Biodiesel Production Catalyzed by a Methanol-Tolerant Lipase A from *Candida antarctica* in the Presence of Excess Water

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**ABSTRACT:** In this article, biodiesel was prepared using a novel free liquid lipase A from *Candida antarctica* (CALA) as a catalyst in the presence of excess water. The methanol tolerance of CALA was investigated. The effect of reaction conditions, including the molar ratio of soybean oil to methanol, water load, CALA load, reaction temperature, and reaction time, was evaluated. Reaction thermodynamics and kinetics were also analyzed. Results showed that free liquid lipase CALA showed excellent methanol tolerance in the reaction system using one-step addition of methanol and can be used to prepare biodiesel with water load of 12–14%. The influence of three transesterification variables on biodiesel yield was water load > temperature > time. The transesterification conditions were optimized by response surface methodology as follows: CALA load 5%, substrate molar ratio (soybean oil/methanol) 1:7, water load 14%, reaction time 26 h, and temperature 38 °C. The maximum biodiesel yield (92.4 ± 0.8%) was obtained under optimal conditions. The activation energy for biodiesel formation was 52.58 kJ/mol. Kinetic parameters *Km* and *Vmax* were 4.84 × 10⁻¹ mol/L and 6.85 × 10⁻² mol/(L·min), respectively. The mechanism of CALA-catalyzed transesterification was also proposed.

1. INTRODUCTION

Biodiesel, composed of fatty acyl methyl esters or fatty acyl ethyl esters, is a clean, nontoxic, biodegradable, and renewable green fuel, which was first proposed in the 1900s. However, biodiesel did not attract much attention until the crisis of fossil fuel in the early 1980s. Recently, with the gradual exhaustion of fossil fuel and the significant enhancement of people’s awareness of environmental protection, biodiesel has been used as an alternative for fossil fuel in many countries. According to the characteristic of raw materials, biodiesel can be prepared by the transesterification of triglycerides (TGs) in vegetable oil with alcohol or the esterification of free fatty acids (FFAs) with alcohol. And the performance and emission values of biodiesels from vegetable oils, for example, soybean oil, were nearly the same as those of diesel fuels.

Chemical catalysts and enzymes are often used to prepare biodiesel. Compared with chemical catalysts, enzymes are used for biodiesel production due to their mild reaction conditions, high selectivity, high specificity, and cleaner process. Among these enzymes, two forms (immobilized enzymes and free enzymes) are often used for biodiesel production. Although the immobilized enzymes can improve the stability of the enzyme, the high cost and strong adsorption of the by-product glycerol make the large-scale application of immobilized enzymes limited. Recently, due to the lower cost compared to that of the immobilized enzymes, simple operation, low reaction temperature, and high catalytic activities, free liquid enzymes have shown potential in transesterification.

In the enzymatic reaction for biodiesel production, alcohol has an inhibitory effect on lipase, which seriously affects the activity of the enzyme. In order to solve this problem, organic solvents and stepwise addition method of methanol can be used. For example, Cervero et al. achieved 90% biodiesel yield using a method of three-step addition of alcohol, which was 42% higher than that obtained by the one-step addition method. However, these methods drastically increased the complexity of experimental operations and boosted the costs of operations in large scale and industry. Therefore, the development of novel methanol-resistant lipase has become a new focus.

Free liquid lipase A from the yeast *Candida antarctica* (CALA) is a serine hydrolase that has a typical catalytic triad composed of Ser184, His366, and Asp334. Recently, CALA has been used to concentrate DHA and EPA from fish oil, prepare high-enantiomer β-amino esters, and synthesize monoglycerides (MGs). However, the informa...
tion on the application of free liquid CALA for biodiesel production was seldom available.

In this work, in order to decrease the cost of biodiesel preparation and enhance reaction efficiency, free liquid CALA was used as a catalyst to catalyze the transesterification of soybean oil with methanol. The methanol tolerance of CALA was also investigated. The effects of substrate ratio, water load, CALA load, temperature, and time on the CALA-catalyzed transesterification were evaluated and optimized using response surface methodology (RSM). The thermodynamics and kinetics of the reactions were evacuated. The mechanism of the CALA-catalyzed transesterification was also proposed.

2. RESULTS AND DISCUSSION

2.1. Effect of Reaction Progress on the CALA-Catalyzed Transesterification for Biodiesel Production. Figure 1A shows that with reaction progress, biodiesel yield rapidly increased in 8 h. The maximum biodiesel yield (93.8 ± 1.6%) was obtained at 36 h. And the contents of TG and diglyceride (DG) in the reaction product at 36 h were 5.2 ± 0.5% and 1.0 ± 0.1%, respectively. MG was not found in the reaction product within the first 8 h. After that, FFA disappeared, which suggested that liquid CALA had little catalytic effect on the hydrolysis of oil in the reaction system.

