Transesterification for Biodiesel Production Catalyzed by Combined Lipases: Optimization and Kinetics

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Preparation of biodiesel from waste cooking oil catalyzed by combined lipases in tert-*butanol medium was investigated. Several crucial parameters affecting biodiesel yield were optimized by response surface methodology, such as dosage of combined lipases of Novozym 435 and Lipozyme TLIM, weight ratio of Novozym 435 to Lipozyme TLIM, amount of* tert-*butanol, reaction temperature, and molar ratio of oil to methanol. Under the optimized conditions, the highest biodiesel yield was up to 83.5% The proposed model on biodiesel yield had a satisfactory coefficient of* R^2 *(= 94.02%), and was experimentally verified. The combined lipases exhibited high-operational stability. After 30 cycles (300 h) successively, the activity of combined lipases maintained 85% of its original activity. A reaction kinetic model was proposed to describe the system and deduced to be a pseudo-first-order reaction, and the calculated activation energy was 51.71 kJ/mol.* © 2009 American Institute of Chemical Engineers *AIChE J,* 56: 1659–1665, 2010

Keywords: biodiesel, combined lipase, response surface methodology, optimization, kinetics, waste cooking oil

Introduction

Biodiesel, as an alternative fuel, is derived from renewable vegetable oils and animal fats. It consists of the monoalkyl esters formed by a catalyzed transesterification reaction of the triglycerides in the oils or fats with a monohydric alcohol. Compared with petroleum-based diesel, biodiesel has many favorable advantages, such as environmental friendly, renewable and nontoxic.[1] So far, the most common way to produce biodiesel is transesterification, which refers to the reactions of transesterification catalyzed by acid- (alkali-) catalyst, enzyme catalyst or under supercritical conditions. Basha et al.[2] reviewed biodiesel production, combustion, performance and emissions based on 130 articles published from 1980 to 2008.

It is demonstrated that when the content of free fatty acid (FFA) in feedstock is higher than 3%, the alkali catalysts for transesterification are not recommended for biodiesel production. Because a large amount of soap byproduct during the reaction, the reaction process with alkaline creates a serious problem of product separation and ultimately lowers the yield substantially.[3,4] Although homogeneous acid catalyzed process such as sulfuric acid catalyzed method can be employed to produce biodiesel from waste cooking oil (WCO) with high FFA content, the process is operated at high-temperature, and gives rise to seriously environmental and corrosive problems. In addition, the homogeneous acid catalyst is difficult to be recycled resulting in relatively high production cost.[5] Conversely, FFA contained in the WCO can be easily converted into biodiesel with enzyme catalyst, which is fit for feedstock oil with high FFA component.[6] Enzyme-catalyzed process can overcome these aforementioned problems caused by alkali and acid catalyzed processes, but it has not been industrialized because of the high cost of enzyme and low-reaction rate. To lower down the cost of enzyme, one efficient way is to use the combined lipases as biocatalyst, of which one is a rather cheap

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commercial lipase (i.e., Lipozyme TLIM, which price is $800 per kilogram), and can replace some amount of the expensive enzyme (i.e., Novozym 435, which price is $9,500 per kilogram.

It has also been demonstrated that lipases give high-synthesis activity and good stability in the hydrophobic solvent like n-hexane; however, the hydrophilic compounds including substrate (alcohol) or byproduct (glycerol) are immiscible in hydrophobic reaction medium.[7] The problem of solubility results in the absorption of polar molecules onto the hydrophilic support, which leads to low transesterification rate.[8] To solve this problem, *tert*-butanol is used as an ideal cosolvent. With a certain amount of *tert*-butanol as the reaction medium, both methanol and byproduct glycerol are soluble, so the negative effects caused by methanol and glycerol on lipase catalytic activity could be totally eliminated.[9]

However, few studies have been conducted to address the biodiesel production catalyzed by the combined lipases of Novozym 435 and Lipozyme TLIM in *tert*-butanol medium, and its kinetic model study is rarely reported so far. Hence, the main purposes of this work are: (1) to optimize the crucial variables using response surface methodology (RSM). The tested variables are amount of the combined lipases, weight ratio of Novozym 435 to Lipozyme TLIM, amount of *tert*-butanol, molar ratio of oil to methanol and reaction temperature, (2) to evaluate the commercial viability of the process through investigating the stability of the combined lipases of Novozym 435 and Lipozyme TLIM, and (3) to propose a kinetic model on the transesterification for biodiesel production catalyzed by combined lipases in *tert*-butanol medium. The raw material chosen was WCO considered as inexpensive feed stock, which will be, therefore, expected to help the biodiesel to be competitive in price as compared with the use of raw materials from food grade vegetable oils.

