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Enzymatic production of sterculic acid from the novel Phoenix tree seed oil: Optimization and kinetic study

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SUMMARY: Phoenix tree (*Firmiana simplex*) seed oil is a novel oil which is rich in sterculic acid. Sterculic acid, a cyclopropene fatty acid, can be used as the inhibitor of the stearoyl-CoA desaturase system and mammary carcinomas growth. In this work, Lipozyme TLIM-catalyzed hydrolysis of the novel Phoenix tree seed oil was used to prepare sterculic acid. High temperature GC-FID and the degree of hydrolysis (DH) were used to monitor the reaction progress. Effects of reaction variables on the hydrolysis were evaluated and optimized using response surface methodology. Results showed that sterculic acid can be successfully prepared from the novel seed oil, and the effect of reaction variables on the hydrolysis decreased in the order of reaction time > enzyme load > temperature. A high yield of fatty acids (DH, 98.2±0.8%) can be obtained under optimized conditions (45 ºC, mass ratio of water to oil 10:1, enzyme load 10%, and 18 h). The Arrhenius equation for the hydrolysis was LnV_0 = 9.12─4721/T. The activation energy was 39.25KJ/mol. The kinetic values for Vmax, K′m were 0.232mol/(L·min) and 0.084 mol/L, respectively.

KEYWORDS: *Enzymatic hydrolysis; Kinetic; Lipozyme TLIM; Phoenix tree seed oil; Response surface methodology; Sterculic acid*

RESUMEN: ***Producción enzimática de ácido estercúlico a partir del nuevo aceite de semillas del árbol fenix: optimización y estudio cinético.*** El aceite de semilla del árbol fenix (*Firmiana simplex*) es un nuevo tipo de aceite rico en ácido estercúlico. Este es un ácido graso ciclopropeno, que se puede utilizar como inhibidor del sistema de estearoyl-CoA desaturasa y del crecimiento de los carcinomas mamarios. En este trabajo, se utilizó la hidrólisis catalizada por Lipozyme TLIM del nuevo aceite de semilla de árbol fenix para preparar ácido estercúlico. Se utilizaron GC-FID a alta temperatura y el grado de hidrólisis (DH) para monitorizar el progreso de la reacción. Los efectos de las variables de reacción de hidrólisis se evaluaron y optimizaron utilizando la metodología de superficie de respuesta. Los resultados mostraron que el ácido estercúlico puede prepararse con éxito a partir del nuevo aceite de semilla, y el efecto de las variables de reacción en la hidrólisis disminuyó en el orden de tiempo de reacción> carga enzimática> temperatura. Se pueden obtener altos rendimientos de ácidos grasos (DH, 98,2 ± 0,8%) en condiciones optimizadas (45 ºC, relación masa de agua a aceite 10: 1, carga enzimática 10% y 18 h). La ecuación de Arrhenius para la hidrólisis fue LnV_0 = 9,12 – 4721/T. La energía de activación fue 39,25KJ/mol. Los valores cinéticos de Vmax, K/m fueron 0,232 mol/(L · min) y 0,084 mol/L, respectivamente.

PALABRAS CLAVE: *Aceite de semilla de árbol de fénix; Ácido estercúlico; Cinética; Hidrólisis enzimática; Lipozyme TLIM; Metodología de superficie de respuesta*

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1. INTRODUCTION

Sterculic acid is one kind of cyclopropene fatty acid, which can be used as an inhibitor of the stearoyl-CoA desaturase system to inhibit the synthesis of monoenic fatty acids (James *et al*., 1968; Jeffcoat and Pollard, 1968; Reiser and Raju, 1964; Wältermann and Steinbüchel, 2000). Sterculic acid also can inhibit mammary carcinoma growth in rats (Khoo *et al*., 1991). In our previous work (Sun and Li, 2016), we found that Phoenix tree seed oil is rich in sterculic acid (S, 23.2%). The Phoenix tree, also named Chinese parasol or *Firmiana simplex*, is a tall, deciduous tree which has been used as an ornamental plant grown on both sides of roads and courtyards in many provinces of China, for example, Hebei, Shanxi, Fujian, and Taiwan. It can also be found in Europe, Japan, Korea, and USA (Tang *et al*., 2010; Upson and Cullen, 2012; Woo *et al*., 2016). Many active components can be obtained from the stem, leaf, flower, and wood fiber of the Phoenix tree (Kim *et al*., 2015; Son *et al*., 2015; Woo *et al*., 2015). The root bark of the Phoenix tree had been used to treat rheumatism, asthma, fractures, and tumors (Bai *et al*., 2005). The seeds of the Phoenix tree have also been used as traditional Chinese herbs for promoting digestion and treating stomachache (Woo *et al*., 2016). However, no available information about sterculic acid preparation using Phoenix tree seed oil was found.

