Amano Lipase PS-catalyzed Hydrolysis of Pine Nut Oil for the Fatty Acids Production Using Deep Eutectic Solvent as Co-solvent

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Abstract: Free fatty acids (FFAs) are the important material used in food, personal care, emulsifiers, adhesives and surfactants. In order to enhance the preparation of FFAs, the effects of reaction variables, optimization, thermodynamic property for the Amano lipase PS catalyzed hydrolysis of pine nut oil (PNO) using deep eutectic solvents (DESs) as co-solvents were studied. The results showed that FFAs could be successfully prepared from pine nut oil through Amano lipase PS catalyzed hydrolysis using Choline chloride:Urea (ChCl:U, 1:2, mol/mol) as co-solvent. Under the optimal conditions (reaction temperature 46°C, water amount 38%, DES addition 43%, lipase dosage 7.6%, reaction time 13 h), the maximum content of FFAs in the products and degree of hydrolysis (DH) of oil were up to 89.1 ± 1.9% and 92.7 ± 2.2%, respectively. The effects of reaction variables on the hydrolysis increased in the order of DES addition < reaction temperature < reaction time < lipase dosage < water amount. The thermodynamics (Arrhenius equation) for the triglycerides hydrolysis was \( V = 4289.39 \times \exp(-22942.09/RT) \) with the activation energy (\( E_a \)) of 22.94 kJ/mol. The Gibbs free energy (\( \Delta G \)), enthalpy (\( \Delta H \)) and entropy (\( \Delta S \)) were 81.50 ± 2.64 kJ/mol, 20.18 ± 0.12 kJ/mol and -184.59 ± 0.36 J/mol/K, respectively. The lipase in the aqueous DES could be directly re-used for 3 times.

Key words: fatty acid, Amano lipase PS, hydrolysis, pine nut oil, deep eutectic solvent (DES)

1 Introduction

Fatty acids (FAs) are important materials in various industrial fields such as emulsifiers, adhesives, surfactants, food, and personal care, existing naturally as triglycerides (TGs) in oils and fats. There are many routes to release FAs from TGs such as acidic hydrolysis, alkaline hydrolysis, high-temperature steam hydrolysis and enzymatic hydrolysis. Among these routes, enzymatic hydrolysis is a preferable way to promote the quality of the hydrolyzed product. The enzymatic hydrolysis is performed under milder condition (lower temperature and pressure) in comparison with acidic or alkaline hydrolysis. Thus, it can avoid some undesirable reactions and certain disadvantages, which will affect the color of products. Moreover, enzymatic hydrolysis requires no chemical reagents, what’s more, the reusability of enzyme.

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are enzymes that catalyze hydrolysis of ester bonds of triacylglycerols to diacylglycerols (DAGs), monoacylglycerols (MAGs), glycerol and FAs. The hydrolysis could be performed in emulsion, organic aqueous medium, ionic liquid system. In the recent research, lipase showed good activity in deep eutectic solvents (DESs). DESs, a new type of ionic liquids (ILs), are usually prepared by the complexation of the hydrogen-bond acceptors (HBAs) and the hydrogen-bond donors (HBDs) with a special proportion. The melting point of DESs are much lower than those of the individual components mainly due to the formation of intermolecular hydrogen bonds. In comparison to the traditional ILs, the advantages of DESs are cheap, biodegradable, low toxicity or non-toxicity, and very easy to synthesis. According to the report of Zeng et al., lipases showed good activity on the esterification of glycerol and oleic acid in DESs. Recently, it has been reported that Amano lipase PS presented better hydrolysis activity in aqueous DESs determined by hydrolysis of p-nitrophenyl palmitate (p-NPP) to p-nitrophenol (p-NP). However, no study has yet been reported on the hydrolysis of vegetable oil catalyzed by Amano lipase PS.

The present study was aimed to investigation the hydrolysis of pine nut oil...
sis reaction of pine nut oil (PNO) catalyzed by Amano lipase PS using DES as co-solvent. The influence of parameters on the hydrolysis of PNO was studied, while response surface methodology (RSM) was used to assay the most significant factors, such as DES addition, reaction temperature, reaction time, lipase dosage and water amount. The thermodynamics and reuse for the Amano lipase PS catalyzed hydrolysis of PNO were also studied.

2 Experimental

2.1 Materials and chemical reagents

Amano lipase PS (free enzyme) from Burkholderia cepacia (BCL) was purchased from Sigma-Aldrich (Shanghai, China), the activity was ≥ 30000 U/g at pH 7.0 and 50°C. PNO was provided friendly by Liangyun Food Technology (Dalian) Co. Ltd. Choline chloride (ChCl), urea (U), glycerol (Gly), ethylene glycol (EG), 1,2-propylene glycol (1,2-PG), lactic acid (LA), and carboxic acid (CA) were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd (Tianjin, China). Silica G used TLC plate preparation was purchased from Qingdao Ocean Chemical Factory (Qingdao, China). Oleic acid, glycerol monooleate, glycerol dioleate, and glycerol trioleate were purchased from Sigma-Aldrich (Shanghai, China). All other reagents are of analytical or high-performance liquid chromatography grade.