Materials and Methods

Materials

WCO was obtained locally. Acid value of WCO was determined according to American Organization of Analytical Chemists (AOAC) International Method Cd 3a– 63. Percentage of free fatty acids (FFAs) was calculated using oleic acid as a factor. The density of the sample was determined at 25°C by using Anton paar DMA 4500 density meter. The saponification value was determined according to Malaysian Palm Oil Board (MPOB) official test method in 2004. The aforementioned methods were detailed by Akbar et al.[10] The tested physicochemical properties of WCO were: acid value (AV) 84.15 ± 0.15 mg-KOH/g-oil, saponification value (SV) 176.96 ± 0.22 mg KOH/g oil, density (25°C) 0.90 ± 0.01 g/cm^3, and free fatty acid (FFA) 42.08 ± 0.07%.

Novozym 435 and Lipozyme TLIM were purchased from Nov Norvisk (Bagsvaerd, Denmark). The standards of methyl esters with the chromatographic purities of 99.9% were employed to purchase from Sigma-Aldrich (St. Louis, Missouri, USA), such as methyl myristate, methyl palmitate, methyl stearate, methyl heptadecanoate, methyl oleate, methyl linoleate, and methyl linolenate. N-hexane, methanol, and *tert*-butanol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and are with analytic grade.

Experimental design and optimization

The preliminary single factor experiments indicated that the amount of combined lipases of Novozym 435 and Lipozyme TLIM lipases, their weight ratio, molar ratio of methanol to oil, amount of *tert*-butanol and reaction temperature were the significant five variables for biodiesel production catalyzed by combined lipases from WCO. Therefore, RSM was used to optimize the aforementioned parameters. A five-level-five-factor center composite rotation design (CCRD) with 10 star points, including 10 replicates at the center point leading to 36 trials, was employed to fit a second-order response surface. The general form of the second-order polynomial equation is presented in Eq. 1

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum\sum \beta_{ij} X_i X_j \tag{1}$$

where Y is biodiesel yield (dependent variables). β_0 is constant coefficient. β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction term, respectively. X_i and X_j stand for the variables (independent variables). Subscript i and j are the number from 1 to 5.

The fitting degree of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Quadratic polynomial equations were attained by holding one of the independent variances at a constant value and changing the level of the other variables.

The coded and uncoded (actual) levels of the five variables were listed in Table 1.

Kinetic model

Ping Pong Bi Bi model has been extensively used to demonstrate the kinetic mechanism of transesterification for biodiesel production catalyzed by enzyme.[11,12] In this study, the proposed kinetic mechanism was based on the decrease of substrate concentration (including the oil substrate (C_A), and the methanol (C_B)), and the reaction rate presented in the following Eq. 2 was derived

$$r_A = kC_A^{\alpha} C_B^{\beta} \tag{2}$$

where r_A is the reaction rate of WCO, k is reaction rate constant, C_A is the concentration of WCO feedstock, mol/L, α is the reaction order of WCO, C_B is the concentration of methanol, mol/L, and β is the reaction order of methanol.

kC_B^{β} is constant due to the excessive presence of methanol in the reaction, therefore, Eq. 2 is simplified as Eq. 3

$$r_A = k_1 C_A^{\alpha} \tag{3}$$

where

$$k_1 = kC_B^{\beta} \tag{4}$$

It is assumed that x is the biodiesel yield from WCO, and the initial concentration of WCO is C_{A0}, the concentration (C_A) of WCO at any specific time is derived in Eq. 5

Table 1. Coded and Actual Levels of Five Variables

Variable	Coded level of variable				
	−2	−1	0	1	2
Amount of combined lipases (%), X_1	2	3	4	5	6
Weight ratio of Novozym 435 to Lipozyme TLIM (w/w), X_2	20/80	35/65	50/50	65/35	80/20
Molar ratio of methanol to oil, X_3	3:1	6:1	9:1	12:1	15:1
Amount *of tert*-butanol (%, v/v), X_4	20	35	50	65	80
Reaction temperature (°C), X_5	30	40	50	60	70

(Conditions: feedstock oil 2 g, reaction time 10 h).