The aim of this work was to prepare sterculic acid by enzymatic hydrolysis of the novel Phoenix tree seed oil. Traditionally, fatty acids can be prepared from natural oils and fats using chemical catalysts (acid or base) (Murty *et al*., 2002; Satyarthi *et al*., 2011). However, according to the previous report (Aued-Pimentel *et al*., 2004), the structure of cyclopropene in sterculic acid can be destroyed by acid. When a base is used as catalyst, acidification is necessary to prepare fatty acids. Compared with chemical catalysts, biocatalysts have shown many advantages for polyunsaturated fatty acid preparation (Goswami *et al*., 2011; Hasan *et al*., 2006; Leng *et al*., 2008; Mendes *et al*., 2012; Santos *et al*., 2013). Therefore, in this work, Lipozyme TLIM was selected and used for sterculic acid preparation. The hydrolysis process was monitored by high temperature GC-FID and the degree of hydrolysis (DH). The effect of reaction variables on the enzymatic hydrolysis of Phoenix tree seed oil was studied and optimized using response surface methodology (RSM). The activation energy and kinetics for the hydrolysis reaction were also evaluated.

2. MATERIALS AND METHODS

2.1. Materials

Phoenix tree (*Firmiana simplex*) seeds were supplied by the Xintai Nursery Filed (Jiangsu, China). Lipozyme TLIM (from *Thermomyces lanuginose*, TLL) was obtained from Novozymes A/S (Bagsvaerd, Denmark). Potassium hydroxide and sodium hydroxide were purchased from Tianjin Kemiou Chemical Reagent Co. Ltd (Tianjin, China). Other chemicals were of analytical grade.

2.2. Preparation of Phoenix tree seed oil

Phoenix tree seeds were dried and impurities such as pebbles and leaves were removed. Then, the seeds were crushed using a pulverizer and passed through a 40 mesh sieve to obtain seed powder. Next, Phoenix tree seed powder was extracted using n-hexane in a 1000 mL beaker which was placed in an ultrasonic bath. The supernatant was then centrifuged and the solvent was removed using a rotary evaporator. The oil obtained was used for the hydrolysis reaction.

2.3. Hydrolysis reaction of Phoenix tree seed oil

Hydrolysis reactions were conducted using 250 mL three-necked flasks. The mixture of Phoenix tree seed oil and water (ratio of water to oil 10:1, w/w) was prepared using a magnetic stirrer (200 rpm) at different temperatures (30–55 °C) in oil bath. The reaction was started by the addition of Lipozyme TLIM (10%, relative to the weight of all substrates). The samples were taken out at specified time intervals, dissolved and centrifuged.

2.4. Analysis methods

High temperature GC (Agilent 7980B) equipped with a DB-1H capillary column (30 m×0.25 mm, 0.1 μm of film thickness) and flame ionization detector (FID) was also used to evaluate the hydrolysis product formation during the hydrolysis. The detector and injector temperatures were set at 350 °C and 400 °C, respectively. Initial column temperature was 100 °C, programmed to 360 °C at the rate of 20 °C/min. Helium was used as carrier gas at a flow rate of 1 mL/min. Acid values were analyzed according to the AOCS Official Method Cd 3d-63 (Firestone *et al*., 1998).

2.5. Evaluation of the degree of hydrolysis (DH)

DH (%) was calculated from the following equation:

$$DH = \frac{AV_t - AV_0}{SV - AV_0} \times 100\% \quad (1)$$

Where AV_0 is the initial acid value of Phoenix tree seed oil, and AV_t is the acid value of Phoenix tree seed oil at time t. SV is the saponification value of Phoenix tree seed oil.