2.2. Effect of Substrate Ratio on Biodiesel Production. Figure 2 shows that the maximum biodiesel yield (88.1 ± 1.5%) was achieved when the mole ratio of methanol was 7 times that of oil, which was due to the increase of methanol, promoting the forward reaction for biodiesel preparation. However, with further increase of the mole ratio of methanol, biodiesel yield decreased from 88.1 ± 1.5% of 1:7 (oil/methanol) to 49.0 ± 0.8% of 1:12, which was related to the presence of excessive methanol in the reaction system, which can decrease enzymatic activities. A similar inhibitory effect of methanol on the lipase activity can also be found in previous reports.

In order to overcome the deactivation of lipase in the excess methanol, a two-step addition method of methanol (7 times methanol was first added into the reaction system, and the residual methanol was added after 4 h) was studied. The results showed that when the substrate molar ratio was 1:9, the maximum biodiesel yield using the two-step addition method was 73.2 ± 0.3% at 48 h. Furthermore, the ratio of oil to methanol was 1:9, 75.1 ± 0.9% of biodiesel yield was obtained at 48 h using the two-step addition method. However, these biodiesel yields were all lower than that of 1:7 molar ratio using the one-step addition method (88.1 ± 1.5% at 48 h). These results showed that 1:7 molar ratio using the one-step addition method was a good choice for the liquid CALA-catalyzed reaction.

Interestingly, the optimal ratio of oil to methanol, 1:7 (mol/mol), was higher than that of previous reports (oil-to-methanol, mol/mol), 1:33 and 1:4. Moreover, the methanol tolerance of liquid CALA was also better than that of other lipases, such as lipase @ZIF-67 (oil-to-methanol molar ratio 1:6, biodiesel yield 80.6 ± 2.0%),37 lipase from Pseudomonas fluorescens38 (oil-to-methanol molar ratio 1:3, biodiesel yield 83.8%), recombinant lipase from Rhizomucor miehei (oil-to-methanol molar ratio 1:3, biodiesel yield 86.6%),5 and liquid recombinant lipase 2 from Candida rugosa18 (oil-to-methanol molar ratio 1:3, biodiesel yield 95.3%). Overall, free liquid CALA had excellent tolerance to methanol for biodiesel production using the one-step addition of methanol.

2.3. Effect of Water Load on Biodiesel Production. Figure 3 shows that when the water load was less than 12% [water/(oil and methanol), w/w], biodiesel yield gradually increased from 0 to 93.8 ± 1.5%, which was ascribed to the fact that with the increase of water load, the emulsification of the
reaction system was enhanced and more activation interfaces are formed. The maximum biodiesel yield (93.8 ± 1.5%) was achieved when the water load was 12%. When the water load was >12%, the biodiesel yield decreased, which was attributed to the fact that excessive water can dilute the concentrations of CALA and methanol. Meanwhile, the presence of water clusters in the CALA activity center can change its structure and decrease its activity. Furthermore, the hydrolysis of oil was improved in the presence of excessive water (Figure 1c), which resulted in the increase of by-products and the decrease of biodiesel production. The optimal water load of the liquid CALA-catalyzed transesterification was 12%, which was 0.5% (water/oil, w/w) higher than that catalyzed by the lipase Callera Trans L40 and 10% (water/oil, w/w) higher than that catalyzed by lipase NS81006. Besides, free liquid CALA also had better stability at high water load than some immobilized enzymes. such as lipase @ZIF-67 (biodiesel yield 70% at the optimal water load of 10%), lipase PS (biodiesel yield 65% at the optimal water load of 3.8%), lipozyme TL-IM (biodiesel yield 69% at the optimal water load of 4%), and so forth. However, liquid lipase from Mucor miehei showed high catalyst activity at a high initial water load (25%).

2.4. Effect of CALA Load on Biodiesel Production. Figure 4 shows that with enzyme load increasing from 1 to 7%, the maximum biodiesel yield increased from 62.2 ± 0.8 to 93.0 ± 1.3% (Figure 4a). Likewise, the initial transesterification rate also increased from 0.025 to 0.466 mol/(L·min) (Figure 4b). However, when the CALA load was >7%, no significant changes in transesterification rate and biodiesel yield were found (Figure 4a). A linear relationship \( Y = 7.35x - 4.85, R^2 = 0.936 \) of CALA load and the initial reaction rate was found (Figure 4b), which indicated that the CALA-catalyzed transesterification was mainly controlled by kinetics and the effect of external mass transfer can be negligible. A similar external mass transfer effect can be minimized using agitation in other enzymatic reactions.

