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RESEARCH PAPER

Optimization of Enzymatic Synthesis of L-ascorbyl Palmitate by Solvent Engineering and Statistical Experimental Designs

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Abstract Statistical experimental designs combined with solvent engineering for optimization of enzymatic synthesis of L-ascorbyl palmitate were developed. First, the composition of the solvent for co-dissolving polar and apolar substrates was determined. The co-solvent mixture of *tert*-pentanol: DMSO at a ratio of 9:1 (v/v) and the optimal biocatalyst were obtained. Then, the Plackett-Burman design was implemented to screen the variables that significantly influence the conversion. The method of steepest ascent was used to approach the proximity of optimum. After determining the Plackett-Burman and steepest ascent designs, the optimum values were determined by central composite design under response surface methodology. The statistical analysis showed that the optimum reaction conditions (temperature 50°C, enzyme concentration 5.8 g/L, and substrate molar ratio 11:1, stirring rate 160 rpm, amount of molecular sieve 50 g/L, time 18 h) led to the maximum conversion (66.44%) and production concentration (20.63 g/L). A very satisfactory conversion (64.74%) and production concentration (20.13 g/L) could be achieved in short time (6 h).

Keywords: lipase, ascorbyl palmitate, co-solvent, optimization, statistical experimental designs, model

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1. Introduction

Ascorbyl esters are hydrophobic and biodegradable antioxidants and have several applications in the food, cosmetic, and pharmaceutical fields [1-3]. L-ascorbyl palmitate (L-AP) is an important ascorbyl ester. In the past, L-AP has been synthesized by chemical methods [4]. However, due to the steady growing demand for environmental friendly process, the synthesis of L-AP by lipase-catalyzed reactions under mild conditions, has become a current commercial interest.

Numerous reports have shown that lipase is a good biocatalyst in non-aqueous media [5-7]. It has been showed that the activity and stability of lipase in an organic solvent is highly correlated with the solvent's hydrophobicity (Log P). Lipase is able to maintain high activity in organic solvent with high log *P*-value [8,9]. However, when the reaction substrates had multiple hydroxyl groups, such as L-ascorbic acid (L-AC), this resulted in lower solubility of lipase in organic solvent and high log *P* values. Thus, the major challenge is to find out a solvent, which can dissolve ascorbic acid and retain the lipase activity, simultaneously [10]. To overcome the challenge, it is possible to choose a co-solvent mixture for lipase-catalyzed synthesis of L-AP.

The use of experimental statistical designs has become more common in several sciences, such as analytical chemistry, engineering environmental chemistry and bioprocess [11-13]. However, there have been no studies in literature that address the enzymatic synthesis of L-AP in co-solvent mixture, which has been analyzed using experimental statistical designs.

The goal of this present study was to develop a co-solvent mixture for synthesis of L-AP, to optimize the reaction conditions for L-AP, and to model the lipase-

catalyzed esterification reaction between L-AC and palmitic acid in optimal co-solvent mixture by statistical experimental designs and statistical analysis.

2. Materials and Methods

2.1. Materials

Novozym 435 (lipase B from *Candida antarctica*, immobilized on a macroporous acrylic resin) and Lipozyme TL IM (lipase from *Thermomyces lanuginosus*, immobilized on silica granulation) were purchased from Novo Nordisk, *Aspergillus niger* lipase, *Rhizopus chinensis* lipase (RCL) were purchased from Novo Nordisk A/S, Denmark. Palmitic acid, L-AC, DMSO, *tert*-pentanol, acetone, acetonitrile and n-hexane of AR grade, and 4A molecular sieves were purchased from Sinopharm Chemical Reagent Co. Ltd. China.

2.2. Preliminary experiments

In all preliminary experiments, the reaction mixtures were stirred at 160 rpm, 50ºC, and 18 h. All experiments were carried out in 25 mL conical flasks on orbital shakers; into each conical flask was placed L-ascorbic acid and palmitic acid in a molar ratio of 1:3, with 10 mL of solvent, 31.05 mol/L of L-ascorbic acid concentration (based on solvent), 5 g/L of enzyme concentration (based on solvent), and 50 g/L molecular sieve 4A.