2.6. Experimental design

A 3-level-3-factor Box–Behnken experimental design was applied to evaluate the interaction effect of reaction variables on the hydrolysis. The factors and levels used in experimental design were as follows: reaction time (3, 14 and 25 h), reaction temperature (30, 45 and 60 °C), enzyme load (1%, 8% and 15%; relative to the weight of total substrates) (Table 1).

2.7. Statistical analysis

Design-Expert 8.0 was used to analyze the experimental data, which can generalize second-order polynomial model as follows:

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

Where Y is the predicted response (DH); X_i and X_j represent the independent variables. Regression coefficients β_0, β_i, β_{ii}, and β_{ij} are the intercept, linear, quadratic, and interaction terms, respectively.

All experiments were performed at least in triplicate. Results were expressed as average ± SEM. A two-way analysis of variance (ANOVA) was used. Statistical significance was considered at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Reaction progress of the hydrolysis of Phoenix tree seed oil

Figure 1 shows that during the hydrolysis process, the triacylglycerol (TAG) of Phoenix tree seed oil was firstly converted to diacylglycerol (DAG), and then DAG was hydrolyzed to monoacylglycerol (MAG), and finally fatty acids can be produced by the hydrolysis of MAG, DAG and TAG. The low content of MAG (~3%) during the hydrolysis progress indicated that MAG can be quickly converted to free fatty acids when MAG was formed. And the relative high DAG content (~20% at 2 h) indicated that the formation rate of DAG is higher than that of the hydrolysis of DAG. These results showed that the limitation step for the hydrolysis of the oil was attributed to the first step (the formation of DAG by the hydrolysis of TAG). Figure 1 also shows that the content of fatty acids in the product sharply increased with the increase in hydrolysis time from 0 to 12 h, and at 24 h, and the hydrolysis reaction reached equilibrium at 24 h. Similar results can also be found in the chemical (Fe-Zn double-metal cyanide)-catalyzed hydrolysis of oil (Satyarthi *et al.*, 2011).

TABLE 1. Box–Behnken design and results of hydrolysis of Phoenix tree seed oil as affected by reaction time, reaction temperature, and enzyme load

Trial[a]	X_1 (h) Reaction time	X_2 (°C) Reaction temperature	X_3 (%) Enzyme load[b]	DH (%)
1	14 (0)	60 (1)	1 (-1)	42.92±1.2
2	3 (-1)	30 (-1)	8 (0)	67.33±2.1
3	14 (0)	45 (0)	8 (0)	92.01±2.5
4	14 (0)	30 (-1)	1 (-1)	50.37±1.6
5	14 (0)	45 (0)	8 (0)	93.71±1.9
6	14 (0)	45 (0)	8 (0)	96.09±1.4
7	14 (0)	60 (1)	15 (1)	70.27±2.4
8	14 (0)	45 (0)	8 (0)	91.28±1.8
9	3 (-1)	45 (0)	15 (1)	68.39±2.1
10	3 (-1)	45 (0)	1 (-1)	42.64±1.6
11	14 (0)	30 (-1)	15 (1)	78.89±1.3
12	25 (1)	45 (0)	15 (1)	99.98±0.4
13	25 (1)	30 (-1)	8 (0)	93.96±1.2
14	3 (-1)	60 (1)	8 (0)	63.10±1.5
15	14 (0)	45 (0)	8 (0)	98.42±0.6
16	25 (1)	45 (0)	1 (-1)	94.59±1.4
17	25 (1)	60 (0)	8 (0)	90.69±1.3

[a] Numbers were run at random.
[b] Enzyme load (%, based on the weight of total substrates)
All the experiments were carried out with a substrate ratio 10:1 (water/oil, w/w).

3.2. Effect of reaction temperature

Reaction temperature played an important role in the hydrolysis of Phoenix tree seed oil, which influenced the mass transfer of the reaction system and catalytic activity of Lipozyme TLIM. To evaluate the effect of the reaction temperature on the hydrolysis of Phoenix tree seed oil, experiments were performed at different temperatures from 30 °C to 55 °C and the results are shown in the Figure 2.

