthesis of L-ascorbyl laurate. The effect of different enzyme amount and reaction temperature on esterification of L-ascorbyl laurate at constant reaction time (6 h) and substrate molar ratio (1:2) is shown in Fig. 3. At any given temperature from 25 to 65 °C, an increase in enzyme amount led to higher molar conversion (>80%), indicating that the enzyme amount is one of the most important factors affecting L-

Table 3
Analysis of variance for joint test

Factor	Degrees of freedom	Sum of squares	Probability > F[a]
Time (x_1)	5	248.66	0.5044[b]
Temperature (x_2)	5	849.57	0.0493
Enzyme amount (x_3)	5	1103.35	0.0219
Substrate molar ratio (x_4)	5	2298.89	0.0013

[a] Probability > F = level of significance.
[b] Not significant at $p = 0.05$.

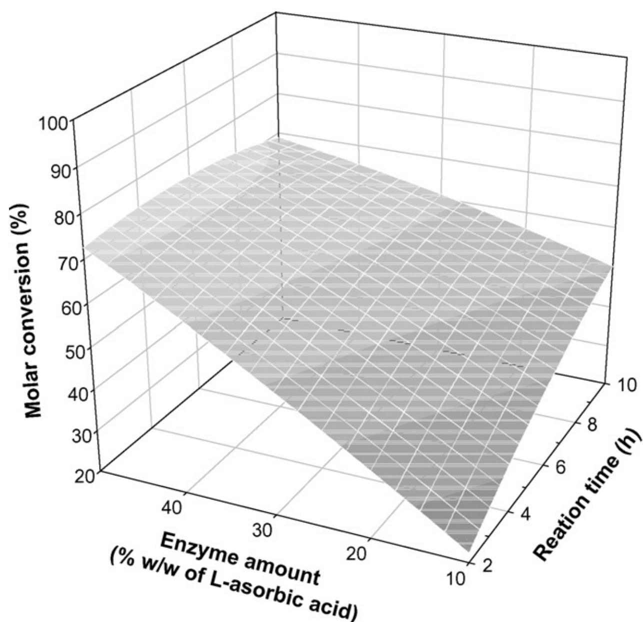

Fig. 2. Response surface plot showing the mutual effect of reaction time and enzyme amount on molar conversion of L-ascorbyl laurate. Other synthesis parameters (substrate molar ratio and reaction temperature) were constant at 0 levels.

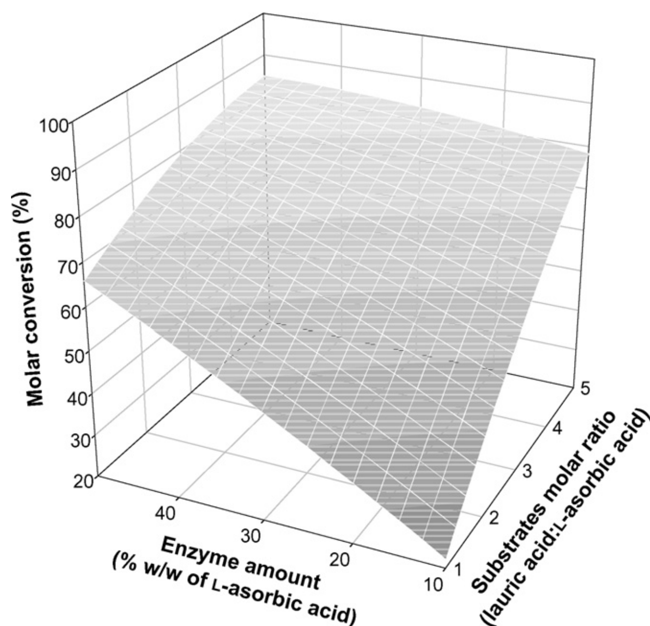

Fig. 4. Response surface plot showing the mutual effect of substrate molar ratio and enzyme amount on molar conversion of L-ascorbyl laurate. Other synthesis parameters (reaction temperature and reaction time) were constant at 0 levels.