2.3. Analysis methods

2.3.1. Products quantification

Quantitative analysis of reactants and products were conducted by HPLC system from Shimadzu, Japan. A reversed-phase column (Sepax BR-C18, 250 mm × 4 mm, 5 μm) was used. A 10 μm volume of the proper dilution of the reaction mixture was injected. A mixture of methanol/ water/ acetic acid, 95/5/0.1 (v/v/v), was used as eluent at 36ºC with a flow rate of 1 mL/min. A SPD-20AVP UV-Vis detector set at 254 nm was used to detect products. The mole conversion was defined as: the molar of the L-AP/the mole of L-AC at the beginning of reaction × 100%.

2.3.2. Determinations of L-AC solubility

To determine the solubility of L-AC in various solvents, 0.15 g of L-AC was dissolved in 6.0 mL of acetonitrile, acetone, *tert*-pentanol, n-hexane, and 0.45 g of L-AC in 6.0 mL of DMSO. These solutions were incubated at 50ºC for 3 h. Samples were analyzed by HPLC with 0.1% oxalic acid solution, in the mobile phase, and detected as same as the section of 2.3.1 products quantification.

2.4. Statistical experimental designs

2.4.1. Plackett-burman design

Plackett-Burman (PB) Statistical experimental design is very useful and widely employed in the screening of major variables that have significant effects on the response. This design gave an out put of 15 experimental runs with 5 independent variables (Table 2). Each experiment was performed in triplicate at the central point, which was carried out in order to determine the experimental error. The main effect of each variable was calculated as the difference between the average of measurements made at the high value (1) and the low value (-1). The parameters selected for the experiment were temperature, enzyme concentration, stirring rate, mole ratio, and amount of molecular sieve, respectively. Reaction time was kept constant at 18 h (time fixed after a previously reaction progress). All the experiments were performed in triplicate and the average of conversion of L-AC was used as the response (dependant variable).

2.4.2. Steepest ascent design

To determine the neighborhood of the optimum response, the significant variables screened by the PB design were optimized in terms of conversion by use of a steepest ascent design to determine the central points of each variable.

2.4.3. Central composite design

Central composite design (CCD) under the response surface methodology (RSM) was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables. A central composite design 2^2 was carried out to adjust the substrates' molar ratio and enzyme concentration. According to the CCD for 2 variables, 11 experimental runs (3 runs at center point) were fitted to the following second order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where, Y was the predicted response, β_0 was the intercept term, β_i was the liner term, β_{ii} was the square term, β_{ij} was the interaction term, and X was independent variables. The developed regression model was evaluated by analyzing the regression coefficients, analysis of variance (ANOVA), and *P* and *F* values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2. The statistical software package statistica 8.0 (statsoft Inc, USA) was used to identify the experimental design, as well as to generate a regression model, and to predict the optimal levels of the selected variables, which were obtained

by solving the regression equation and analyzing the response surface contour.

3. Results and Discussion

3.1. Preliminary experiments

3.1.1. Esterification in mono-solvent

The enzyme activity in organic solvent is often correlated with the solvent hydrophobicity of the solvent (log P) [9,14]. The value of log *P*, a partition coefficient of solvents between water and octanol, is known as not only the widely used parameter to describe solvent polarity, but also a good indicator for enzymatic synthesis [15]. The stronger the hydrophobicity of solvent with a higher log *P* is, the lower the solubility of reaction substrates with multiple hydroxyl groups [16]. Five solvents (DMSO, tert-pentanol, acetone, acetonitrile and n-hexane) with different hydrophobicity were tested. The results are shown in Table 1 together with the conversion of L-AC, the solubility of L-AC and their log *P* values [14].

As depicted in Table 1, n-hexane had the highest hydrophobicity and DMSO had the lowest hydrophobicity. No esterification occurred in these 2 solvents. In acetone and acetonitrile, the conversion was poor. *Tert*-pentanol seemed to be the best solvent. It was interesting to observe the relationship between the conversion and the solubility of L-AC. DMSO had the highest solubility of L-AC and there was no ester formed. This could be explained by the strong polarity of the pure DMSO, which rapidly deactivated the biocatalyst [17]. On the other hand, the lipase had higher enzyme activity in n-hexane (log *P* >3) [18]. However, no L-AP was formed because the L-AC solubility in it was almost negligible. Thus, both the enzyme activity and the substrate solubility played an important role in the esterification. Among the 5 solvents, *tert*-pentanol was the most suitable medium, but the L-AC solubility remained low. The comparatively lower solubility of L-AC in the solvent made it difficult to obtain notable conversions. Thus, the use of co-solvent mixture system seemed to be necessary for enhancing the conversion of L-AC in a short time period.