Figure 2A shows that the DH of Phoenix tree seed oil increased with the increase in reaction temperature from 30 °C to 45 °C. However, when the temperature was above 45 °C, the DH of Phoenix tree seed oil decreased, which may be ascribed to the deactivation of Lipozyme TLIM due to the adsorption of glycerol on the immobilized lipase surface and higher temperature (>45 °C) in the excess water system. Therefore, the optimum temperature for maximum DH was 45 °C, which was lower than that of the Lipozyme TLIM-catalyzed hydrolysis of oil in a supercritical carbon dioxide medium (60 °C) (Prado and Saldaña, 2013), which was attributed to the difference in hydrolysis media.

The initial hydrolysis rate (V_0, mol/(L·min)) of Phoenix tree seed oil, defined as the initial hydrolysis degree per unit time, can be obtained from six experimental points of the hydrolysis degree-time profile corresponding to low DH (15% or less) at different reaction temperatures. Activation energy can be calculated by the Arrhenius law described as follows:

$$LnV_0 = LnA - \frac{E_a}{RT} \quad (3)$$

Where A represents the Arrhenius constant, and Ea represents the activation energy. R and T are gas constant and absolute temperature (K), respectively.

A good linearity between LnV_0 and 1/T was found (Figure 2B). The value of Ea calculated by equation (3) was 39.25 KJ/mol, which was higher than the Ea value (0.97-34.5 KJ/mol) of general lipase-catalyzed reactions (Phuah *et al.*, 2012). The differences can be ascribed to the greater steric hindrance of sterculic acid. Therefore, the Arrhenius equation for the hydrolysis of Phoenix tree seed oil can be re-written as $LnV_0 = 9.12 - 4721/T$.

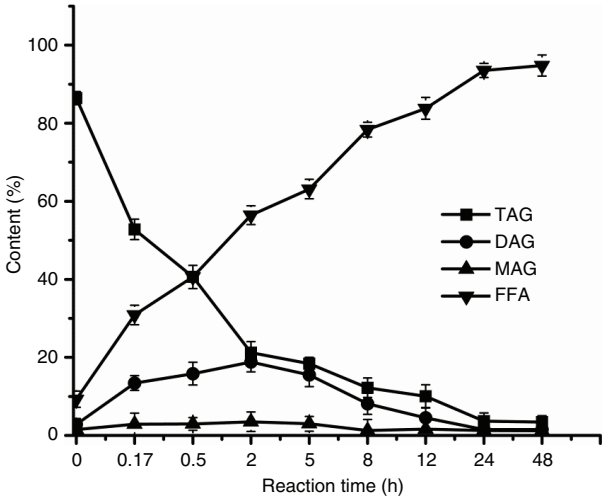

FIGURE 1. Reaction progress in Lipozyme TLIM-catalyzed hydrolysis of Phoenix tree seed oil. Hydrolysis conditions: 10% enzyme load (relative to the weight of all substrates), 10:1 (w/w) mass ratio of water to oil, 200 rpm, and 45 °C.

3.3. Effect of mass ratio of water to oil

To explore the effect of mass ratio of water to oil on the hydrolysis reaction, experiments with different mass ratios were carried out (Figure 3).

