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Optimized enzymatic synthesis of ascorbyl esters from lard using Novozym 435 in co-solvent mixtures

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ABSTRACT

A mild and efficient method for the conversion of fatty acid methyl esters from lard into ascorbyl esters via lipase-catalyzed transesterification in co-solvent mixture is described. A solvent engineering strategy was firstly applied to improve fatty acid ascorbyl esters production. The co-solvent mixture of 30% t-pentanol:70% isooctane (v/v) was optimal. Response surface methodology (RSM) and central composite design (CCD) were employed to estimate the effects of reaction parameters, such as reaction time (12–36 h), temperature (45–65 °C), enzyme amount (10–20%, w/w, of fat acid methyl esters), and substrate molar ratio of fatty acid methyl esters to ascorbic acid (8:1–12:1) for the synthesis of fatty acid ascorbyl esters in co-solvent mixture. Based on the RSM analysis, the optimal reaction conditions were determined as follows: reaction time 34.32 h, temperature 54.6 °C, enzyme amount 12.5%, substrate molar ratio 10.22:1 and the maximum conversion of fatty acid ascorbyl esters was 69.18%. The method proved to be applicable for the synthesis of ascorbyl esters using Novozym 435 in solvent.

1. Introduction

In recent years there has been a large demand for ascorbyl esters for extensive use in food, cosmetics, and medicine on account of their improved miscibility and solubility. The effectiveness against the cytotoxicity of lipid peroxidation [1] and the ability to scavenge hydroxyl free radicals [2] make these lipophilic derivatives excellent antioxidants. Being fat-soluble, they can concentrate into the lipid domains of biological systems and protect cell membranes and low-density lipoprotein (LDL) against oxidation. Syntheses of ascorbyl esters by chemical methods have been attempted in the past [3,4]. Due to the steady growing demand for natural materials, the biosynthesis of such esters by lipase-catalyzed reactions has become a current commercial interest. The enzymatic method is characterized by mild reaction conditions and high regioselectivity. Another advantage is that enzymatically synthesized ascorbyl esters can be qualified as natural additives.

Numerous reports have been published on the synthesis of ascorbyl esters using lipase, which is one of the most widely used enzymes in organic synthesis and industrial processes [5–9]. Acetone, t-amyl alcohol, and t-butanol are the most preferred solvents. Unfortunately, lipases are highly inactivated in such organic solvents, often resulting in poor synthetic yields. By product methanol is hydrophilic and insoluble in the oils, so it is easily adsorbed onto the surface of the immobilized lipase also leading to the negative effect on lipase activity and operational stability. Recently, it has been established that ionic liquids can successfully replace the organic solvents in the synthesis of ascorbyl esters [10]. Even though ionic liquids do not denature lipases, and the reactions proceed to higher conversions, a number of issues including the cost involved on large-scale usage are to be addressed before using them in industry. Until then, organic solvents will remain in use in the enzymatic synthesis of ascorbyl esters.

Research on synthesis of ascorbyl fatty acid ester using unsaturated and saturated fatty acid as an acyl donor in organic solvent catalyzed by lipase has been reported [10–12]. However, the use of lard in the synthesis of ascorbyl fatty acid ester has not been explored. Lard is abundantly available in China. It contains a mixture of monounsaturated, polyunsaturated, and saturated fatty acid. The major fatty acids are palmitic acid (26.61%) and oleic acid (43.18%) [13].

In this study, fatty acid methyl esters from lard will be used as a source of fatty acids in the transesterification with ascorbic acid using commercial immobilized lipase (Fig. 1). A solvent engineering strategy was applied to search for appropriate reaction medium for ascorbyl esters production and a co-solvent mixture was developed. Then optimal conditions in co-solvent mixtures for the enzymatic conversion were investigated by

Abbreviations: RSM, response surface methodology; CCD, central composite design; LDL, low-density lipoprotein.