Table 1. Log P values, conversion and solubility of L-AC in different solvents

Solvent	Log *P*	Conversion of L-AC (%)	Solubility of L-AC (g/L)
DMSO	-1.30	0	59.51 ± 0.13
Acetone	-0.26	42.48 ± 0.12	2.61 ± 0.06
Acetonitrile	-0.36	33.82 ± 0.1	0.61 ± 0.02
Tert-Pentanol	1.30	45.67 ± 0.79	0.85 ± 0.03
n-Hexane	3.50	0	0

3.1.2. Esterification in co-solvent mixture

In order to find the suitable reaction media for the production of L-AP, esterification in the *tert*-pentanol/DMSO solvent system was investigated due to the highest solubility in DMSO and the highest conversion in *tert*-pentanol of L-AC. The log *P* values of mixed solvents could be estimated by the following formula [14]:

$$\log P = x_1 \log P_1 + x_2 \log P_2$$

Table 2. Matrix of the PB experimental design (coded and real values) for L-AP

Runs	Temperature (°C)	Enzyme concentration (g/L)	Stirring rate (rpm)	Mole ratio*	Amount of molecular sieve (g/L)	Conversion (%)
1	1(65)	-1(2.5)	1(200)	-1(1)	-1(25)	11.95
2	1(65)	1(7.5)	-1(120)	1(5)	-1(25)	56.26
3	-1(35)	1(7.5)	1(200)	-1(1)	1(75)	31.26
4	1(65)	-1(2.5)	1(200)	1(5)	-1(25)	57.15
5	1(65)	1(7.5)	-1(120)	1(5)	1(75)	63.83
6	1(65)	1(7.5)	1(200)	-1(1)	1(75)	24.24
7	-1(35)	1(7.5)	1(200)	1(5)	-1(25)	54.62
8	-1(35)	-1(2.5)	1(200)	1(5)	1(75)	38.17
9	-1(35)	-1(2.5)	-1(120)	1(5)	1(75)	26.27
10	1(65)	-1(2.5)	-1(120)	-1(1)	1(75)	7.96
11	-1(35)	1(7.5)	-1(120)	-1(1)	-1(25)	23.22
12	-1(35)	-1(2.5)	-1(120)	-1(1)	-1(25)	12.23
13	0(50)	0(5)	0(160)	0(3)	0(50)	54.17
14	0(50)	0(5)	0(160)	0(3)	0(50)	53.69
15	0(50)	0(5)	0(160)	0(3)	0(50)	54.53

*Palmitic acid: L-AC.

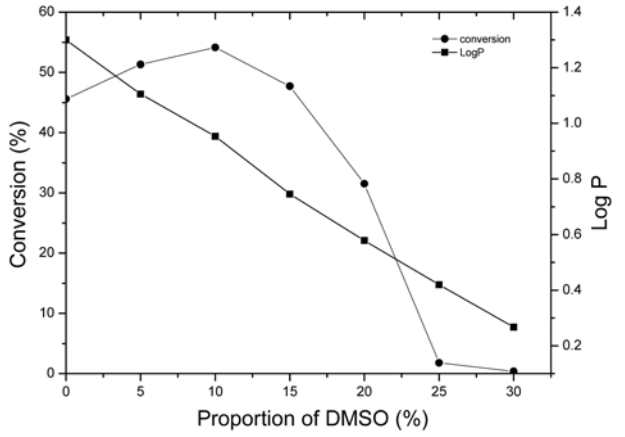

Fig. 1. Effect of the content of DMSO on esterification in *tert*-pentanol/DMSO. Reaction conditions: 160 rpm, 50°C, 18 h, 75 mmol L-AC, 225 mmol palmitic acid, 5 g/L lipase, 50 g/L 4A molecular sieve in 10 mL of mixed solvent. The symbols (●, ■) indicate the conversion of L-AC and the log P values of mixed solvents, respectively.