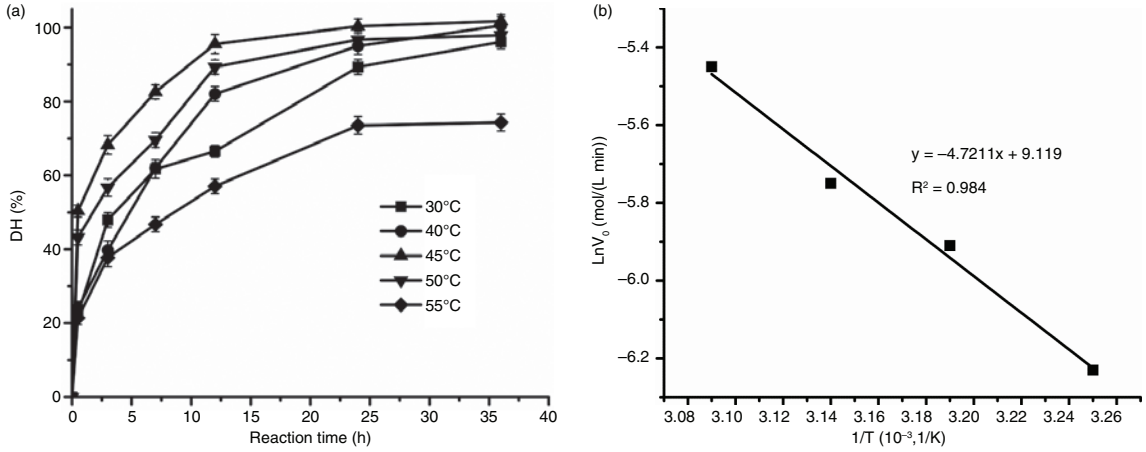

FIGURE 2. (A) Effect of reaction temperature on the hydrolysis of Phoenix tree seed oil. Reaction conditions: 5% enzyme load (relative to the weight of all substrates), 10:1 (w/w) mass ratio of water to oil, and 200 rpm. (B) The relationship between initial hydrolysis rate and reaction temperature (K), reaction conditions were the same as Fig.2 (A).

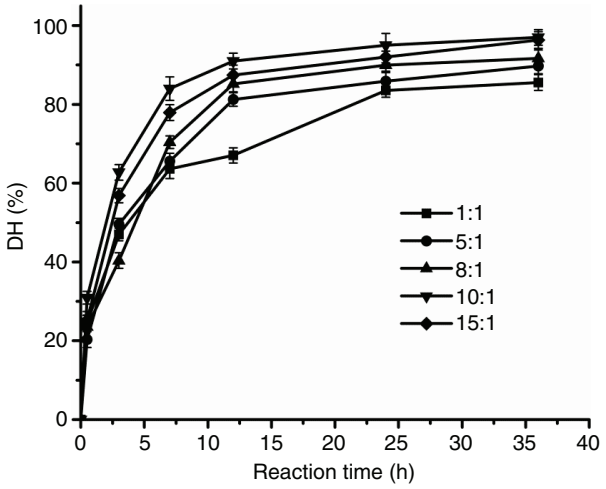

FIGURE 3. Effect of mass ratio of water to oil on the hydrolysis of Phoenix tree seed oil with 5% enzyme load (relative to the weight of substrates) at 45 °C and 200 rpm.

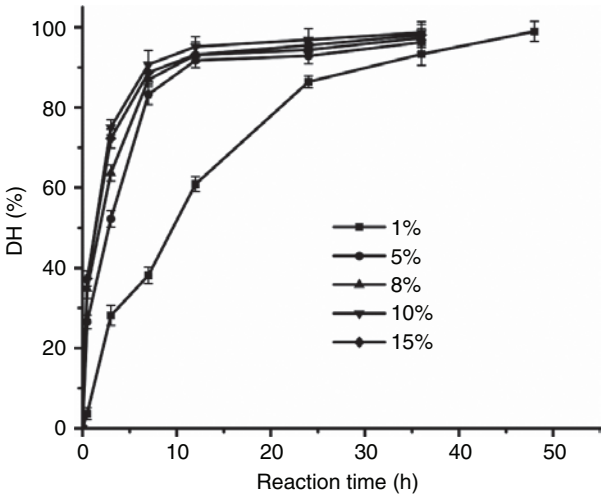

FIGURE 4. Effect of enzyme load on the hydrolysis of Phoenix tree seed oil at 45 °C, 10:1 (w/w) mass ratio of water to oil, and 200 rpm.

With the increase in mass ratio of water to oil from 1:1 to 10:1 (w/w), the DH of Phoenix tree seed oil increased significantly to its maximum (~96%) after the reaction reached equilibrium. When the mass ratio of water to oil increased to 15:1, the maximum DH of Phoenix tree seed oil was the same as that of 10:1. However, the initial hydrolysis rate (0.0041 mol/(L·min)) of 15:1 is lower than that (0.0038 mol/(L· min)) of 10:1. These results showed that enough water is necessary for the hydrolysis of Phoenix tree seed oil. Similar effect of water on the lipase-catalyzed hydrolysis of oil can also be found in previous reports (Chen *et al.*, 2014; Prado and Saldaña, 2013).