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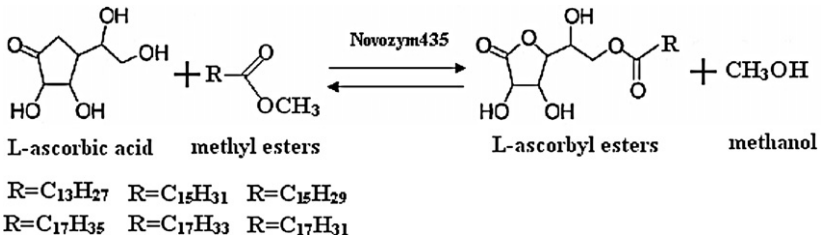

Fig. 1. Lipase-catalyzed transesterification of ascorbic acid and methyl esters of lard using immobilized Novozym 435 in co-solvent mixture.

using central composite rotatable design and response surface methodology.

2. Materials and methods

2.1. Materials

Novozym435 (lipase B from *Candida antarctica*, a nonspecific lipase immobilized on a macroporous acrylic resin with a specific activity 10,000 PLU/g and water content 1–2% (w/w)) was purchased from Novozymes A/S Bioindustrial, Inc. (Bagsvaerd, Denmark). L-ascorbic acid (purity > 99%) was from Sinopharm chemical reagent Co. Ltd. (Shanghai, China). The refined bleached and deodorized lard was kindly provided by Oil Processing Company of Lianyungang (China), with the composition of C14:0 (1.5%), C16:0 (26%), C16:1 (2.5%), C18:0 (13%), C18:1 (45%), and C18:2 (12%). Methyl esters of lard were synthesized in our laboratory by the lipase-mediated transesterification between lard and methanol. These esters were purified and used as acyl donors in the preparation of ascorbyl esters. All other chemicals used were of analytical grade or chromatography grade.

2.2. Procedure for lipase-catalyzed transesterification in co-solvent mixture screening

The reaction was initiated by mixing 24.2 mg of the immobilized lipase (Novozym435) and 10 mL of co-solvent mixtures (analytical grade), 0.1 mmol of ascorbic acid, and 0.8 mmol of acyl donor in 100 mL screw-capped flask. The total volume of the reaction mixture was 10 mL. The reactions were carried out on a rotary shaker at 50 °C and 180 rpm, for 24 h. Samples (1 mL) taken from the reaction mixture, were filtered through an anhydrous sodium sulfate column to remove any moisture and enzyme particles and analyzed by HPLC.

2.3. General procedure for lipase-catalyzed transesterification in optimization reaction

Ascorbic acid (0.1 mmol) and different molar ratios of acyl donor were added into 10 mL co-solvent mixture of 30% t-pentanol:70% isooctane (v/v), followed by different amounts of enzyme (10–20%, w/w, of acyl donor). The mixtures containing ascorbic acid, acyl donor and Novozym® 435 were stirred in a rotary shaker (180 rpm) at designed reaction temperatures. After reaction, 1 mL of the reaction mixture was sampled and the solid enzyme and any possible residual water (solvents contained) were removed in an anhydrous sodium sulfate column. All samples were analyzed by HPLC.

2.4. HPLC analysis of the samples

Ascorbyl esters were analyzed on an Agilent 1100 series HPLC, equipped with an Eclipse XDB-C18 column (5 μm, 4.6 mm × 250 mm, Agilent) and an UV detector at 254 nm. The column temperature maintained 30 °C. Elution was performed with acetonitrile/methanol/water (40/45/15, v/v/v, containing 0.1% TFA) at a flow-rate of 1 mL/min for 45 min. Percentage conversion was calculated based on the number of moles of ascorbic acid added, which was the limiting substrate. The composition of the mixed esters of ascorbic acid obtained was determined and calculated by standard samples of ascorbyl fatty acid esters by external standard method. The conversion was calculated as followed: total molar numbers of ascorbyl fatty acid esters/molar numbers of ascorbic acid added × 100%.