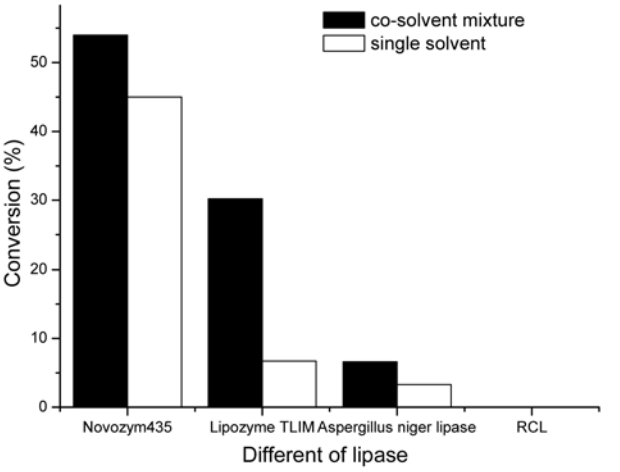

Fig. 2. The comparison of conversion of different types of lipase synthesis of L-AP in optimal co-solvent mixture and single *tert*-pentanol. Reaction conditions: 160 rpm, 50°C, 18 h, 75 mmol L-AC, 225 mmol palmitic acid, and 5 g/L lipase, 50 g/L 4A molecular sieve in 10 mL of solvent.

Where, x_1 and x_2 were the mole fractions of 2 solvents, respectively. The conversion of L-AC in *tert*-pentanol containing 0 ~ 30% DMSO was tested (Fig. 1), the log *P* values of which were within a large range. From Fig. 1, it was interesting to note that L-AP was remarkably formed in *tert*-pentanol / DMSO system, until the proportion of DMSO was 10%. Later, with the increase of proportion of DMSO, the conversion sharply declined. The conversion was negligible at DMSO percentage higher than 30%, probably due to the inactivation of the lipase by DMSO, resulting in disruption of the hydration shell on the lipase surface [17]. Furthermore, the addition of DMSO (10%) to the medium gave rise to a notable acceleration of the process, resulting in a 54% conversion to L-AP. According to above mentioned formula, we were able to determine the log P of the mixture, which was 0.95. This increased substantially the solubility of L-AC, thus allowing the esterification to proceed. As the reaction medium was composed primarily of *tert*-pentanol (where most lipase was significantly stable), the inactivation of the lipase was greatly reduced. The advantage of the co-solvent mixture was that it allowed rational changes in the polarity of the reaction medium and therefore in the molecular interactions between solvents and reactants. As a result, the given product was accumulated by a rational change in the interactions without drastic changes in the environment of the lipase.

3.1.3. Different types of lipase catalyzed esterification in co-solvent mixture and single solvent

In order to explore whether the co-solvent mixture (*tert*-pentanol:DMSO = 9:1) has extensive applicability or not, the synthesis of L-AP by different types of lipase in co-solvent mixture and single solvent (*tert*-pentanol) were investigated. As can be seen from Fig. 2, the conversion in co-solvent mixture was higher than that in *tert*-pentanol. This showed that this co-solvent mixture could be used as a prospective medium for enzymatic synthesis of L-AP. Furthermore, it could be concluded that the different lipase sources may also take some effect on the conversion. Considering the conversion of L-AC, Novozym 435 seemed to be the best biocatalyst. Therefore, Novozym 435 was used in the subsequent steps of this work.

Based on the previous research [19,20], the reaction time was kept constant at 18 h in the statistical experiment designs. In order to acquire optimum conditions for the synthesis of L-AP, statistical experimental designs were used to further optimize reaction conditions.