3.4. Effect of enzyme load

Enzyme load will affect mass transfer and initial reaction rate. In order to evaluate the effect of enzyme load on the hydrolysis of Phoenix tree seed oil, different enzyme loads from 1% (relative on the weight of all substrates) to 15% were added to the reaction. The DH of Phoenix tree seed oil increased with the increase in enzyme load from 1% to 10% (Figure 4). However, when the enzyme load exceeded 10%, the initial DH of Phoenix tree seed oil decreased. When the hydrolysis reached equilibrium with a higher enzyme load (≥5%), the maximum DH of Phoenix tree seed oil were similar (~96.5%). These results can be explained by the fact that sufficient enzyme is necessary for the hydrolysis. However, excessive enzyme also leads to aggregate and great mass limitation, which results in the low hydrolysis rate at the highest enzyme load (15%). A similar effect of high enzyme load on the reaction can also be found in other reactions (Albasi *et al.*, 1999; Al-Zuhair *et al.*, 2003; Sun and Chen, 2015).

3.5. Kinetic study

The linear relationship between the reaction rate and enzyme loading (Figure 5A) showed that the effect of external mass transfer limitation on the hydrolysis reaction can be neglected, and the hydrolysis was controlled by kinetics. In order to obtain the kinetic parameter of the hydrolysis reaction, several experiments were performed by varing oil concentration (0.02 mol/L-0.3 mol/L). The initial reaction rate firstly increased to the maximum (V_0 = 0.120 mol/(L·min)) (Figure 5B) and then decreased with the increase in oil concentration. According to Ping-pong Bi-Bi, the initial reaction rate can be described as follows:

$$V_0 = V_{max}/(1+K_A/[A] + K_B/[B] (1+[A]/K_{IA})) \quad (4)$$

Due to the presence of significantly excess water, the contention of H_2O can be regarded as a constant. Hence, the initial reaction rate can be described as:

$$V_0 = V_{max}[A]/([A]+K'm) \quad (5)$$

Where V_0 is the initial hydrolysis rate; Vmax is the maximum reaction rate; [A] and [B] are Michanelis-Meton constants of oil and water, respectively; K_{IA} is the inhibition contant of oil, and K′m is the apparent Michaelis-Meton.

A good Lineweaver-Burk plot was obtained between the reciprocal of the initial rate versus the reciprocal concentration of oil (Figure 5C). The value for Vmax and K′m were calculated as 0.232 mol/(L*min) and 0.084 mol/L, respectively.