2.5. Experimental design

A 3-level-4-factor CCD was employed in this study, requiring 30 experiments [14]. The independent variables are studied at five different levels, and the variables and their coded levels used for the study are shown in Table 1. All the experiments were done in triplicate and the average of obtained percent molar conversion was taken as the dependent variable or response (Y). The second-order polynomial coefficients were calculated and analyzed using the "Design Expert" software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second-order polynomial equation (1) is

$$Y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij} \quad (1)$$

where Y is response (percent molar conversion); b_0, b_i, b_{ii}, and b_{ij} are constant coefficients and x_i is the uncoded independent variables. The goodness of the model fit was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Quadratic polynomial equations were attained by holding one of the independent variances at a constant value and changing the level of the other variables.

3. Results and discussion

3.1. Effect of reaction medium

One important requirement for achieving high ascorbyl esters production is to find an optimal reaction medium with respect to the substrate and the product solubility, and the enzyme sta-

Table 1
Level and code of variables chosen for central composite rotatable design.

Variables	Uncoded symbol	Coded level				
		−2	−1	0	1	2
Substrate molar ratio (acyl donor to ascorbic acid)	x_1	6:1	8:1	10:1	12:1	14:1
Enzyme amount/%	x_2	5	10	15	20	25
Temperature/°C	x_3	35	45	55	65	75
Reaction time/h	x_4	0	12	24	36	48

Table 2
Lipase-catalyzed transesterification of ascorbic acid and fatty acid methyl esters in different co-solvent mixtures.

Co-solvent mixtures (v/v)	Conversion (%)	Co-solvent mixtures (v/v)	Conversion (%)	Co-solvent mixtures (v/v)	Conversion (%)
Acetone:10% n-hexane	8.2	t-Pentanol:10% n-hexane	8.5	t-Butanol:10% n-hexane	8.1
Acetone:20% n-hexane	9.6	t-Pentanol:20% n-hexane	9.3	t-Butanol:20% n-hexane	8.6
Acetone:30% n-hexane	10	t-Pentanol:30% n-hexane	11.4	t-Butanol:30% n-hexane	9.7
Acetone:50% n-hexane	11.7	t-Pentanol:50% n-hexane	17.8	t-Butanol:50% n-hexane	11.5
Acetone:70% n-hexane	13.8	t-Pentanol:70% n-hexane	22.4	t-Butanol:70% n-hexane	18.7
Acetone:80% n-hexane	10.6	t-Pentanol:80% n-hexane	18.9	t-Butanol:80% n-hexane	8.5
Acetone:10% n-heptane	8.6	t-Pentanol:10% n-heptane	9.7	t-Butanol:10% n-heptane	9.1
Acetone:20% n-heptane	9.5	t-Pentanol:20% n-heptane	10.9	t-Butanol:20% n-heptane	9.6
Acetone:30% n-heptane	12.6	t-Pentanol:30% n-heptane	12.4	t-Butanol:30% n-heptane	11.4
Acetone:50% n-heptane	14.9	t-Pentanol:50% n-heptane	14.7	t-Butanol:50% n-heptane	13.9
Acetone:70% n-heptane	15.6	t-Pentanol:70% n-heptane	25.6	t-Butanol:70% n-heptane	18.3
Acetone:80% n-heptane	15.7	t-Pentanol:80% n-heptane	13.4	t-Butanol:80% n-heptane	11.2
Acetone:10% isooctane	8.9	t-Pentanol:10% isooctane	12.7	t-Butanol:10% isooctane	10.2
Acetone:20% isooctane	10.1	t-Pentanol:20% isooctane	13.8	t-Butanol:20% isooctane	11.6
Acetone:30% isooctane	14.8	t-Pentanol:30% isooctane	15.1	t-Butanol:30% isooctane	13.4
Acetone:50% isooctane	16.8	t-Pentanol:50% isooctane	23.3	t-Butanol:50% isooctane	18.9
Acetone:70% isooctane	17.8	t-Pentanol:70% isooctane	31.6	t-Butanol:70% isooctane	26.8
Acetone:80% isooctane	16.2	t-Pentanol:80% isooctane	22.6	t-Butanol:80% isooctane	19.8

bility. Ascorbic acid and methanol are generally soluble in polar organic solvents, like acetone, t-butanol, t-pentanol which have a strongly inactivating effect on lipase [15]. In previous studies, we have demonstrated that hexane and isooctane are the more suitable solvents for lipase-catalyzed transferesterification of oil [16,17]. Therefore, in this study solvent engineering method was applied. According to solvent engineering method, we chose co-solvent mixtures as reaction medium. It was composed of a high log *P* solvent which can provide a suitable environment for enzyme catalysis and a low log *P* solvent which can be used for dissolving methanol, such as co-solvent mixtures of t-pentanol and isooctane.