2.5. Effect of Transesterification Temperature on Biodiesel Production. Appropriate temperature can accelerate the transesterification rate and increasing biodiesel yield. When the temperature was ≤40 °C, biodiesel yield gradually increased with the increase of temperature (Figure 5a). Meanwhile, the initial transesterification rate increased significantly from 0.8 × 10^{-2} to 3.1 × 10^{-2} mol/(L·min), which was mainly attributed to the fact that transesterification is endothermic and mass transfer limitation can be reduced at high temperature. The maximum biodiesel yield (84.0 ± 1.2%) was achieved at 40 °C. However, when the temperature was >40 °C, biodiesel yield decreased from 84.0 ± 1.2% at 40 °C to 18.2 ± 1.6% at 60 °C, which was due to the inactivation of liquid CALA. A linear relationship between the initial reaction rate \( [\ln V_0, \text{mol/(L·min)}] \) with temperature \( (1/T, 1/K) \) \( (Y = 6.327x - 16.826, R^2 = 0.970) \) was found (Figure 5b). Therefore, according to Arrhenius equation \( \text{eq 1} \), the activation energy \( (E_a) \) of biodiesel formation was calculated as 52.58 kJ/mol.

\[
\ln V_0 = \ln A - \frac{E_a}{T}
\]  

(1)

In \( \text{eq 1}, V_0, T, \text{and A} \) are the initial transesterification rate (mol/(L·min)), reaction temperature (K), and pre-exponential factor, respectively.

2.6. Model Fitting and Response Surface Analysis. The above results of reaction variables (reaction time, substrate ratio, water load, lipase load, and reaction temperature) show that the effects of reaction time, water load, and reaction temperature on biodiesel yield were significant. Therefore, the interaction effect of these three reaction variables was evaluated and optimized by RSM to achieve the highest possible biodiesel yield (Table 1).

Multiple regression fitting of experimental data in Table 1 was conducted by RSM, and the quadratic multinomial regression equation of biodiesel yield and independent

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**Figure 3.** Effect of water load on the biodiesel yield. Conditions: temperature, 30 °C; substrate ratio, 1:7 (oil/methanol); CALA load, 5 wt %.

**Figure 4.** Effect of CALA load on the biodiesel yield (a) and the initial reaction rate (b). Conditions: temperature, 30 °C; substrate molar ratio, 1:7 (oil/methanol); water load, 12 wt %.
variables $X_1$ (reaction time), $X_2$ (water load), and $X_3$ (reaction temperature) was obtained as eq 2.

Biodiesel yield = $84.96 + 4.64X_1 + 32.03X_2 - 17.43X_3$
+ $3.20X_1X_2 - 3.79X_1X_3 - 10.22X_2X_3$
− $9.34X_1^2 - 33.13X_2^2 - 25.42X_3^2$

(2)

The regression equation variance analysis and significance test in Table 2 suggested that the predicted value and the actual value were highly consistent ($R^2 = 0.946$). Meanwhile, the model was significant ($p < 0.05$) and was proper for the analysis of and prediction for biodiesel yield. In addition, the effect of three variables on biodiesel yield decreased in the order of water load > temperature > reaction time (Figure 6). According to the 3D surface plots, the highest biodiesel yield ($92.4 \pm 0.8\%$) could be achieved under optimized conditions: 38 °C, water load 14%, 26 h, CALA load 7 wt %, and substrate molar ratio 1:7.

2.7. Kinetic Constants of the CALA-Catalyzed Transesterification for Biodiesel Production. According to a previous report, the kinetic mechanism of the CALA-catalyzed transesterification corresponds to the Ping-Pong Bi−Bi mechanism. The kinetic equation of bisubstrates competitive inhibition is as the eq 3.

$$V_0 = \frac{V_m[A][B]}{[A][B] + [B]K_m^A + [A] \left( 1 + \frac{[A]}{K_m^A} \right) K_m^B}$$

(3)

In eq 3, $V_0$ and $V_m$ are the initial transesterification rate and the maximum transesterification rate (mol/(L·min)), respectively. $[A]$ and $[B]$ are the concentrations of substrate A and substrate B (mol/L), respectively. $K_m^A$ and $K_m^B$ are the Michaelis–Menten constants. $K_m^A$ is the inhibition constant of substrate A. The relationship between methanol concentrations ([A]) with the initial rate ($V_0$) is shown in Figure 7.