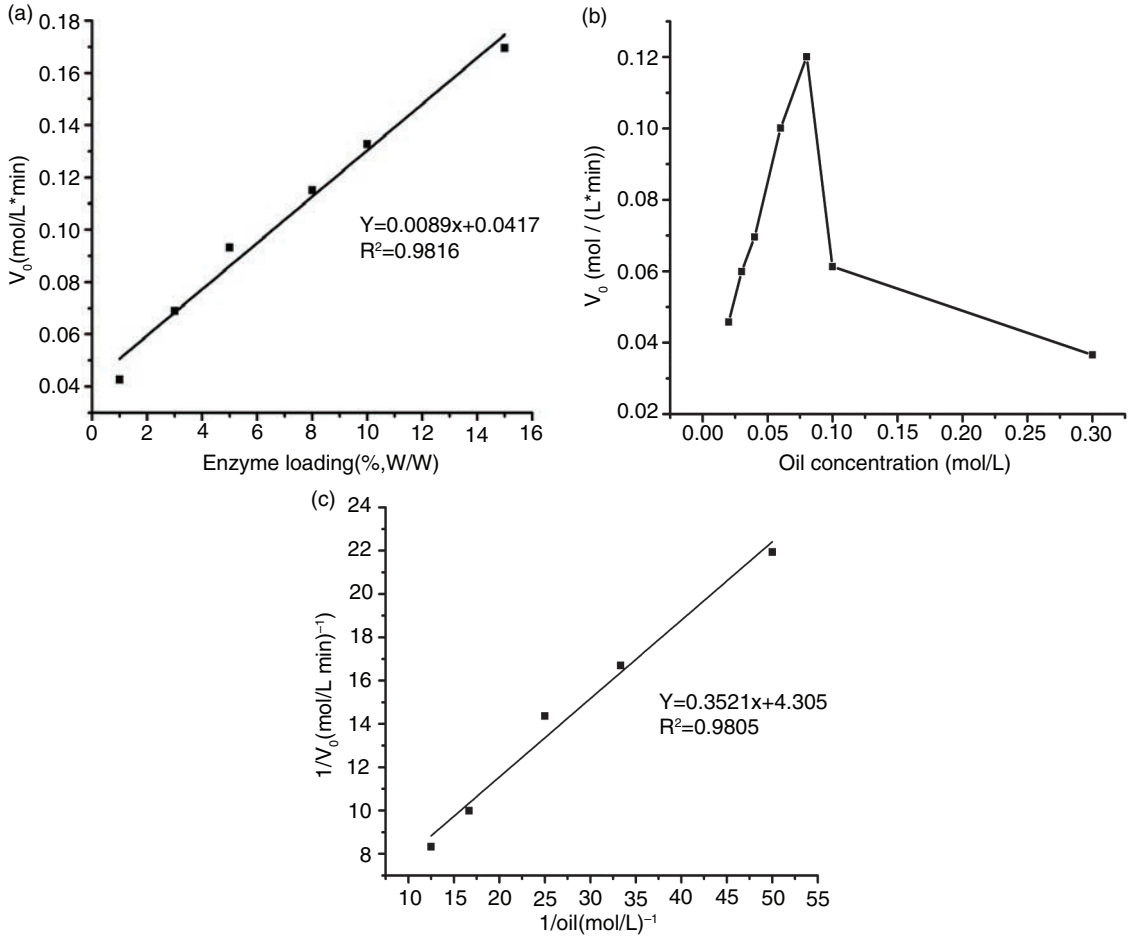

Figure 5. (A) Effect of enzyme loading on initial reaction rate. (B) Relationship between the initial reaction rate with oil concentration at lower oil concentrations. (C) Reciprocal initial rate versus reciprocal oil concentration. Reaction conditions for (A): oil:water = 1:10 (w/w), 45 °C. Reaction conditions for (B) and (C): oil:water = 0.02-0.3 (mol/L), 40 °C, enzyme loading 10% (relative to the weight of total substrates).

Table 2. Analysis of variance (ANOVA) for quadratic model to the DH of Phoenix tree seed oil.

Sourse	Sum of squares	Degrees of freedom	Mean square	F value	Prob > F[a]
Model	6039.73	9	671.08	20.61	0.0003
X_1	2372.64	1	2372.64	72.85	< 0.0001
X_2	69.43	1	69.43	2.13	0.1876
X_3	946.60	1	946.60	29.06	0.0010
$X_1 X_2$	0.23	1	0.23	0.01	0.9360
$X_1 X_3$	103.64	1	103.64	3.18	0.1176
$X_2 X_3$	0.34	1	0.34	0.01	0.9214
X_1^2	0.07	1	0.07	0.00	0.9645
X_2^2	1032.89	1	1032.89	31.71	0.0008
X_3^2	1368.90	1	1368.90	42.03	0.0003
Residual	227.98	7	32.57		
Lack of fit	193.09	3	64.36	7.38	0.0416
Total	6267.71	16			
R^2=0.9636		R^2_{Adj}=0.9169			