The results were shown in Table 2, the conversion of ascorbic acid in t-pentanol co-solvent mixture > that in t-butanol co-solvent mixture > that in acetone co-solvent mixture. For each co-solvent mixture, there is a consistent trend of increasing conversion with increasing amount of the co-solvent up to the point where the solubility limit of the methanol affect the lipase activity. However, mixtures with different co-solvents having the same solvent ratio did not result in the same conversion. For example, at solvent ratio of 3:7 of isooctane to a co-solvent, the conversion changed from 17.8% with acetone as the co-solvent to 31.6% with t-pentanol as the co-solvent. The highest conversion (31.6%) was found in the co-solvent mixtures of 30% t-pentanol:70% isooctane, which was higher than that in t-pentanol (29.5%) The result indicates that a mixture of t-pentanol with isooctane at a ratio of 3:7 is optimal for lipase catalyzed synthesis of fatty acid ascorbyl esters.

3.2. Optimized synthesis of lipase-catalyzed ascorbyl esters

3.2.1. Model fitting

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material. All 30 of the designed experiments were conducted for optimizing the four individual parameters in the current central composite design. The backward regression procedure for "Design Expert" 7.0.0 was employed to fit the second-order polynomial Eq. (1) to the experimental data percent molar conversions (Table 3). The results were analyzed by multiple regression analysis. Among the various treatments, maximum molar conversion was recorded under the experimental conditions of substrate molar ratio (acyl donor to ascorbic acid) 10:1, enzyme amount 10%, temperature 55 °C and reaction time 24 h. According to the "Design Expert" 7.0.0 output of backward regression procedure, the second-order polynomial Eq. (2) was given below:

$$Y = 67.97 + 11.10x_1 + 2.69x_2 + 0.23x_3 + 4.60x_4 + 0.049x_1x_2 - 0.60x_1x_3 + 1.81x_1x_4 - 0.34x_2x_3 - 1.68x_2x_4 - 0.89x_3x_4 - 5.42x_1^2 - 5.47x_2^2 - 5.00x_3^2 - 3.32x_4^2 \quad (2)$$

In order to determine whether or not the second-order polynomial model (Eq. (2)) is significant, it is necessary to conduct ANOVA analysis. The *P*-values were used as a tool to check the significance of each coefficient, which also indicated the interaction strength of each parameter. The smaller the *P*-values are, the bigger the significance of the corresponding coefficient [18]. Here, the *P*-value of the model was smaller than 0.0001, which indicated that the model

Table 3
Central composite design matrix of the four variables in coded units along with the experimental values of ascorbyl esters conversion.

Trials no.	x_1	x_2	x_3	x_4	Conversion %
1	−1	−1	−1	−1	30.12
2	1	−1	−1	−1	52.26
3	−1	1	−1	−1	34.97
4	1	1	−1	−1	58.47
5	−1	−1	1	−1	29.08
6	1	−1	1	−1	48.25
7	−1	1	1	−1	41.61
8	1	1	1	−1	54.31
9	−1	−1	−1	1	39.07
10	1	−1	−1	1	64.74
11	−1	1	−1	1	43.06
12	1	1	−1	1	68.57
13	−1	−1	1	1	38.65
14	1	−1	1	1	63.26
15	−1	1	1	1	33.06
16	1	1	1	1	63.73
17	−2	0	0	0	27.81
18	2	0	0	0	69.05
19	0	−2	0	0	40.16
20	0	2	0	0	56.26
21	0	0	−2	0	43.91
22	0	0	2	0	56.31
23	0	0	0	−2	–
24	0	0	0	2	68.17
25	0	0	0	0	62.73
26	0	0	0	0	65.22
27	0	0	0	0	70.19
28	0	0	0	0	69.09
29	0	0	0	0	71.07
30	0	0	0	0	69.52

Table 4
Results of regression analysis of central composite design experiment.