Figure 5 shows that when the conversion rate of methanol ([A]) was >4.83 mol/L (i.e., when the molar ratio of oil to methanol was <1:7), the initial transesterification rate ($V_0$) decreased sharply due to the inhibition effect of methanol. Therefore, the kinetic constants $V_m$ and $K_m^A$ of the transesterification were evaluated at low methanol concentrations (<4.83 mol/L). Keeping the amount of soybean oil constant and varying the concentration of methanol from 1.66 to 4.83 mol/L, the transesterification could be regarded as a pseudo-first-order reaction, as shown in eq 4.

$$V_0 = \frac{V_m[A]}{[A] + K_m^A}$$

(4)

In eq 4, $V_0$ and $V_m$ are the methanol concentration (mol/L) and the apparent Michaelis constant, respectively. According to the Lineweaver–Burk double reciprocal method, eq 5 was obtained from Figure 7b as follows.

$$\frac{1}{V} = \frac{7.070}{[A]} + 1.460$$

(5)

Consequently, $K_m^A$ and $V_m$ of free liquid CALA-catalyzed transesterification were calculated as 0.484 mol/L and 6.85 × 10⁻² mol/(L·min), respectively.

2.8. Transesterification Mechanism Catalyzed by CALA for Biodiesel Production. The catalytic triad of CALA consisted of serine (Ser184), aspartate (Asp334), and histidine (His366). According to the structure of CALA and the catalytic characteristic of lipase, the mechanism of free liquid CALA-catalyzed transesterification of soybean oil with
methanol is proposed in Figure 8. TG first enters the active site of CALA, where Ser184 is activated by His366. Meanwhile, the Ser184 attacks the carbonyl carbon atom of TG (step 1). And the tetrahedral intermediate 1 is formed. Next, the intermediate 1 is dissociated. The rest of TG, at the same time, accepts the proton from His366 and then leaves from the active site in the form of DGs (step 2). Afterward, methanol enters the active site of CALA, attacks the carbonyl carbon atom bound to Ser184, and contributes one proton to His366 to form the intermediate 2 (step 3). Finally, with the collapse of intermediate 2, biodiesel (FAME) is formed and CALA activity.

Table 2. Results Obtained from Analysis of Variance

| source    | sum of squares | degrees of freedom | mean square | F-value | P-value (Prob > F) |
|-----------|----------------|--------------------|-------------|---------|--------------------|
| model     | 17 619.20      | 9                  | 1957.70     | 13.6    | 0.0012             |
| X1: reaction time | 272.63    | 1                  | 272.63      | 1.89    | 0.2111             |
| X2: water load | 7677.53   | 1                  | 7677.50     | 53.34   | 0.0002             |
| X3: temperature | 2501.19   | 1                  | 2501.20     | 17.38   | 0.0042             |
| X1X2      | 9.55          | 1                  | 9.55        | 0.07    | 0.8041             |
| X1X3      | 37.06         | 1                  | 37.06       | 0.26    | 0.6274             |
| X2X3      | 438.17        | 1                  | 438.17      | 3.04    | 0.1245             |
| X1^2      | 167.75        | 1                  | 167.75      | 1.17    | 0.3161             |
| X2^2      | 3567.42       | 1                  | 3567.40     | 24.78   | 0.0016             |
| X3^2      | 2425.12       | 1                  | 2425.12     | 16.85   | 0.0045             |
| residual  | 1007.55       | 7                  | 143.94      |         |                    |
| lack of fit | 782.72       | 3                  | 260.91      | 4.64    | 0.0861             |
| pure error | 224.83       | 4                  | 56.21       |         |                    |
| total     | 18 626.70     | 16                 |             |         |                    |

R² = 0.9459

Figure 6. Interaction effect of reaction time, temperature, and water load on the biodiesel yield: (a) the interaction effect of temperature with reaction time; (b) the interaction effect of reaction time with water load; (c) the interaction effect of temperature with water load. Reaction conditions: substrate molar ratio, 1:7 (oil/methanol); CALA load, 7 wt %.

Figure 7. The relationship between the initial transesterification rate (V₀) and methanol concentration ([A]) at low methanol concentrations (a); Lineweaver–Burk (b). Conditions: 40 °C; soybean oil, 3 mmol; water load, 15 wt %; CALA load, 5 wt %.
site is recovered (step 4). MG and glycerol (Gl) are also formed in the same way.