$$C_A = C_{A0}(1 - x) \tag{5}$$

Therefore

$$r_A = -\frac{d\,C_A}{dt} = \frac{d(C_{A0}(1-x))}{dt} = C_{A0}\frac{dx}{dt} = k_1 C_A^{\alpha} \tag{6}$$

Combining Eq. 5 with Eq. 6, Eq. 7 can be obtained

$$\frac{dx}{dt} = \frac{k_1}{C_{A0}} C_A^{\alpha} = \frac{k_1}{C_{A0}}[C_{A0}(1-x)]^{\alpha} = k_2[C_{A0}(1-x)]^{\alpha} \tag{7}$$

where

$$k_2 = \frac{k_1}{C_{A0}} \tag{8}$$

Using integral method, Eq. 9 was derived from Eq. 7

$$\ln\frac{dx}{dt} = \alpha\ln[C_{A0}(1-x)] + \ln k_2 \tag{9}$$

Equations 9 and 7, the kinetic parameters, k_1, k_2 and α were calculated by the linear regression using the software OriginPro 7.0 (product of Origin Lab, USA).

Gas chromatography (GC) analysis for biodiesel

The method of determining fatty acid methyl esters (FAMEs) was detailed in the previous work.[13] A GC-9790 gas chromatograph (Fuli Analytical Instrument Co., Ltd., China) equipped with an INNOWAX capillary column (30 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA), and a flame ionizing detector (FID) was used. Helium was used as a carrier gas. Temperature programming was performed. The column temperature was kept at 180°C for 2 min, heated to 230°C at 3°C/min, and then maintained at 230°C for 1 min. The temperatures of the injector and detector were set at 230 and 280°C, respectively.

Methyl heptadecanoate was served as the internal standard for GC and dissolvent in n-hexane solution with a concentration of 2 mg/mL. The retention times of standard chemicals methyl laurate, methyl palmitate, methyl heptadecanoate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate were 5.5, 11.6, 13.1, 15.8, 16.2, 17.2 and 18.8 min, respectively. After transesterification reaction finished, aliquots of 100 μL samples were withdrawn from the reaction mixture and centrifuged at 10,000 rpm for 1 min. 5 μL of supernatant was sampled and diluted with 295 μL n-hexane hexane, and then 300 μL methyl heptadecanoate was added as the internal standard solution. The final volume of the samples was 600 μL. GC analyses were done by injecting 1 μL sample into the column. Biodiesel yield was defined as the produced FAMEs amount divided by the initial amount of oil (g/g), and calculated with Eq. 10

$$\text{Biodiesel yield }(\%) = \frac{A_{\text{sample}} \times f_0}{A_{\text{int}\,ernal} \times W_{\text{int}\,ernal}} \qquad f_0 = \frac{W_{\text{sample}} \times A_{\text{int}\,ernal}}{W_{\text{int}\,ernal} \times A_{\text{sample}}} \tag{10}$$

where A_{sample} is the peak area of FAMEs in sample f_0 is the response factor, $A_{internal}$ is the peak area of the internal standard, $W_{internal}$ is the weight of the internal standard g, W_{sample} is the weight of the sample g.

Table 2. Central Composite Rotation Design and Experimental Data

Trials	X_1	X_2	X_3	X_4	X_5	Biodiesel yield (Y, %)
1	−1	−1	−1	−1	1	64.5 ± 0.05
2	−1	−1	−1	1	−1	67.2 ± 0.01
3	−1	−1	1	−1	−1	48.8 ±0.07
4	−1	−1	1	1	1	63.8 ± 0.04
5	−1	1	−1	−1	−1	71.6 ± 0.08
6	−1	1	−1	1	1	76.9 ± 0.02
7	−1	1	1	−1	1	53.8 ± 0.02
8	−1	1	1	1	−1	61.1 ± 0.02
9	1	−1	−1	−1	−1	68.9 ± 0.05
10	1	−1	−1	1	1	80.1 ± 0.06
11	1	−1	1	−1	1	58.1 ± 0.03
12	1	−1	1	1	−1	64.8 ± 0.07
13	1	1	−1	−1	1	62.4 ± 0.05
14	1	1	−1	1	−1	72.5 ± 0.05
15	1	1	1	−1	−1	53.7 ± 0.07
16	1	1	1	1	1	69.0 ± 0.03
17	−2	0	0	0	0	65.9 ± 0.04
18	2	0	0	0	0	64.2 ± 0.04
19	0	−2	0	0	0	65.7 ± 0.03
20	0	2	0	0	0	69.9 ± 0.03
21	0	0	−2	0	0	53.5 ± 0.05
22	0	0	2	0	0	50.5 ± 0.03
23	0	0	0	−2	0	65.3 ± 0.02
24	0	0	0	2	0	79.5 ± 0.04
25	0	0	0	0	−2	68.2 ± 0.07
26	0	0	0	0	2	74.5 ± 0.07
27	0	0	0	0	0	80.7 ± 0.11
28	0	0	0	0	0	79.4 ± 0.02
29	0	0	0	0	0	81.1 ± 0.13
30	0	0	0	0	0	81.0 ± 0.05
31	0	0	0	0	0	80.5 ± 0.04
32	0	0	0	0	0	83.5 ± 0.12
33	0	0	0	0	0	82.1 ± 0.20
34	0	0	0	0	0	78.9 ± 0.06
35	0	0	0	0	0	79.4 ± 0.10
36	0	0	0	0	0	82.8 ± 0.09