ascorbyl laurate formation. Additionally, the enzyme (Novozym® 435) used in this study was active at lower temperature (around 25 °C) compared to those obtained in several previous works [1,6,9], and this may be because the reaction environment changed from hydrophobic to hydrophilic solvents. Fig. 4 represents the effect of varying enzyme amount and substrate molar ratio on the formation of L-ascorbyl laurate at 6 h and 45 °C. At the lowest substrate molar ratio (1:1) with the lowest enzyme amount (10%), the molar conversion was only 20%. The highest enzyme amount (50%) and substrate molar ratio (1:5) could result in the molar conversion reaching a maximum level of over 80%. This indicated that the molar conversion was greatly affected by enzyme amount (x_3) and substrate molar ratio (x_4) and was in good agreement with the analysis outcome of joint test for different variables utilized in this study (see Table 3). This is an indicator of effectiveness and economical performance.

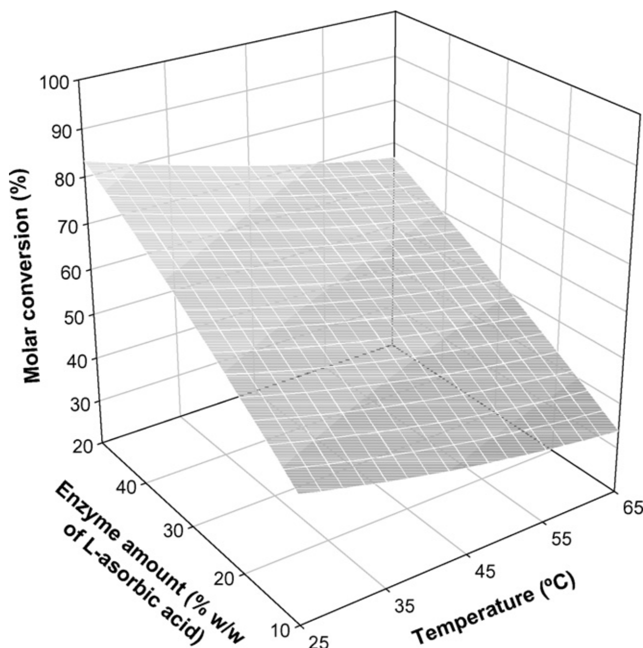

Fig. 3. Response surface plot showing the mutual effect of reaction temperature and enzyme amount on molar conversion of L-ascorbyl laurate. Other synthesis parameters (substrate molar ratio and reaction time) were constant at 0 levels.

The relationships between reaction factors and response can be better understood by examining the planned series of contour plots (Fig. 5) generated from the predicted model [Eq. (II)] by holding the enzyme amount (20, 30, 40%) and substrate molar ratio (1:2, 1:3, 1:4) constant. Fig. 5A–C represent the same substrate molar ratio (1:2); and A, D, and G represent the same enzyme amount (20%). Such an application could be adopted to study the synthesis variables simultaneously in a five-dimensional space. For an economically feasible process development, an enzyme quantity can be selected by chance for calculating optimum parameters. All nine contour plots exhibited similar behavior in that the predicted molar conversion increased with the enzyme amount and substrate molar ratio. Similarly, the reaction temperature around 25 °C gave higher percent molar conversion than 35 and 45 °C. The reaction with 40% enzyme and substrate molar ratio 1:4 (Fig. 5) could result in reaching the predicted high molar conversion (~95%) at a shorter reaction time. There is a trade off between enzyme amount and time; however, enzyme can be reused leading to cost savings.