3.2. Statistical experimental designs

3.2.1. Screening of independent variables using PB design

After determining the preliminary experimental conditions that afforded high conversion, PB design was carried out (Table 2), evaluating the effect of temperature, enzyme concentration, stirring rate, substrates mole ratio, and amount of molecular sieve by means the pare to chart (Fig. 3). The main effects of each independent variable were presented in Fig. 3, which served as a measure to view individual variable's contributions on the conversion. This was estimated based on the difference between the averages of the measurement made at the high level (1) and the low level (-1) of each variable. An inspection of this figure showed that substrates mole ratio and enzyme concentration presented apositive significant effects ($p < 0.05$) on the L-AP

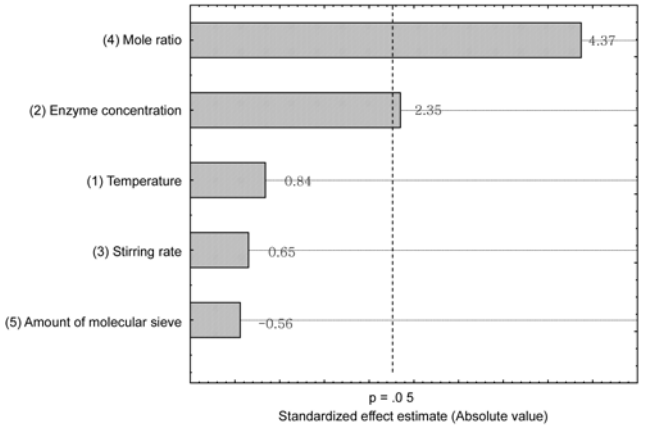

Fig. 3. Pareto chart of the effects of independent variables on the conversion.

yield. On the other hand, the stirring rate, temperature and amount of molecular sieve did not present positive significant effects.

Since PB design on its own did not determine the exact level of variables, but rather provide information about effect of each variable. Based on these, the factors that positively contributed to the conversion i.e. substrates mole ratio and enzyme concentration were further screened using steepest ascent design and CCD under RSM so as to get the optimum levels.

3.2.2. Steepest ascent design for determination of center point

The steepest ascent method was utilized to determine the appropriate direction of changing variables by increasing or decreasing the levels of the variables for enhancing conversion. As shown in Table 3, the highest level of conversion (64.21%) was achieved at the third step. Thus, an appropriate central point for the further optimization step (CCD design) was chosen: Enzyme concentration (5 g/L) and substrate molar ratio (9:1).

Table 3. Experimental design of the steepest ascent and corresponding results

Runs	Enzyme concentration (g/L)	Mole ratio*	Conversion of L-AC (%)
1	1	1	9.2
2	3	5	53.4
3	5	9	64.2
4	7	11	60.3

*Palmitic acid: L-AC.

3.2.3. Optimization of variables by CCD under RSM

Taking into the account of the results obtained in PB and Steepest ascent design, CCD under RSM was used to determine the optimum conditions of the two significant factors, keeping the temperature (50°C, stirring rate (160 rpm), and amount of molecular sieve (50 g/L). The experiments were planned to obtain a quadratic model consisting of 8 experimental runs and 3 experimental runs at center point. Enzyme concentration was 5 g/L and substrate mole ratio was 9:1 at center point (Table 4). The ranges of enzyme concentration and substrate mole ratio were 1.5 ~ 8.5 g/L and 3.34 ~ 14.65 (Table 4). The CCD matrix along with range and levels of two independent variables, experimental and predicted values of conversion, residual were showed in Table 4, while adequacy and the fitness were evaluated by ANOVA (Table 4), the regression equation coefficients were calculated, and the data were fitted to a second-order polynomial equation. The regression equation obtained for conversion was as follows:

$$Y(\text{conversion}) = 18.24 + 9.50E - 0.73\ E^2 + 3.63\ M - 0.14\ M^2 - 0.089\ E\ M$$

Where, the conversion of L-ascorbic acid (Y) was a function of enzyme concentration (E) and the substrates mole ratio (M).