3. CONCLUSIONS

Free liquid CALA was successfully used as the catalyst for biodiesel production. And liquid CALA showed excellent methanol tolerance in the reaction system using the one-step addition of methanol. CALA also showed good catalysis performance in the presence of excess water (14%). The effect of transesterification variables on biodiesel yield was water load > temperature > time. The transesterification variables were optimized by RSM as CALA load 5%, molar ratio of oil to methanol 1:7, water load 14% at 38°C for 26 h. Under these conditions, biodiesel yield was 92.4 ± 0.8%. Moreover, the activation energies (Ea) for biodiesel formation was 52.58 kJ/mol, and reaction kinetic values \( K_m' \) and \( V_{max} \) were 0.484 mol/L and \( 6.85 \times 10^{-2} \) mol/(L·min), respectively. These results provide a promising approach for biodiesel production in green and renewable reaction systems.

4. EXPERIMENTAL SECTION

4.1. Materials. Free liquid CALA was purchased from Novozymes (Denmark). Soybean oil was provided by Yihai Jiali Co., Ltd. (Shanghai, China). Methanol (analytical grade) was from Fuyu Fine Chemical Co., Ltd. (Tianjin, China). Standards, including oleic acid, methyl oleate, MG, DG, and TG, were purchased from Sigma Co., Ltd. (Shanghai, China). Hexane was chromatographic grade and was from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China).

4.2. Enzymatic Transesterification. Soybean oil (oil-to-methanol, 1:3–1:12, mol/mol) and water (4 ≈ 35 wt %, based on the total mass of soybean oil and methanol) were weighed and preheated to 20–60°C in a water bath with a thermostatic magnetic stirrer (agitation speed 500 rpm). Then, free liquid CALA (1 ≈ 10 wt %) and methanol were added into the reaction mixture. Sample (20 μL) was withdrawn at regular intervals. Hexane (2 mL) and anhydrous sodium sulfate (1 g) were used to dissolve the sample and remove water, respectively. Finally, the sample was centrifuged for 2 min, and the upper solution was used for gas chromatograph (GC) analysis. The biodiesel yield was calculated from eq 6.

\[
\text{Biodiesel yield (\%)} = \frac{m_{\text{FAME}}}{m_{\text{TG}} + m_{\text{DG}} + m_{\text{MG}} + m_{\text{FAME}} + m_{\text{FA}}} \times 100\%
\]

In eq 6, \( m_{\text{FAME}} \), \( m_{\text{TG}} \), \( m_{\text{DG}} \), \( m_{\text{MG}} \), and \( m_{\text{FA}} \) are the mass of FAME, TG, DG, MG, and FA (g), respectively, from the GC results adopting an external standard method.

4.3. GC Analysis. The biodiesel yield and product composition were analyzed by GC (Agilent 7890B) with a flame ionization detector (FID) equipped with a DB-1ht column (30 m × 250 μm × 0.10 μm). The temperature of the oven was first increased from 170 to 200°C at 2°C/min and then from 200 to 290°C at 20°C/min, and then it was further increased from 290 to 320°C at 6°C/min and remained at 320°C for 1 min; finally, it was increased from 320 to 360°C at 20°C/min and remained at 360°C for 7 min. The total run time was 34.5 min. A volume of 1 μL of the solution was injected. The temperature of both the injector and the FID was 350°C. The flow rates of hydrogen, nitrogen, and air were 30, 4, and 300 mL/min, respectively.
4.4. RSM Design. The effect of reaction conditions (reaction time, water load, temperature) on the biodiesel yield was designed and evaluated by Design Expert 8.0.5 (Table 1). The data were analyzed using the regression equation obtained from the model as follows

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ij} X_i^2 + \sum_{i=1, j=1+i}^{3} \beta_{ij} X_i X_j \]  

(7)

where \( Y \) is the biodiesel yield; \( X_i \) and \( X_j \) are reaction conditions; \( \beta_0 \) is the intercept; \( \beta_i, \beta_{ij}, \) and \( \beta_{ij} \) are the coefficients of the model.

4.5. Statistical Analysis. All experiments were conducted at least in triplicate, and the experimental data were analyzed by SPSS 20.0. All results were expressed as average ± SD, and \( p < 0.05 \) indicated that the difference between the values was significant.

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The authors declare no competing financial interest.

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■ ABBREVIATIONS

CALA, lipase A from Candida antarctica; \( E \), activation energies; GC, gas chromatograph; MG, monoglyceride; GI, glycerol; DG, diglyceride; TG, triglyceride; FFA, free fatty acid

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