Parameters	Estimate	Standard error	*F*-value	*P* > *F*
Intercept	69.97	1.94	17.68	<0.0001
x_1	11.10	0.97	131.30	<0.0001
x_2	2.69	0.97	7.71	0.0149
x_3	0.23	0.97	0.056	0.8168
x_4	4.60	1.15	16.10	0.0013
x_1x_2	0.049	1.19	1.731E−003	0.9674
x_1x_3	−0.60	1.19	0.26	0.6185
x_1x_4	1.81	1.19	2.32	0.1496
x_2x_3	−0.34	1.19	0.081	0.7799
x_2x_4	−1.68	1.19	2.01	0.1777
x_3x_4	−0.89	1.19	0.56	0.4678
x_1^2	−5.42	0.92	34.74	<0.0001
x_2^2	−5.47	0.92	35.45	<0.0001
x_3^2	−5.00	0.92	29.56	<0.0001
x_4^2	−3.32	1.19	7.81	0.0144

was statistically significant and suitable for use in this experiment. The *P*-value of "lack of fit" was 2.74 ($P > 0.01$), which indicated that "lack of fit" was insignificant relative to the pure error. The coefficient of determination (R^2) was 0.9465, which indicated that the accuracy of the polynomial model were adequate. The regression coefficients and the corresponding *P*-values were presented in Table 4. From the *P*-values of each model term, it could be concluded that substrate molar ratio (x_1), enzyme amount (x_2) and reaction time (x_4) were the most important factors that represented a statistically significant overall effect ($P < 0.05$) on the conversion of ascorbyl esters. However, reaction temperature (x_3) showed a less significant effect ($P > 0.05$) on the enzymatic synthesis of ascorbyl esters. The interaction terms (x_1x_2, x_1x_3, x_1x_4, x_2x_3, x_2x_4, x_3x_4) all are insignificant ($P > 0.05$) and the quadratic terms are significant ($P < 0.0001$ or $P < 0.05$). These results showed that the effects of above various factors on ascorbyl esters conversion were not simple linear relationship.

3.2.2. Mutual effect of parameters

Fig. 2 shows the effect of enzyme amount, reaction time, and their mutual interaction on ascorbyl esters synthesis at 55 °C and 10:1 substrate molar ratio. At the designed range of enzyme amount

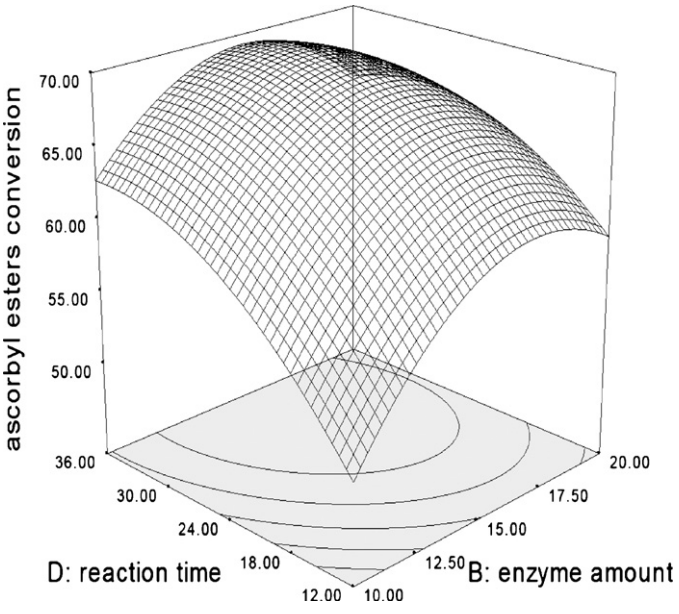

Fig. 2. Response surface plot representing the effect of enzyme amount, reaction time and their reciprocal interaction on ascorbyl esters synthesis. Temperature and substrate molar ratio is constant at 55 °C and 10:1, respectively.