The F value and P value were used to check the significance of each coefficient, which also indicated the

Table 4. Experimental design using central composite design of two independent variables with their actual and coded values showing the experimental conversion, predicted conversion, and residual

Runs	Enzyme concentration (g/L)	Mole ratio	Experimental conversion (%)	Predicted conversion (%)	Residual
1	-1.00 (2.5)	-1.00 (5)	53.2	50.90	2.30
2	-1.00 (2.5)	1.00 (13)	58.55	57.77	0.78
3	1.00 (7.5)	-1.00 (5)	58.50	59.52	-1.02
4	1.00 (7.5)	1.00 (13)	60.28	62.82	-2.54
5	-1.41 (1.5)	0.00 (9)	48.38	50.60	-2.22
6	1.41 (8.5)	0.00 (9)	62.73	60.26	2.47
7	0.00 (5)	-1.41 (3.34)	55.52	56.48	-0.96
8	0.00 (5)	1.41 (14.65)	64.87	63.67	1.20
9	0.00 (5)	0.00 (9)	63.59	64.60	-1.01
10	0.00 (5)	0.00 (9)	64.26	64.60	-0.34
11	0.00 (5)	0.00 (9)	65.95	64.60	1.35

Table 5. Results of the ANVOA analysis of optimization of the conversion

Source	Sum of squares	Degree of freedom	Mean square	F	P
Enzyme concentration (L)	93.32	1	93.32	63.10	0.015
Enzyme concentration (Q)	118.62	1	118.62	80.20	0.012
Mole ratio (L)	51.78	1	51.78	35.01	0.027
Mole ratio (Q)	28.92	1	28.92	19.55	0.048
1 L by 2 L	3.19	1	3.19	2.15	0.28
Lack of Fit	26.75	3	8.92	6.03	0.15

interaction strength between independent variables. The lager the magnitude of the F value and the smaller the P value, the more significant was the corresponding coefficient [21]. It was observed that the linear and the square effect of enzyme concentration and mole ratio were significant at the level of $p < 0.05$. Furthermore, the interactive term between enzyme concentration and mole ratio shown in the ANOVA analysis from Table 5 were not significant ($p > 0.05$). Linear and quadratic effects of parameters were significant, meaning that they could act as limiting variable and little variation in their concentration would alter the conversion to a considerable extent. The value of determinations coefficient R^2 was 0.902. It indicated that 90.2% variability of response could explain, indicating a good agreement between experimental and predicted values. The "lack of fit" measured the failure of the model to represent data in the experimental domain at points which were not included in the regression. The non-significant "lack of fit" also indicated that the model was a good fit.

The 3D response surface plot was the graphical representations of the regression equation to investigate the interaction among variables and to determine the optimum values of each factor for maximum conversion (Fig. 4). The results showed the conversion was considerably affected by varying enzyme concentration and substrate mole ratio. The predicted conversion decreased at the higher and lower values of ranges for both enzyme concentration and mole ratio. Maximum conversion was obtained near the center points of the response surface. The maximum conversion of about 66.01% was predicted at the enzyme concentration and mole ratio of about 5.8 g/L and 11:1.

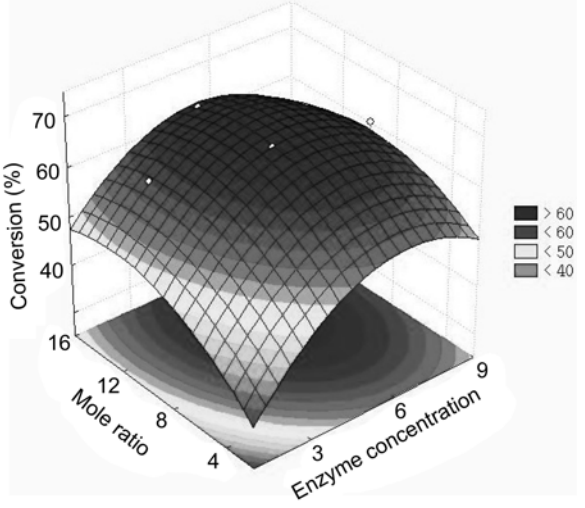

Fig. 4. 3D response surface shows the effect of enzyme concentration and mole ratio on the conversion.

3.2.4. Optimum conditions and model verification

According to the preliminary experiment and statistical experimental designs, the optimum reaction with the maximum predicted conversion was suggested as 66.01% at 18 h, temperature 50°C, enzyme amount 5.8 g/L, substrate molar ratio 11:1, stirring rate 160 rpm, and amount of molecular sieve 50 g/L.

The adequacy of the predicted model was examined by performing three additional independent experiments at the suggested optimum synthesis conditions. The predicted conversion was 66.01% and the actual experimental values were 64.13, 68.77, and 66.42%. The mean was 66.44%. The results of a chi-square test (P-value = 0.9, degrees of freedom = 2) indicated that the observed values were essentially the same as the predicted values and that the generated model adequately predicted the conversion [22].