Table 3. Analysis of Variance (ANOVA) for Regression Equation

Sources	D.F.	Sum of squares	Mean of square	*F*-value	Possibility (*P*-value)
Model	20	3373.05	168.65	11.78	0.0001**
(Linear)	5	891.19	178.24	12.45	0.0001**
(Quadratic)	5	2302.25	460.45	32.17	0.0001**
(Cross Product)	10	179.61	17.96	1.25	0.33477
Residual error	15	214.69	14.31		
(Lack of fit)	6	194.14	32.36	14.18	0.38
(Pure error)	9	20.54	2.28		
R-square		94.02%			
Adj. R-square		86.04%			

**Significant at 1% level.
D.F.: degree of freedom.
Sum of squares: the sum of squared predicted values in a standard regression model.
Mean of squares: the average of squared predicted values in a standard regression model.

Statistics analysis

All trials were carried out in triplicate and the data were analyzed by the software SAS 9.0 (product of SAS Institute, Inc., Cary, NC, USA).

Results and Discussion

RSM model fitting

As stated earlier, the crucial parameters (amount of the combined lipases of Novozym 435 and Lipozyme TLIM, weight ratio of Novozym 435 to Lipozyme TLIM, molar ratio of methanol to oil, volume amount of *tert*-butanol and reaction temperature) affecting biodiesel yield were optimized through RSM. The statistical combination of the independent variables in coded and actual values along with the experimental response (biodiesel yield, %) was presented in Table 2.

In order to determine whether or not the quadratic model was significant, it was necessary to conduct ANOVA analysis (Table 3), in which the regression sum of square was subdivided into two parts that attributed to linear regression and quadratic model, respectively.

The *P*-value was used as the tool to check the significance of each coefficient, which also indicates the interaction strength of each parameter. The smaller the *P*-value is, the bigger the significance of the regression coefficient is. In other words, the higher the *F*-value for the model ($F_{\text{model}} > 5$), the lower probability the value for the model ($P < 0.005$), which also indicates the bigger significance of the fitted model. In this study, the *P*-value of the model is $P \leq 0.001$, suggesting that the model is suitable for the experimental prediction of biodiesel yield. The *P*-value of "lack of fit" is 0.38 ($P < 0.01$), indicating that "lack of fit" is insignificant relative to the pure error. The coefficient of determination ($R^2 = 94.02\%$) implies that the accuracy and general availability of the polynomial model were adequate.

The regression coefficients and the corresponding *P*-values are shown in Table 4. From the *P*-value of each term, it is concluded that the regression coefficients of the linear term X_3, X_4, and the quadratic term X_1*X_1, X_2*X_2, X_3*X_3, X_4*X_4 and X_5*X_5, have a significant effect on biodiesel yield. Among them, X_4*X_4 and X_5*X_5 are significant at 5% level, while the others are significant at 1% level.