3.3. Attaining optimum condition

The optimal synthesis condition for L-ascorbyl laurate was analyzed by the ridge max analysis [12] which computes the estimated ridge of maximum response for increasing radii from the center of original design. The outcome of ridge max analysis shows that higher enzyme amount will result in higher molar conversion of L-ascorbyl laurate with different conditions of all other parameters (Table 4). Therefore, according to the ridge max analysis outcome, the optimum reaction condition with the maximum predicted molar conversion was suggested as 93.2 ± 5.6% at 6.7 h, 30.6 °C, 34% enzyme amount, and 1:4.3 sub-

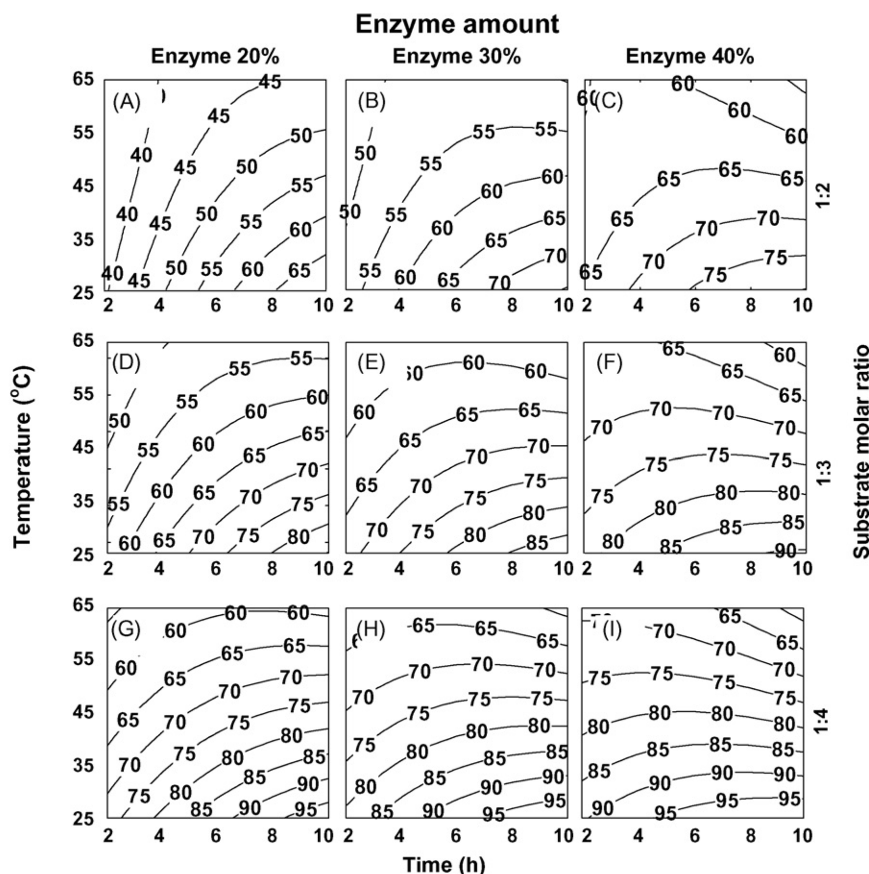

Fig. 5. Contour plots showing the mutual interaction of various reaction parameters on percent molar conversion of L-ascorbyl laurate. Enzyme amount was estimated by weight of L-ascorbic acid. The numbers inside the contour plots indicate molar conversions at given reaction conditions.

Table 4
Estimated ridge of maximum response for variable molar conversion

Coded radius	Estimated response (corporation)	Standard error	x_1 (h)	x_2 (°C)	x_3 (% w/w, by wt. of L-ascorbic acid)	x_4 (Lauric acid/L-ascorbic acid)
0	68.13	4.26	6.00	45.00	30.00	3.00
0.2	73.17	4.14	6.13	42.91	31.75	3.29
0.4	78.07	3.85	6.25	40.25	33.04	3.55
0.6	82.99	3.69	6.37	37.20	33.82	3.81
0.8	88.01	4.18	6.51	33.93	34.13	4.05
1.0	93.22	5.64	6.68	30.55	34.02	4.28

strate molar ratio at the distance of the coded radius 1.0 as shown in Table 4.