2.2 DESs synthesis

DESs were synthesized according to the previous report[12]. HBA and HBD with a certain molar proportion was heated and stirred in an incubator at the setting temperature until a colorless homogeneous liquid was formed, typically 2 h. (a) ChCl:EG (1:2, mol/mol), 80°C. (b) ChCl:EG (1:1, mol/mol), 80°C. (c) ChCl:U (1:2, mol/mol), 70°C. (d) ChCl:Gly (1:2, mol/mol), 70°C. (e) ChCl:1,2-PG (1:3, mol/mol), 100°C. (f) ChCl:1,2-PG (1:2, mol/mol), 100°C. (g) ChCl:CA (1:3, mol/mol), 80°C. (h) ChCl:LA (1:1, mol/mol), 80°C.

2.3 Hydrolysis of pine nut oil in aqueous DESs

10 g PNO, the proper amount of water and DES were placed into a baker (250 mL), then the mixture was stirred (300 rpm) and heated in a water bath. When the temperature reached to the given temperature, the hydrolysis was initiated by adding the lipase into the baker. The reaction reached to the selected time intervals, 5 mL of product mixture was withdrawn immediately, and the hydrolysis was stopped by adding 20 mL actone/methanol (3:1, v/v). The mixture was centrifuged at 5000 rpm for 5 min, then the upper layer, dried with anhydrous Na2SO4, was used to profile the product.

2.4 Reusability of the resulted aqueous DES containing lipase

The reusability of Amano lipase PS was assessed in the hydrolysis of pine nut oil in DES aqueous solution for FFAs production. The reactions were performed under the optimal conditions. After each reaction run, the product mixture was cooled to ambient temperature (about 1 h), then separated into two phases. The upper layer (liquid) was poured out completely as much as possible. The lower layer, containing lipase, was reused for the subsequent batch hydrolysis by adding appropriate amount of PNO.

2.5 Analysis of hydrolysis product profile

A gas chromatograph (Agilent 7890B, Shanghai China) equipped with a flame ionization detector (FID) and a DB-IHT capillary column (30 m × 0.25 mm, 0.1 μm of film thickness, J&W Scientific, USA) was used to determine the relative content of FFAs, monoacylglycerols (MAGs), diacylglycerols (DAGs), and triacylglycerols (TAGs) in the hydrolysis products according to the reports of Palla et al.[14] and Chung et al.[15] with some modification. The hydrolysis product samples (0.5 mL) were dissolved (vibrating violently) in n-hexane (2 mL), the mixture was centrifuged at 1500 rpm for 5 min, then the n-hexane layer containing FFAs, MAGs, DAGs and TAGs was obtained. The n-hexane layer was washed with 1 mL of distilled water for six times dried over anhydrous Na2SO4 for GC analysis. The detector temperature was maintained at 350°C. The oven temperature programming was set as follows: 100°C to 200°C at the rate of 50°C/min; to 290°C at the rate of 15°C/min; to 320°C at the rate of 40°C/min for 8 min; finally to 360°C at the rate of 20°C/min for 6 min. The flow rate of carrier gas N2 was set at 4.4 mL/min. The external standard method was used to quantify the hydrolysis products (FFAs, MAGs, DAGs, and TAGs). The calibration curves were established using oleic acid, glycerol monooleate, glycerol dioleate, and glycerol trioleate as standards of FFAs, MAGs, DAGs, and TAGs. FFAs content and degree of hydrolysis (DH) of TAGs were defined as Eq. (1) and Eq. (2), respectively. DH of TAGs could indicate the conversion of TAGs during the hydrolysis.

\[
\text{FFAs content (\%) } = \frac{C_{\text{FFAs}}}{C_{\text{FFAs}} + C_{\text{MAGs}} + C_{\text{DAGs}} + C_{\text{TAGs}}} \times 100\% \tag{1}
\]

\[
\text{DH (\%) } = \frac{C_{\text{TAGs}0} - C_{\text{TAGs}}}{C_{\text{TAGs}0}} \times 100\% \tag{2}
\]

where, \(C_{\text{FFAs}}, C_{\text{MAGs}}, C_{\text{DAGs}}\), and \(C_{\text{TAGs}}\) were concentrations (mmol/mL) of FFAs, MAGs, DAGs, and TAGs, respectively. \(C_{\text{TAGs}0}\) and \(C_{\text{TAGs}}\) were concentrations (mmol/mL) of TAGs before and after hydrolysis, respectively.

2.6 Fatty acid composition

PNO was methylated according to the AOCS Official Method Ce 2–66[16]. The separation of the FFAs from the
hydrolyzate of PNO by TLC was carried out on Silica G plate (20 × 20 cm, 0.20-0.25 mm). A sample (200 μL) was dissolved in Chloroform/methanol (2:1, v/v; 800 μL), mixed by orbital shaker (Lab dancer, IKA@-Werke Gmbh & Co. KG Germany) for 2 min, centrifuged at 1500 rpm for 3 min, and then 200 μL of the organic layer was spotted onto the TLC plate. The plate was developed in a TLC tank using the mixture of hexane: diethyl ether: acetic acid (70:30:1, v/v/v) as developing solvents. After development, the plate was dried under vacuum. The TLC band of hydrolyzate was visualized under UV light after spraying with a 0.2% ethanolic solution of 2,7-dichlorofluorescein sodium salt. The FFAs band was scraped and put into a 50-mL flask, then 5 mL of methanolic solution of boron trifluoride (15% as BF₃) was added and kept reflux at 80°C in water bath for 3 min. 3 mL of n-hexane was added to the boiling mixture through the condenser and boiled for 1 min. Then, the flask was removed from the water bath, and 20 mL saturated NaCl solution was added in. The flask was vibrated vigorously for 0.5 min, followed by the addition of more saturated NaCl solution. The flask was allowed to stand for 5 min and then 1.5 mL of the upper n-hexane layer containing methyl esters dried by anhydrous sodium sulfate was transferred into a test tube for GC analysis.