Using the designed experimental data (Table 2), the polynomial model for predicting the biodiesel yield is regressed and shown in the following (in terms of coded factors)

$$\begin{aligned} Y = {} & 80.62 + 0.77 * X_1 + 0.55 * X_2 - 4.04 * X_3 + 4.25 * X_4 \\ & + 1.36 * X_5 - 3.49 * X_1 * X_1 - 2.09 * X_1 * X_2 \\ & + 0.9 * X_1 * X_3 + 0.81 * X_1 * X_4 - 0.04 * X_1 * X_5 \\ & - 2.80 * X_2 * X_2 - 0.04 * X_2 * X_3 + 0.15 * X_2 * X_4 \\ & - 0.85 * X_2 * X_5 - 6.75 * X_3 * X_3 + 0.94 * X_3 * X_4 \\ & + 0.79 * X_3 * X_5 - 1.65 * X_4 * X_4 + 1.78 * X_4 * X_5 \\ & - 1.91 * X_5 * X_5 \end{aligned} \tag{11}$$

Many parameters can influence the biodiesel yield from WCO. Equation 11 shows that biodiesel yield had a complex relationship with independent variables that encompassed both first- and second-order polynomials, and had more than one maximum point. The best way to express the effect of one parameter on the biodiesel yield in the experimental

Table 4. Results of Regression Analysis of a Full Second-Order Polynomial Model

Term	Coefficients estimated	Pr > |t|
Intercept	80.62	
X_1	0.77	0.3366
X_2	0.55	0.4873
X_3	− 4.04	0.0001**
X_4	4.25	0.0001**
X_5	1.36	0.0990131
X_1*X_1	− 3.49	0.0001**
X_1*X_2	− 2.09	0.0433
X_1*X_3	0.90	0.3564
X_1*X_4	0.81	0.4038
X_1*X_5	− 0.04	0.9689
X_2*X_2	− 2.80	0.0007*
X_2*X_3	− 0.04	0.9689
X_2*X_4	0.15	0.8761
X_2*X_5	− 0.85	0.3830
X_3*X_3	− 6.75	0.0001**
X_3*X_4	0.94	0.3373
X_3*X_5	0.79	0.4181
X_4*X_4	− 1.65	0.0261*
X_4*X_5	1.78	0.0801
X_5*X_5	− 1.91	0.0119*

*Significant at 5% level.
**Significant at 1% level.

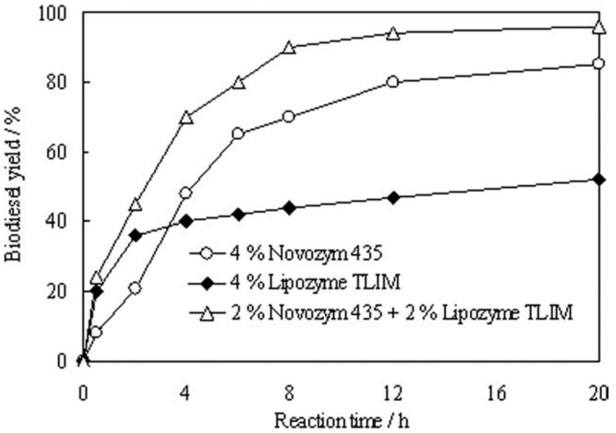

Figure 1. The positive synergistic effect of combined lipases for biodiesel production.

(Conditions: soybean oil 2 g, amount of combined lipases 4 wt % (based on oil weight), weight ratio of Novozym 435 to Lipozyme TLIM 1:1 (w/w), reaction temperature 50°C, molar ratio of methanol to oil 5:1, and amount of *tert*-butanol 50% (based on oil volume, v/v)).

range is to generate response surface plots of the equation (contour plot and response surface curve are not shown). According to the obtained contour plots, the significance of the mutual interactions between the independent variables could be estimated. The optimum conditions were: amount of the combined lipases 4 wt % (based on oil weight), weight ratio of Novozym 435 to Lipozyme TLIM 1:1 (w/w), temperature 50°C, molar ratio of methanol to oil 9:1, and amount of *tert*-butanol 50% (based on oil volume, v/v). Under the optimized conditions, biodiesel yield could be achieved up to 83.5% after reaction10 h. It was demonstrated that in the case of feedstock with high FFA content, the combined lipases of Novozym 435 and Lipozyme TLIM (1:1, w/w) presented higher catalytic activity in comparison with the single lipase Novozym 435 reported by Lai et al.[14] who demonstrated that only 68% biodiesel yield from rice bran oil with 54% FFA content was obtained under the optimum conditions of Novozym 435 dosage 5 wt % (based on oil weight), oil to methanol molar ratio 1:10, reaction temperature 50°C, reaction time 4 h, and *tert*-butanol amount 50% (based on oil volume, v/v). The probable reason is that the combined lipases of Novozym 435 and Lipozyme TLIM present positive synergistic effect resulting in high-conversion rate of biodiesel. As known, the enzymatic transesterification process for biodiesel production involves three steps, i.e., from triglyceride (TG) to diglyceride (DG), from diglyceride to monoglyceride (MG), and from monoglyceride to glycerol (GL). In our preliminary experiments (see Figure 1), for transesterification process of Novozym 435 single lipase, it was demonstrated that the reaction step from MG to GL was faster than the steps from TG to DG, and DG to MG, and the slowest step from DG to MG was the rate-limiting step. While for Lipozyme TLIM single lipase, the rate-limiting step was the step from TG to DG, which indicated that combined lipases of Novozym 435 and Lipozyme TLIM could eliminate the rate-limiting step in biodiesel production. Therefore, combined lipase can enhance the biodiesel yield and increase the reaction rate.