We compared the previous report [1], who used immobilized lipase from *C. antarctica* (Novozym® 435) to synthesize 6-O-palmitoyl L-ascorbic acid in 2-methyl-2-butanol with 68% ester yield in 8 h, at 55 °C, and with 1:5 substrate molar ratio with our results. Although our target product and solvent type was different from Humeau et al. [1], Song et al. [14], Song et al. [15] and Lv et al. [16], however, it was interesting to find that all investigations obtained similar results with 33–48% of L-ascorbyl ester molar conversion which represented at least 45% lower product yields than the results obtained in our study. Comparing the enzymatic synthesis of L-ascorbyl laurate (C_{12}) to the previous report [6], our results showed ~20% higher molar conversion and 41 h shorter reaction time than their *t*-butanol system. Obviously, from the RSM and ridge max statistical analysis results, our optimum reaction condition involved higher molar conversion yield and lower reaction temperature than their report.

3.4. Model verification

The adequacy of the predicted model was examined by performing three additional independent experiments at the suggested optimum synthesis conditions. The predicted value was 93.2 ± 5.6% molar conversion and the actual experimental value was 92.6 ± 3.8%. A chi-square test (p value = 0.982, degrees of freedom = 5) indicated that the observed values were essentially the same as the predicted values and that the generated model adequately predicted the percent molar conversion [13].

4. Conclusions

The enzymatic synthesis of L-ascorbyl laurate by means of direct esterification of L-ascorbic acid and lauric acid in acetonitrile was investigated in the present work. Also, the optimum reaction condition was studied by central composite rotatory design and response surface methodology. Based on the results of four parameters evaluation (joint test), only reaction time exhibited minor effect on the

process which might because of the equilibrium was reached. As the experiment was designed to utilize 34 wt% enzyme amount and 1:4.3 substrate molar ratio at 30.6 °C for 6.7 h, a ∼93% of maximum molar conversion was obtained. Thus, the optimization of lipase-catalyzed synthesis for L-ascorbyl laurate by Novozym® 435 was successfully developed by CCRD and RSM.

Acknowledgement

This research was supported by the National Science Council (NSC95-2313-B-212-011-MY3), Taiwan, the Republic of China.

References

[1] C. Humeau, M. Girardin, D. Coulon, A. Miclo, Biotechnol. Lett. 17 (1995) 1091–1094.
[2] X.Y. Liu, F.L. Guo, Y.C. Liu, Z.L. Liu, Chem. Phys. Lipids 83 (1996) 39–43.
[3] N.V. Perrpicone, U.S. Patent 5. 409,693 (1993).
[4] S. Palma, P.L. Nostro, R. Manzo, D. Allemandi, Eur. J. Pharm. Sci. 16 (2002) 37–43.
[5] C. Humeau, M. Girardin, B. Rovel, A. Miclo, J. Mol. Catal. B: Enzym. 5 (1998) 19–23.
[6] Y. Yan, U.T. Bornscheuer, R.D. Schmid, Biotechnol. Lett. 21 (1999) 1051–1054.
[7] R.W. Ahmad, W.A. Anderson, M. Moo-Young, Enzyme Microb. Technol. 23 (1998) 438–450.
[8] W.-D. Chiang, S.-W. Chang, C.-J. Shieh, Process Biochem. 38 (2003) 1193–1199.
[9] L.-H. Tang, T. Zhang, M.M. Shehate, Y.-F. Sun, Biotechnol. Appl. Biochem. 32 (2000) 35–39.
[10] W.G. Cochran, G.M. Cox, Experimental Designs, John Wiley & Son Inc., New York, 1992.
[11] Y. Watanabe, S. Adachi, K. Nakanishi, R. Matsuno, Food Sci. Technol. Res. 5 (1999) 188–192.
[12] SAS, SAS user guide, SAS Institute Inc., Cary, NC, 1990.
[13] L. Ott, An Introduction to Statistical Methods and Data Analysis, PWS-Kent Publishing Company, Boston, 1988.
[14] Q.-X. Song, D.-Z. Wei, W.-Y. Zhou, W.-Q. Xu, S.-L. Yang, Biotechnol. Lett. 26 (2004) 1777–1780.
[15] Q.X. Song, Y. Zhao, W.Q. Xu, W.Y. Zhou, D.Z. Wei, Bioprocess Biosyst. Eng. 28 (2006) 211–215.
[16] L.-X. Lv, S.-Y. Chen, Y.-Q. Li, J. Sci. Food Agric. 88 (2008) 659–666.