The analysis of fatty acid methyl esters was performed on a gas chromatograph (GC) (Agilent 6890N) equipped with a flame ionization detector (FID) and a BPX-70 capillary column (30 m × 0.25 mm, 0.25 μm of film thickness) (SGE Technologies Co. Ltd., Australia). The column, injector, and detector temperatures were set at 180, 230, and 300°C, respectively. The flow rate of carrier gas N₂ with a split ratio of 1:20 was set at 70 mL/min. The FAME peaks of all fatty acids in the sample were identified by comparison with reference to the retention times of FAME standards (Sigma-Aldrich, Steinheim, Germany) at the same conditions. The relative content of each fatty acid was calculated according to the peak area of a fatty acid to the total peak area (the peaks of all fatty acids in the sample).

2.7 Experimental design

The experimental design and statistical analysis were created and performed using Design-Expert 8.0 (Stat-Ease, Inc, Minneapolis, USA). Response surface methodology with a five-factor, three-level Box-Behnken design (BBD) was utilized to study the effect of the hydrolysis parameters on the FFA contents in the products. Five independent factors, namely temperature (X₁, °C), water amount (X₂, %), DESs amount (X₃, %), lipase dosage (X₄, %), and reaction time (X₅, h) were used, and three levels were applied to each factor (Table 2). The ranges of the variables investigated were selected according to the preliminary experiments. The response was FFA content in the product mixture. The five replicates of the center points assay the repeatability of the designed method and used to examine the experimental error. The relationships between the response and the variables were expressed as a quadratic polynomial regression model. The mathematical model proposed for the response of Y was:

\[ Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \sum_j \beta_{ij} X_i X_j \]

where \( X_i \) and \( X_j \) represent the independent variables; \( \beta_0, \beta_i, \beta_{ij} \) are the model intercept, linear term coefficient, quadratic term coefficient, and the interaction regression coefficient, respectively. The fit of the model was analyzed by coefficients of determination (R²) and the analysis of variance (ANOVA) using Design-Expert 8.0 (Stat-Ease Inc., Minneapolis, USA).

2.8 Statistical analysis

All the experiments were carried out at least in triplicate. Figure preparation and nonlinear fitting of thermodynamic data using Arrhenius equation were performed using Origin 9.0 software (OriginLab Co., Northampton, USA).

3 Results and Discussion

The fatty acid compositions of the PNO were 16:0 (42.3 ± 0.02%), 18:0 (28.0 ± 0.03%), 9c-18:1 (25.33 ± 0.10%), 5c,9c-18:2 (2.76 ± 0.01%), 9c,12c-18:2 (44.27 ± 0.06%), 5c, 9c,12c-18:3 (pinolenic acid, 16.43 ± 0.08%), 9c,12c, 15c-18:3 (1.46 ± 0.02%), 11c,14c-20:2 (1.15 ± 0.19%). Our results were similar to the reports by Xu et al. and Destaillats et al.¹⁷

3.1 Screening of the DESs

DESs have a direct effect on enzymatic activity and stability in lipase-catalyzed hydrolysis¹⁶. In order to screen suitable DES for preparing FFAs from PNO, the effect of DESs using as co-solvent on the lipase-catalyzed hydrolysis of PNO was investigated (Fig. 1). Using ChCl:U (1:2) and ChCl:Gly (1:2) as co-solvent for the hydrolysis, DH and FFAs content were both higher relatively (DH, 83.34 ± 1.51% and 76.34 ± 1.48% respectively; FFAs contents, 67.78 ± 1.98% and 46.34 ± 1.48%, respectively). When ChCl:EG (1:2), ChCl:EG:1:1, ChCl:1,2-PG (1:2), and ChCl:1,2-PG (1:3) were used as co-solvents, DHs were higher, while FFAs contents were lower (DHs, 86.3 ± 1.6%, 80.4 ± 1.8%, 67.2 ± 1.3%, and 65.5 ± 1.9%, respectively; FFAs contents, 26.4 ± 1.87%, 18.9 ± 1.7%, 26.1 ± 1.8%, and 25.7 ± 1.7%, respectively). DHs using ChCl:LA (1:1) and ChCl:CA (1:3) as co-solvent were both lower than 10%. The results implied that the composition of DESs might affect the catalytic efficiency of Amano lipase PS, and this phenomenon was also reported in the previous research¹⁵,¹⁶. According to DH and FFAs content in the hydrolysis product, ChCl:U (1:2) was selected as the co-solvent for the following experiments.
Hydrolysis degree of TAG and FFAs contents in the product mixture during the Amano lipase PS catalyzed hydrolysis of pine nut oil in aqueous deep eutectic solvents. Pine oil 10 g, Amount of DESs 50% (based on the mass of pine oil), water addition 30% (based on the mass of pine oil), lipase dosage 5% (based on the mass of pine oil), temperature 60°C, Time 8 h. a, ChCl:EG (1:2, mol/mol); b, ChCl:EG (1:1, mol/mol); c, ChCl:U (1:2, mol/mol); d, ChCl:Gly (1:2, mol/mol); e, ChCl:1,2-PG (1:3, mol/mol); f, ChCl:1,2-PG (1:2, mol/mol); g, ChCl:CA (1:3, mol/mol); h, ChCl:La (1:1, mol/mol).