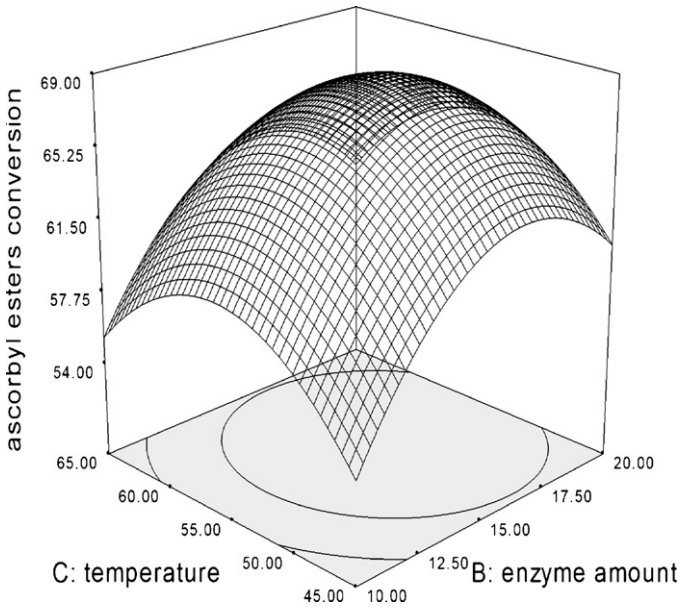

Fig. 3. Response surface plot representing the effect of reaction temperature, enzyme amount and their reciprocal interaction on ascorbyl esters synthesis. Reaction time and substrate molar ratio is constant at 24 h and 10:1, respectively.

from 10% to 20%, the augmentation of ascorbyl esters resulted in a linear increase in reaction time from 0 to 24 h, and then changed little. An increase of enzyme amount increased the molar conversion of ascorbyl esters at a constant reaction time within an enzyme amount of about 17%, and then decreased.

Fig. 3 depicts the effect of enzyme amount, temperature and their reciprocal effect on ascorbyl esters synthesis at constant reaction time (24 h) and substrate molar ratio (10:1). At the designed range of temperature from 45 °C to 65 °C, the conversion of ascorbyl esters increased with enzyme amount increasing. This result was consistent with that obtained from Table 4, which indicated that enzyme amount (x_2) has significant effect ($P < 0.05$) on the biosynthesis of ascorbyl esters. At a low temperature, the conversion of ascorbyl esters increased with temperature increasing, and then reduced when temperature was above 55 °C, possibly because of the thermal denaturation of lipase [19], however, the effect of reaction time was lower than the effect of enzyme amount. This result was consistent with that obtained from Table 4, which indicated that reaction temperature (x_3) do not have significant effect ($P > 0.05$) on the biosynthesis of ascorbyl esters.

Fig. 4 represents the effect of varying enzyme amount and substrate molar ratio on the formation of fatty acid ascorbyl esters at 24 h and 55 °C. At the lowest substrate molar ratio (8:1) with the lowest enzyme amount (10%), the conversion was only 40%. The highest substrate molar ratio (12:1) and higher enzyme amount (16%) could result in a maximum conversion of about 70%. This indicated that the conversion was greatly affected by substrate molar ratio and enzyme amount. It also can be observed from Fig. 3, the effect of substrate molar ratio was more significant than that of enzyme amount. This result was consistent with the result from Table 3.

3.2.3. Attaining optimal conditions and model validation

The optimal values of the selected variables were obtained by solving the regression equation (Eq. (2)) through least squares method using Design-Expert 7 software (Table 5). Economically thinking, low enzyme amount was suitable for the transesterification of fatty acid methyl esters with ascorbic acid. According to this, the optimal reaction conditions (predicted value 68.79%) were as follows: 34.32 h, 54.6 °C, 12.5% enzyme amount, and 10.22:1

Table 5
Optimal conditions found by the model.