3.2.5. Reaction progress of the conversion of L-AC at optimum synthesis conditions

In following the reaction progress of the conversion at optimum synthesis conditions, the time course of which was presented in Fig. 5, it could be observed that initial reaction rate was high, leading to high conversion (64.74%) in a short reaction time (6 h). However, the amount of conversion was less than 2% after 6 h. It indicated the reaction seemed to reach a steady state after 6 h at optimum synthesis conditions.

We compared our results with previous studies [20,23-27]. Lerin *et al.* [20] obtained the conversion in L-AP (56%) at 70°C, L-AC to palmitic acid mole ratio of 1:9, stirring rate of 150 rpm, enzyme concentration of 5 wt% (based on the total amount of substrates, L-AC and palmitic acid) at 6 h. Lerin *et al.* [23] obtained the conversion in

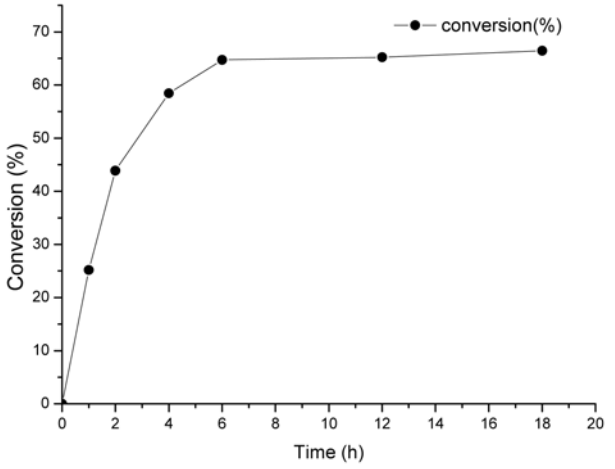

Fig. 5. Time course of the conversion of L-AC at optimum synthesis conditions.

L-AP (27%) under ultrasound irradiation at 70°C, L-AC to palmitic acid molar ratio of 1:9, enzyme concentration of 5 wt% at 3 h of reaction (based on the total amount of substrates). Humeau *et al.* [24] tested Novozym 435 to synthesis L-AP in *tert*-pentanol and obtained the conversion of 56% in 8 h at 55°C. Hsieh *et al.* [25] investigated surfactant-coated lipase synthesis L-AP and achieved the conversion of 47% in 24 h, 50°C, and mole ratio of 1:6 (L-AC : palmitic acid). We obtained a higher mole conversion in our study than the results obtained in these previous studies. Furthermore, although our target product and solvent type were different from Song et al and Lv *et al.* [26-28]; however, it was interesting to find that all investigations similar results with 33 ~ 48% of L-ascorbyl ester mole conversion which represented at 15% lower conversion than the result obtained in our study. Obviously, we obtained a higher mole conversion over a shorter time period, and at a lower reaction temperature than was found in their studies.

4. Conclusion

Solvent engineering was applied to improve the conversion of L-AC and a co-solvent mixture of *tert*-pentanol: the optimum mixture was DMSO at a ratio of 9:1 (v/v) with Novozym 435 as a biocatalyst. It could be concluded that the use of this co-solvent mixture is the best media for synthesis of L-AP based on our results. Optimization of the conditions was carried out by statistical experimental designs including PB design, steepest ascent design and CCD under RSM. Optimal conditions in co-solvent mixture were: temperature 50°C, enzyme concentration 5.8 g/L, substrate molar ratio 11:1, stirring rate 160 rpm, amount of molecular sieve 50 g/L, and 18 h. Reaction progress of the conversion of L-AC at optimum synthesis conditions showed that very satisfactory conversion (64.74%) and production concentration (20.13 g/L) could be achieved in short time (6 h). Validation experiments verified the availability and the accuracy of the model. The predicted values were in agreement with the experimental values. Obviously, statistical experimental designs combined with solvent engineering are useful for synthesis of L-AP by lipase and statistical analysis was proved to be a useful and powerful tool in developing optimal conditions for L-ascorbyl palmitate synthesis.

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