3.2 Preliminary experiments

For an efficient industrial process, the reaction time is of importance. Thus, the reaction progress, meaning the production of the FFAs content and DH during the enzymatic hydrolysis, were studied. The time course of DH and FFAs in the product mixtures are shown in Fig. 2A. The results depicted a rapid increase in DH and FFAs to 83.4 ± 2.1% and 69.5 ± 1.9% at first 8 h, respectively. After 8 h, the hydrolysis rate declined, and at 12 h, DH and FFAs in the product mixture were 86.4 ± 2.2% and 74.9 ± 1.8%, respectively. After 12 h of the reaction, DH and FFAs remained almost constant with reaction time increasing.

In general, the reaction temperature plays an important role in the activity and stability of the enzyme, and thus influences the reaction rate and the conversion of the substrate. The effect of reaction temperature on the Amano lipase PS catalyzed-hydrolysis of pine nut oil is shown in Fig. 2B. In the range of 30°C - 60°C, DH was almost constant and kept at 90%. With the increasing of temperature (beyond 60°C), DH was decreased slightly, which might be due to the denaturation of the lipase protein. FFAs content was about 63% at 30°C, further increased to higher than 70% in the range of 40°C to 60°C, and then decreased rapidly at higher temperature (> 70°C).

Lipase catalyzed hydrolysis is reversible reaction, and water amount in the reaction mixture is an important factor in determining chemical equilibrium of the reaction. The hydrolysis of pine nut oil by Amano lipase PS was investigated in water amount ranging from 5% to 70%, and the results are presented in Fig. 2D. DH was low at water amount 5%, and kept at high value (about 90%) in water amount range from 10% to 50%, but decreased slightly when water amount was higher than 50%. FFAs content increased obviously with water amount increasing from 5% to 10%, kept at higher than 70% in water amount range from 10% to 50% and reached to the maximum value (75%). With the further increasing of water amount (beyond 50%), the FFAs content began to decline. The similar result was shown in the previous report (20). The probable reason was that the excess water could inhibit the hydrolysis by competitive inhibition with substrate for binding to enzyme (20).

The rate of lipase-catalyzed reaction is determined by Lipase dosage, which normally increases with the increasing of lipase dosage. Figure 2E shows the effect of lipase dosage on the hydrolysis of pine nut oil. When lipase dosage ranged from 1% to 5%, DH and FFAs content increased from 66.3 ± 1.8% to 92.5 ± 1.7%, and 37.1 ± 1.9% to 79.4 ± 1.7%, respectively. DH and FFAs content reached plateau value with an increase in lipase dosage beyond 5%.

3.3 Statistical analysis and Models fitting

The experimental conditions and the results of 46 runs with BBD design are presented in Table 1. As shown in Table 1, the FFA content in the product mixture was in the range of 6.55%-88.90%. The FFA content of the quadratic polynomial model fitting in the form of ANOVA is shown in Table 2.

The objective was to obtain as high content of FFAs in the product mixture as possible. RSM was an empirical method used to evaluate the relation between a set of controllable experimental factors and the observed values. Models of the factors and the responses were performed by RSM to predict the highest possible FFAs content in the product mixture. The results obtained for the models are listed in Table 2. The data were analyzed by employing a multiple regression technique to evaluate the true relationship between the variables and the response. Two second-order model for FFAs content (%) in the product mixture was satisfactorily established. Y was the predicted values for the FFAs content (%), and X₁, X₂, X₃, X₄, and X₅ were the coded variables as described in Table 2.

\[
Y = 71.69 + 8.22X₁ + 20.18X₂ - 0.91X₃ + 14.01X₄ + 12.78X₅ + 8.01X₁X₂ + 4.63X₁X₃ - 4.17X₁X₄ - 3.82X₁X₅ + 14.19X₂X₃ + 6.67X₂X₄ + 2.99X₂X₅ - 10.89X₃X₄ - 4.72X₃X₅ + 4.97X₄X₅ - 10.65X₁² - 16.62X₂² - 6.62X₃² - 10.49X₄² - 7.64X₅²
\]

The statistical significance of regression equation was
Amano Lipase PS-Catalyzed Preparation of FFAs in Aqueous DES

Fig. 2  Effects of the reaction time, temperature, water amount, DESs addition and lipase on TAG hydrolysis degree and FFAs content in the product mixture during hydrolysis catalyzed by Amano lipase PS in aqueous DES (ChClU (1:2, mol/mol)). A: pine oil 10 g, DES addition (50%, based on the mass of oil), water amount 30% (based on the mass of oil), lipase dosage 5% (based on the mass of oil), reaction temperature 60°C. B: pine oil 10 g, DES addition (50%, based on the mass of oil), water amount 30% (based on the mass of oil), lipase dosage 5% (based on the mass of oil), reaction time 12 h. C: pine oil 10 g, water amount 30% (based on the mass of oil), lipase dosage 5% (based on the mass of oil), reaction temperature 40°C, reaction time 12 h. D: pine oil 10 g, DES addition (50%, based on the mass of oil), lipase dosage 5% (based on the mass of oil), reaction temperature 40°C, reaction time 12 h. E: pine oil 10 g, DES addition (50%, based on the mass of oil), water amount 30% (based on the mass of oil), reaction temperature 40°C, reaction time 12 h.

checked by F-test, and ANOVA for response surface quadratic polynomial models are summarized in Table 3. The high F-values (4.97) and low P-values (0.0001) indicated that the model was highly significant, and the degree of the fits were better on the border of the independent variables. The lack-of-fit measured the failure of the model to represent the data in the experimental region at points which are not included in the regression. The F-value (4.55) and P-value (0.0502, p > 0.05) of the lack-of-fit for the model (Table 2) showed that the lack-of-fit was not significant relative to the pure error. Therefore, the regression models were successful and could accurately represent the variables chosen in the experimental region.