Thus, it is concluded that the combined lipases transesterification process is better for biodiesel production, which not only enhances the biodiesel yield, due to positive synergistic effect of combined lipases of Novozym 435 and Lipozyme TLIM, but also reduces the total costs of the enzyme since half of the dosage of Novozym 435 (the price is $9,500 per kilogram) is replaced by cheap Lipozyme TLIM (the price is $800 per kilogram).

Validation of the model

The model was processed by partial differential coefficient, and ranged from 0.81% to 1.36%. The point of curved surface was determined to be the center point. Under the optimized conditions, three experiments were conducted to validate the model obtained from RSM under the conditions of the amount of combined lipases 4 wt % (based on oil weight), weight ratio of Novozym 435 to Lipozyme TLIM 1:1 (w/w) reaction temperature 50°C, molar ratio of methanol to oil 9:1, and amount of *tert*-butanol 50% (based on oil volume, v/v). The validation results are from 81.9% to 83.7%, which is in good agreement with the predicted value (81.4%). Therefore, the model is considered to be accurate and reliable for predicting the biodiesel yield from WCO using combined lipases as biocatalyst.

The stability of the combined lipases

The operational stability of an immobilized enzyme without appreciable loss of enzyme activity is very important for the economic viability of a biosynthetic process. The combined lipases of Novozym 435 and Lipozyme TLIM in *tert*-butanol medium were used repeatedly, and high stability was observed (Figure 2).

The combined lipases could maintain 85% of their initial activity after 30 cycles successively (300 h). The probable reason is that *tert*-butanol can dissolve methanol and the byproduct glycerol, and then eliminated the negative effects of methanol and byproduct glycerol on lipase activity. The result was in good agreement with those reported in literature.[7,9,15] It is concluded that the combined lipases Novozym 435 and Lipozyme TLIM can be used at least 30 cycles (300 h) without obvious loss of activity. The transesterification catalyzed by the combined lipases is applicable not only

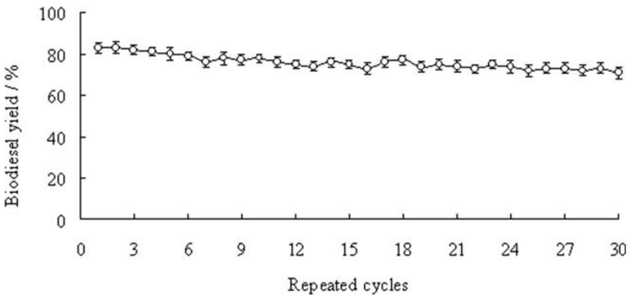

Figure 2. The operation stability of combined lipases.

(Conditions: oil 2 g, amount of combined lipases 4 wt % (based on oil weight), weight ratio of Novozym 435 to Lipozyme TLIM 1:1 (w/w), reaction temperature 50°C, reaction time 10 h, molar ratio of methanol to oil 9:1, and amount of *tert*-butanol 50% (based on oil volume, v/v)).

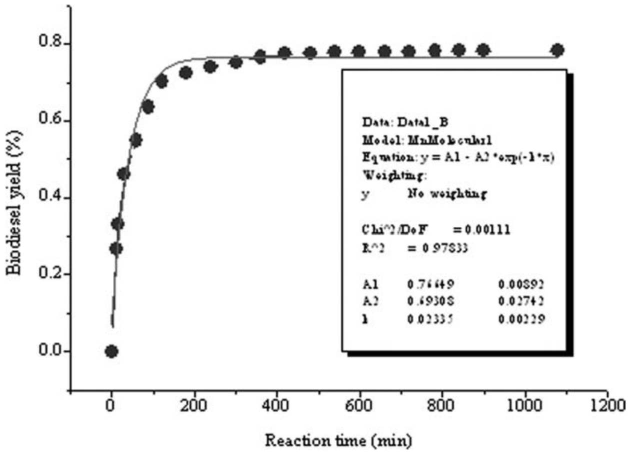

Figure 3. The biodiesel yield at 50°C as the function of reaction time.