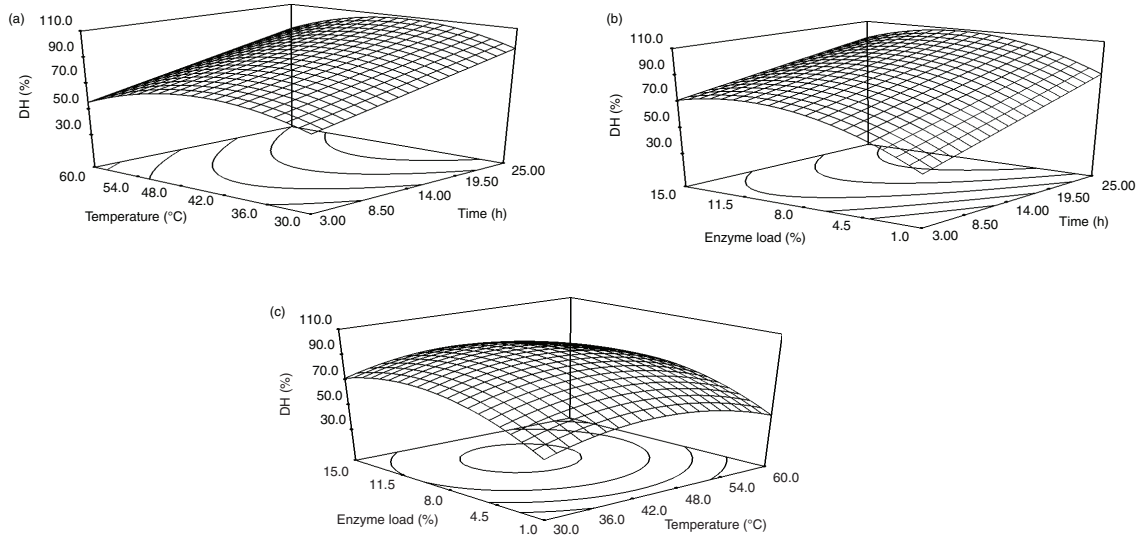

FIGURE 6. 3D surface plots between two variables for the hydrolysis of Phoenix tree seed oil: (A) reaction temperature and reaction time with 8% enzyme load (relative to the total weight of substrates); (B) reaction time and enzyme load at 45 °C; (C) reaction temperature and enzyme load for 14h.

3.6. Response surface analysis and model fitting

RSM is a comprehensiveness of statistical and numerical technology which is used to optimize experimental conditions (Syam *et al*., 2016). The experimental values obtained from the model are shown in Table 1. The data were regressed by employing a quadratic polynomial equation to calculate the relationship between the DH of Phoenix tree seed oil and hydrolysis variables.

The analysis of variance (ANOVA) for the model is shown in Table 2. The low P-value ($P < 0.001$) and high coefficient ($R^2 = 0.9636$) indicated that the model was adequate to explain the relationship between reaction variables and the DH of Phoenix tree seed oil. The order of hydrolysis variables influencing the DH of Phoenix tree seed oil was reaction time > enzyme load > hydrolysis temperature. The regression equation explaining the DH of Phoenix tree seed oil was given as follow:

$$Y\ (\%) = 94.30 + 17.22X_1 - 2.95\ X_2 + 2.95X_3 + 0.24X_1X_2 - 5.09X_1X_3 - 0.29X_2X_3 + 0.13X_1^{\ 2} - 15.66X_2^{\ 2} - 18.03X_3^{\ 2} \quad (6)$$

Figure 6A shows the effect of reaction time, temperature, and their mutual interaction on the hydrolysis of Phoenix tree seed oil with an 8% enzyme load. High DH (>96.4%) of Phoenix tree seed oil can be obtained at long reaction times (14–25 h) and moderate temperatures (36–54°C). Figure 6B shows the 3D surface plot of DH drawn for the interaction of enzyme load and reaction time, which were similar to those of the interaction of reaction time and reaction temperature. Proper enzyme load and longer reaction time were beneficial for the hydrolysis. Low DH at high enzyme load was attributed to the aggregation of excessive enzyme. Figure 6C shows the effect of enzyme load, temperature and their mutual interaction on the hydrolysis reaction. The maximum DH (97.2%) of Phoenix tree seed oil could be achieved at 40–45 °C and 8%–13% enzyme load. The deactivation of Lipozyme TLIM at a higher temperature (>54 °C) can also be found in Figure 6C.

The maximum DH (98.2±0.8%) of Phoenix tree seed oil can be achieved at the following optimized conditions: 45 °C, 18h, 10% enzyme load and 10:1 mass ratio of water to oil. Good agreement between the observed and predicted values indicates the validation of the model. In the hydrolysis product, the content of sterculic acid was similar to that (~23%) of Phoenix tree seed oil.

4. CONCLUSIONS

In this study, sterculic acid was successfully prepared by Lipozyme TLIM-catalyzed hydrolysis of the novel Phoenix tree seed oil, and RSM was used to optimize the hydrolysis reaction. A quadratic regression model between reaction variables (reaction temperature, enzyme load, and reaction time) and the hydrolysis of Phoenix tree seed oil was also achieved. The maximum DH (98.2±0.8%) of Phoenix tree seed oil can be obtained under optimized conditions (45 °C, 18h, 10% enzyme load and 10:1 mass ratio of water to oil). The content of sterculic acid in the fatty acid product was ~23%. The activation energy of the hydrolysis of Phoenix tree

seed oil for fatty acid preparation was 39.25KJ/mol. The kinetic values for Vmax, K/m were 0.232mol/(L·min) and 0.084 mol/L, respectively. These results can provide some available information for the production of fatty acids with cyclopropene. However, the method for sterculic acid preparation was limited by the high cost of the enzyme.

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