The P-values were used as a tool to check the signifi-
### Table 1  BBD arrangement and responses for lipase hydrolysis of PNO using DES as co-solvent.

| Run no | Temperature (°C) | Water content (X₁) (%) | DES addition (X₂) (%) | Lipase dosage (X₃) (%) | Reaction time (X₄) (h) | FFA contents (Y) (%) |
|--------|------------------|------------------------|-----------------------|------------------------|------------------------|---------------------|
| 1      | -1 (20)          | 0 (30)                 | 0 (50)                | -1 (1)                 | 0 (12)                 | 15.54               |
| 2      | -1 (20)          | 0 (30)                 | 0 (50)                | 0 (5)                  | 1 (20)                 | 60.83               |
| 3      | 0 (40)           | 0 (30)                 | 90                    | 0 (5)                  | 1 (20)                 | 56.04               |
| 4      | 0 (40)           | 1 (50)                 | 90                    | 0 (5)                  | 0 (12)                 | 77.48               |
| 5      | 0 (40)           | 1 (50)                 | 0 (50)                | -1 (1)                 | 0 (12)                 | 43.48               |
| 6      | 1 (60)           | -1 (10)                | 0 (50)                | 0 (5)                  | 0 (12)                 | 27.34               |
| 7      | 1 (60)           | 0 (30)                 | 0 (50)                | 0 (5)                  | -1 (4)                 | 48.01               |
| 8      | 0 (40)           | 1 (50)                 | 10                    | 0 (5)                  | 0 (12)                 | 60.02               |
| 9      | 0 (40)           | -1 (10)                | 0 (50)                | 0 (5)                  | -1 (4)                 | 6.55                |
| 10     | 1 (60)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 88.10               |
| 11     | 0 (40)           | 0 (30)                 | 90                    | 0 (5)                  | -1 (4)                 | 53.92               |
| 12     | 0 (40)           | 0 (30)                 | 0 (50)                | 1 (9)                  | 1 (20)                 | 88.90               |
| 13     | 0 (40)           | 0 (30)                 | 90                    | 1 (9)                  | 0 (12)                 | 46.94               |
| 14     | 0 (40)           | 0 (30)                 | 10                    | 0 (5)                  | -1 (4)                 | 60.50               |
| 15     | -1 (20)          | 1 (50)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 58.75               |
| 16     | 0 (40)           | 1 (50)                 | 0 (50)                | 0 (5)                  | -1 (4)                 | 52.27               |
| 17     | 0 (40)           | 0 (30)                 | 10                    | 0 (5)                  | 1 (20)                 | 81.49               |
| 18     | 0 (40)           | 1 (50)                 | 0 (50)                | 0 (5)                  | 1 (20)                 | 86.13               |
| 19     | 0 (40)           | -1 (10)                | 0 (50)                | 1 (9)                  | 0 (12)                 | 19.33               |
| 20     | -1 (20)          | 0 (30)                 | 90                    | 0 (5)                  | 0 (12)                 | 51.59               |
| 21     | -1 (20)          | 0 (30)                 | 0 (50)                | 0 (5)                  | -1 (4)                 | 8.74                |
| 22     | 1 (60)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 1 (20)                 | 84.80               |
| 23     | -1 (20)          | 0 (30)                 | 0 (50)                | 1 (9)                  | 0 (12)                 | 68.36               |
| 24     | 0 (40)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 79.89               |
| 25     | 0 (40)           | -1 (10)                | 0 (50)                | -1 (1)                 | 0 (12)                 | 15.73               |
| 26     | 0 (40)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 78.24               |
| 27     | 0 (40)           | 0 (30)                 | 10                    | 1 (9)                  | 0 (12)                 | 72.69               |
| 28     | 1 (60)           | 1 (50)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 79.35               |
| 29     | 0 (40)           | 0 (30)                 | 10                    | -1 (1)                 | 0 (12)                 | 39.98               |
| 30     | -1 (20)          | -1 (10)                | 0 (50)                | 0 (5)                  | 0 (12)                 | 38.79               |
| 31     | 0 (40)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 62.12               |
| 32     | 1 (60)           | 0 (30)                 | 10                    | 0 (5)                  | 0 (12)                 | 29.44               |
| 33     | 0 (40)           | -1 (10)                | 10                    | 0 (5)                  | 0 (12)                 | 55.72               |
| 34     | 1 (60)           | 0 (30)                 | 0 (50)                | -1 (1)                 | 0 (12)                 | 51.97               |
| 35     | 0 (40)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 65.55               |
| 36     | 0 (40)           | 1 (50)                 | 0 (50)                | 1 (9)                  | 0 (12)                 | 73.75               |
| 37     | 1 (60)           | 0 (30)                 | 90                    | 0 (5)                  | 0 (12)                 | 62.34               |
| 38     | -1 (20)          | 0 (30)                 | 10                    | 0 (5)                  | 0 (12)                 | 37.20               |
| 39     | 0 (40)           | 0 (30)                 | 0 (50)                | 1 (9)                  | -1 (4)                 | 60.60               |
| 40     | 0 (40)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 73.22               |
| 41     | 0 (40)           | 0 (30)                 | 90                    | -1 (1)                 | 0 (12)                 | 57.78               |
| 42     | 0 (40)           | 0 (30)                 | 0 (50)                | 0 (5)                  | 0 (12)                 | 71.12               |
| 43     | 0 (40)           | -1 (10)                | 90                    | 0 (5)                  | 0 (12)                 | 16.40               |
| 44     | 0 (40)           | -1 (10)                | 0 (50)                | 0 (5)                  | 1 (20)                 | 28.47               |
| 45     | 0 (40)           | 0 (30)                 | 0 (50)                | -1 (1)                 | -1 (4)                 | 30.79               |
| 46     | 0 (40)           | 0 (30)                 | 0 (50)                | -1 (1)                 | 1 (20)                 | 39.21               |
Table 2  Analysis of variance (ANOVA) for response surface quadratic model pertaining to the predicted FFAs production.