Trial no	Substrate molar ratio	Enzyme amount/%	Temperature/°C	Time/h	Ascorbyl esters conversion/%
a	10.16:1	15.70	54.50	32.16	70.60%
b	10.22:1	12.50	54.60	34.32	68.79%
c	10.30:1	12.55	48.50	35.28	67.51%
d	10.18:1	13.35	49.60	21.24	64.32%

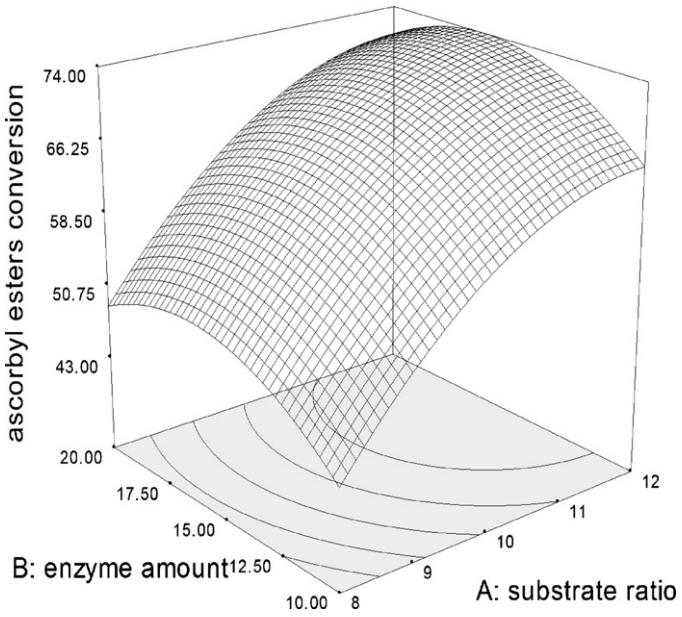

Fig. 4. Response surface plot representing the effect of substrate molar ratio, enzyme amount and their reciprocal interaction on ascorbyl esters synthesis. Reaction temperature and reaction time is constant at 55 °C and 24 h, respectively.

substrate molar ratios. We compared the previous report [20], who used immobilized lipase from *C. antarctica* (Novozym® 435) to synthesize palm-based and soybean-based ascorbyl esters in acetone with 68% (palm oil) and 17% (soybean oil) conversion in 24 h, at 50 °C, and with 1:4 substrate molar ratio with our results. It found that our results were similar with palm-based ascorbyl esters conversion, then higher than soybean-based ascorbyl esters conversion. Comparing with Hsieh et al. [20] and Burham et al. [21], it was interesting to find that all investigations obtained higher conversion at the expenses of higher enzyme amount (170% and 151%, respectively) which represented at least 139% higher enzyme amount than the results obtained in our study. Obviously, from the RSM optimization results, our optimum reaction condition involved higher molar conversion yield and lower reaction enzyme amount than their report.

In order to verify the prediction of the model, the optimal reaction conditions were applied to three independent replicates for ascorbyl esters synthesis. The average ascorbyl esters conversion was 69.18%, a figure well within the estimated value of the model equation. This demonstrated the validation of the RSM model. The good correlation between these results confirmed that the response model was adequate for reflecting the expected optimization. The results also suggested that the models of Eq. (2) are satisfactory and accurate.

4. Conclusion

A solvent engineering method was applied to improve ascorbyl esters synthesis and a co-solvent mixture of t-pentanol with isooctane at a ratio of 3:7 was optimal. Optimization of the reaction conditions for maximal conversion of ascorbyl esters was carried out. RSM was used to optimize the synthesis of ascorbyl esters. The coefficient of determination (R^2) for the model is 93.6%. *P*-value ($P < 0.0001$) demonstrates a very high significance for the regression model. Ascorbyl esters conversion of 69.18% was obtained when optimal conditions for maximal ascorbyl esters synthesis in a co-solvent mixture were: 34.32 h, 54.6 °C, 12.5% enzyme amount, and 10.22:1 substrate molar ratios. Validation experiments verified the availability and the accuracy of the model. The predicted value was in agreement with the experimental value. The study proved the response surface method to be useful for optimization of reaction parameters for ascorbyl esters synthesis by lipase in a co-solvent mixture and statistical analysis is proved to be a useful and powerful tool in developing optimal synthesis conditions.

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