| Source                  | Sum of Squares | Degree of freedom | Mean Square | F Value | P-value | Prob > F | Significance |
|-------------------------|----------------|-------------------|-------------|---------|---------|----------|--------------|
| Model                   | 18504.79       | 20                | 925.24      | 4.97    | 0.0001  | **       |              |
| Reaction temperature ($X_1$) | 1082          | 1                 | 1082        | 5.82    | 0.0235  | *        |              |
| Water amount ($X_2$)    | 6515.72        | 1                 | 6515.72     | 35.03   | <0.0001 | **       |              |
| DES addition ($X_3$)    | 13.23          | 1                 | 13.23       | 0.071   | 0.7919  |          |              |
| Lipase dosage ($X_4$)   | 3141.13        | 1                 | 3141.13     | 16.89   | 0.0004  | **       |              |
| Reaction time ($X_5$)   | 2613.89        | 1                 | 2613.89     | 14.05   | 0.0009  | **       |              |
| $X_1X_2$                | 256.7          | 1                 | 256.7       | 1.38    | 0.2511  |          |              |
| $X_1X_3$                | 85.61          | 1                 | 85.61       | 0.46    | 0.5037  |          |              |
| $X_1X_4$                | 69.63          | 1                 | 69.63       | 0.37    | 0.5461  |          |              |
| $X_1X_5$                | 58.47          | 1                 | 58.47       | 0.31    | 0.58    |          |              |
| $X_2X_3$                | 805.85         | 1                 | 805.85      | 4.33    | 0.0478  | *        |              |
| $X_2X_4$                | 177.93         | 1                 | 177.93      | 0.96    | 0.3374  |          |              |
| $X_2X_5$                | 35.67          | 1                 | 35.67       | 0.19    | 0.6652  |          |              |
| $X_3X_4$                | 474.32         | 1                 | 474.32      | 2.55    | 0.1228  |          |              |
| $X_3X_5$                | 88.95          | 1                 | 88.95       | 0.48    | 0.4956  |          |              |
| $X_4X_5$                | 98.84          | 1                 | 98.84       | 0.53    | 0.4728  |          |              |
| $X_1^2$                 | 990.34         | 1                 | 990.34      | 5.33    | 0.0296  | *        |              |
| $X_2^2$                 | 2410.53        | 1                 | 2410.53     | 12.96   | 0.0014  | **       |              |
| $X_3^2$                 | 382.55         | 1                 | 382.55      | 2.06    | 0.1639  |          |              |
| $X_4^2$                 | 959.69         | 1                 | 959.69      | 5.16    | 0.032   | *        |              |
| $X_5^2$                 | 509.97         | 1                 | 509.97      | 2.74    | 0.1102  |          |              |
| Residual                | 4649.48        | 25                | 185.98      |         |         |          |              |
| Lack of Fit             | 4407.33        | 20                | 220.37      | 4.55    | 0.0502  |          |              |
| Pure Error              | 242.16         | 5                 | 48.43       |         |         |          |              |
| Cor Total               | 23154.27       | 45                |             |         |         |          |              |

* Significant at the 5% level; ** Significant at the 1% level

Table 3  Gibb’s free energy, enthalpy and entropy values of Amano lipase PS catalyzed hydrolysis of PNO at different temperature.

| T (°C) | T (K) | $\Delta G$ (kJ/mol) | $\Delta H$ (kJ/mol) | $\Delta S$ (J/mol/K) |
|--------|-------|----------------------|---------------------|----------------------|
| 40     | 313.15| 77.99                | 20.34               | −184.11              |
| 50     | 323.15| 98.83                | 20.26               | −184.37              |
| 55     | 328.15| 80.76                | 20.21               | −184.49              |
| 70     | 343.15| 83.53                | 20.09               | −184.87              |
| 80     | 353.15| 85.38                | 20.01               | −185.10              |

Mean $81.50 \pm 2.64$ $20.18 \pm 0.12$ $−184.59 \pm 0.36$

PNO: pine nut oil.
cance of each coefficient, which in turn might indicate the pattern of the interaction between the variables. For the model of $Y$ in Table 3, the linear coefficients ($X_2$, $X_4$, $X_5$) and the quadratic term coefficients ($X_2^2$, $X_1^2$, $X_4^2$) were significant with small $P$-values ($p < 0.01$), the linear coefficient ($X_3$), the cross product coefficient of $X_2X_3$ and the quadratic term coefficients ($X_1^2$ and $X_4^2$) were also significant at $p < 0.05$. The reaction variables affecting on FFAs content in the product mixture were in the order of water amount $>$ lipase dosage $>$ reaction time $>$ reaction temperature $>$ DESs addition.

The relationship between independent variables and response can be better understood by examining the planned series of 2D contour plots (Fig. 3) generated from the predicted model. Figure 3a shows the effect of reaction temperature, water amount, and their mutual interaction on FFAs content in the product mixture. At the same temperature, with the increase of water amount, the FFAs content increased firstly, and then decreased. Figure 3b shows the effect of reaction temperature, DES addition, and their mutual interaction on FFAs content in the product mixture. The maximum value of FFAs content appeared in temperature of 40-60°C and DES addition range of 30-70%.

Figure 3c shows the effect of reaction temperature, lipase
dosage, and their mutual interaction on FFAs content in the product mixture. The maximum value of FFAs content appeared in temperature of 36-54°C and lipase dosage of 6-9%. Figure 3d shows reaction temperature, reaction time, and mutual interaction on FFAs content in the product mixture. A suitable increment in reaction temperature and time increased the FFAs content in the product mixture. At the same time, with the increase of reaction temperature, FFAs content in the product mixture increased initially, and then decreased. The maximum value of FFAs content in the product mixture appeared in reaction temperature range of 35-55°C and reaction time of 13-20 h. Figure 3e shows water amount, DESs addition, and their mutual interaction on FFAs content in the product mixture. When DESs addition kept at a constant level, FFAs content in the product mixture increased firstly, and then decreased. The maximum value of FFAs content appeared in water amount of 40-50% and DESs addition range of 40-90%. Figure 3f shows water amount, lipase dosage, and their mutual interaction on FFAs content in the product mixture. The maximum FFAs content appeared in water amount range of 35-50% and lipase dosage of 6-9%. Figure 3g shows water amount, reaction time, and their mutual interaction on FFAs content in the product mixture. The maximum FFAs content appeared in water amount of 35-50% and reaction time range of 12-18 h. Figure 3h shows DESs addition, lipase dosage, and their mutual interaction on FFAs content in the product mixture. With DESs addition increasing, the FFAs content increased initially, and then decreased. Figure 3i shows DESs addition, reaction time, and their mutual interaction on FFAs content in the product mixture. When DES addition kept at a constant level, FFA content increased firstly with reaction time prolonging, then decreased. The maximum FFAs content appeared in DES addition of 35-50% and reaction time range of 14-18h. Figure 3j shows lipase dosage, reaction time, and their mutual interaction on the FFAs content in the product mixture. When lipase dosage kept at a constant level, with reaction time prolonging, FFA content increased. The maximum FFAs content appeared in lipase dosage of 6-9% and reaction time range of 13-18 h.

3.4 Attaining optimum condition and model verification

Reaction conditions optimized by RSM were as follows: reaction temperature 45.95°C, water amount 37.76%, DES addition 43.10%, lipase dosage 7.61%, and reaction time 13.12 h, under which the predicted value of FFAs content was 87.21%. In the actual work, these variables values were corrected as follows: reaction temperature 46°C, water amount 38%, DES addition 43%, lipase dosage 7.6% and reaction time 13 h. Under these conditions, FFAs content in the product mixture was 89.1 ± 1.9% (DH was 92.7 ± 2.2%), which was well accorded with the predicted result. The results indicated the feasibility and accuracy of the quadratic regression model. Furthermore, under the optimal condition without DES, the FFA content in the product was 85.5 ± 1.6% (DH was 90.5 ± 2.0%). The fatty acid composition of FFAs in the hydrolysis product was 16:0 (4.34 ± 0.04%), 18:0 (2.14 ± 0.04%), 9c-18:1 (22.98 ± 0.06%), 5c,9c-18:2 (2.73 ± 0.06%), 9c,12c-18:2 (46.72 ± 0.17%), 5c,9c,12c-18:3 (pinolenic acid, 17.15 ± 0.14%), 9c,12c,15c-18:3 (1.11 ± 0.01%) and 11c,14c-20:2 (1.19 ± 0.02%). The fatty acid composition of the FFAs from hydrolysis was similar to those of the original PNO.

In order to examine the intermediate products in the hydrolysis process, the contents of FFAs, MAGs, DAGs, and TAGs were determined during the Amano lipase PS-catalyzed hydrolysis of PNO under optimal condition. The result is shown in Fig. 4. TAGs content decreased sharply to about 34% at 1 h, and then decreased to 4.6% at 12 h. DAGs increased dramatically until 0.5 h to 21.7%, and then decreased to 4.0% at 12 h. MAGs increased rapidly to 11.9%, and decreased to 2.6% at 12 h. FFAs increased gradually to 88.7% until 12 h, after 12 h, though FFAs still increased slightly, MAGs, DAGs and TAGs decreased very slowly.

3.5 Recycling of the Amano lipase PS

In this research, the lipase was reused, making it more cost effective. When the reaction was finished, the reaction mixture was homogeneous phase (Fig. 5A), which distinctly separated into two phase when cooled to ambient temperature (Fig. 5B). Figure 4C shows the reusability of Amano lipase PS for FFAs production in the hydrolysis of PNO. When the enzyme was used as it fresh state, DH and FFAs content were 92.7 ± 2.2% and 89.1 ± 1.9%, respectively. In the recycle experiments, DH and FFAs contents were de-
Increased continuously: 1st run, 92.5 ± 2.1% and 87.2 ± 1.9%, respectively; 2nd run, 76.1 ± 3.8% and 68.9 ± 4.7%, respectively; 3rd run, 72.0 ± 3.7% and 61.2 ± 4.5%, respectively; 4th run, 56.1 ± 11.3% and 41.4 ± 4.6%, respectively.

The decrease of DH and FFAs content in the product mixture might be due to the accumulation of glycerol and the inactivation of lipase. For preparing FFAs, the lipase could be reused 3 times in our study.

3.6 Thermodynamic analysis for hydrolysis of PNO

Arrhenius equation, described by equation (4) was used to described the effect of temperature on the reaction rate. Different kinetic experiments were performed at different reaction temperature (40°C, 45°C, 55°C, 70°C, and 80°C) with an lipase dosage of 7% (based on PNO mass), water amount 40% (based on the PNO mass), ChCl:U (1:2) addition 40% (based on PNO mass), reaction time <15 min, DH of PNO was no more than 25%.

\[ V = A \times \exp\left( -\frac{Ea}{RT} \right) \]  

where, \( V \) is the initial rate of PNO hydrolysis, \( A \) is a constant (pre-exponential factor), \( Ea \) is activation energy, \( R \) is universal gas constant (8.31 J·mol⁻¹·K⁻¹), \( T \) is absolute temperature (K).

The effect temperature on the initial rate of hydrolysis is shown in Fig. 6. The initial reaction rate increased rapidly with temperature increasing (below 80°C). A nonlinear fit of the experimental data to the proposed model was directly performed using Origin software. As shown in Fig. 6, good correlation coefficient (\( R^2 = 0.9901 \)) was obtained and the estimated value for \( Ea \) was about 22.94 kJ/mol, which was reasonably acceptable and in the range of 0.97–34.5 kJ/mol in the previous report. The \( Ea \) in our study was lower than those in some reports. The \( Ea \) of Lipolase 100T catalyzed-hydrolysis of sunflower oil in a high-pressure reactors was 32.7 kJ/mol. Hou and Sun reported an \( Ea \) of 39.25 kJ/mol for the hydrolysis of phoenix tree oil catalyzed by Lipozyme TLIM in aqueous solution. In the report of Phuah et al., the \( Ea \) for the partial hydrolysis of palm oil catalyzed by Lipase RM IM (Rhizomucor miehei) was 31.35 kJ/mol.

According to transition-state theory, some equations (Eq.
Amano Lipase PS-Catalyzed Preparation of FFAs in Aqueous DES

5-7\textsuperscript{25} was used to evaluate the thermodynamic properties such as changes of Gibbs free energy ($\Delta G$), enthalpy ($\Delta H$) and entropy ($\Delta S$) in the process of Amano lipase PS catalyzed hydrolysis of PNO using DES as co-solvent.

$$\Delta G = \Delta H - T \times \Delta S $$

(5)

$$\Delta H = Ea - R \times T $$

(6)

$$\Delta S = R \ln \left( \frac{A \times N \times h}{R \times T} \right) - R \times \ln \left( \frac{A \times N \times h}{R \times T} \right) - 1 $$

(7)

where, $h$ is Planck constant ($6.63 \times 10^{-34}$ J·s), $N$ is Avogadro constant/number ($6.02 \times 10^{23}$ mol\textsuperscript{-1}).

The thermodynamic parameters were calculated using the Eq. 5-7, the values of $\Delta G$, $\Delta H$ and $\Delta S$ are shown in Table 3. The $\Delta G$ values were in the range of 77.99 – 85.38 kJ/mol with mean of 81.50 ± 2.64 kJ/mol, and much higher than the value (28.61 kJ/mol) of the previous report\textsuperscript{26}. $\Delta H$ values were in the range of 20.01 – 20.34 kJ/mol with average of 20.18 ± 0.12 kJ/mol, and higher than the value (14.91 kJ/mol) in the previous research\textsuperscript{26}. $\Delta S$ values were in the range of -185.10 to -184.11 J/K·mol with mean of -184.59 ± 0.36 J/K·mol, and lower than the value (-45.21 J/K·mol) presented by Ramani et al.\textsuperscript{26}. The negative change in entropy value indicated that the hydrolysis of PNO had become ordered. The positive value of enthalpy of the reaction system revealed the hydrolysis of PNO using DES as co-solvent was endothermic process. The positive Gibb’s energy value showed the Amano lipase PS catalyzed hydrolysis of PNO using DES as co-solvent was no spontaneous\textsuperscript{25}.

4 Conclusion

The FFAs containing pinolenic acid was successfully prepared through Amano lipase PS catalyzed hydrolysis of PNO using ChCl:U (1:2, mol/mol) as co-solvent and the hydrolysis reaction was optimized using RSM. The maximum FFAs content (89.05 ± 1.92%) and DH (92.68 ± 2.24%) of PNO hydrolysis product were achieved under the optimal conditions (temperature 46°C, water amount 38%, DES addition 43%, lipase dosage 7.6%, reaction time 13 h). The content of pinolenic acid in the FFAs product was about 17%. The activation energy, Gibbs free energy, enthalpy and entropy of the hydrolysis of PNO catalyzed by Amano lipase PS using ChCl:U as co-solvent were 22.94 kJ/mol, 20.15 ± 2.64 kJ/mol, 20.18 ± 0.12 kJ/mol and -184.59 ± 0.36 J/mol/K, respectively. The lipase could be reused for three times. The results in our study could provide valuable reference for the preparation of FFAs by lipase catalyzed hydrolysis of oils using DES as co-solvent.

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