(Conditions: oil 2 g, amount of combined lipases 4 wt % (based on oil weight), weight ratio of Novozym 435 to Lipozyme TLIM 1:1 (w/w), reaction temperature 50°C, molar ratio of methanol to oil 9:1, and amount of *tert*-butanol of 50% (based on oil volume, v/v)).

to the batch reaction, but also to the continuous reaction and different reactor configurations.

Kinetic model

The reaction kinetics of the transesterification of WCO with methanol catalyzed by the combined lipases of Novozym 435 and Lipozyme TLIM in *tert*-butanol medium was also investigated. The biodiesel yield was expressed by the concentration decrease of the substrates under the conditions of oil 2 g, the amount of the combined lipases 4 wt % (based on oil weight), weight ratio of Novozym 435 to Lipozyme TLIM 1:1 (w/w)molar ratio of methanol to oil 9:1, and amount of *tert*-butanol 50% (based on oil volume, v/v) at the reaction temperatures of 30, 40, and 50°C, respectively. In this reaction system, the initial concentration of WCO was 0.28 mol/L. The biodiesel yield at 50°C (data at 30 and 40°C, not shown) was depicted as the function of reaction time in Figure 3.

Figure 3 shows that the biodiesel yield increases rapidly within the first 3 h, and slightly increases during the reaction period from 3 h to 10 h. Beyond 10 h, the biodiesel yield keeps relatively stable and shows no significant increment. The possible reason is that the reaction equilibrium is achieved within 10 h. A nonlinear equation is obtained from the experimental data (Figure 2) using OriginPro 7.0 and given in Eq. 12

$$x = 0.76649 - 0.69308\,\mathrm{e}^{-0.02335\mathrm{t}} \tag{12}$$

where x is biodiesel yield, %; t is reaction time, min.

At 50°C, from Eq. 12, it could be obtained

$$\frac{dx}{dt} = 0.016183\,\mathrm{e}^{-0.02335\mathrm{t}} \tag{13}$$

It was easily deduced that the relationship between $\ln\frac{dx}{dt}$ and $\ln[C_{A0}\ (1 - x)]$ was a linearity and shown in Figure 4.

Through OriginPro.7.0, a linear equation (Eq. 14) can be obtained at 50°C

$$\ln\frac{dx}{dt} = 0.98678\ln[C_{A0}(1 - x)] + 4.0556, R^2 = 0.9795 \tag{14}$$

Hence, the reaction kinetic constant at 50°C was obtained in comparison with Eqs. 9 and 14. The reaction rate order (α) equals 0.98678, indicating that the reaction rate order is a pseudo-first-order in this work, and the reaction rate constants of k_1 and k_2 are 11.5440 min^{-1} and 57.7198 L/(mol · min), respectively.

Similarly, the reaction kinetic constants of k_1 and k_2 at 30°C are 3.2489 min^{-1} and 16.2445 L/(mol · min), respectively. The reaction kinetic constants of k_1 and k_2 at 40°C are 7.2156 min^{-1} and 36.0782 L/(mol · min), respectively.

According to the Arrhenius equation (Eq. 15)

$$k_1 = A\exp\left(\frac{-E_\alpha}{RT}\right) \tag{15}$$

where k_1 is the reaction rate constant min^{-1}, A is the pre-exponential factor related to the state of the reaction, E_α is the activation energy, R is the gas law constant J/(mol · K), and T is temperature K.

In order to get the values of E_α and A, a fitting straight line was obtained to fit the experimental data at 30°C, 40°C, and 50°C. The straight line fitting the Arrhenius equation was presented in Figure 4, and the linear equation was obtained as Eq. 16

$$\ln k_1 = -\frac{E_a}{R} \times \frac{1}{T} + \ln A \tag{16}$$

According to Figure 5, a linear equation (Eq. 17) was obtained through OriginPro. 7.0

$$\ln k_1 = (-6219.4) \times \frac{1}{T} + 21.751, R^2 = 0.9832 \tag{17}$$

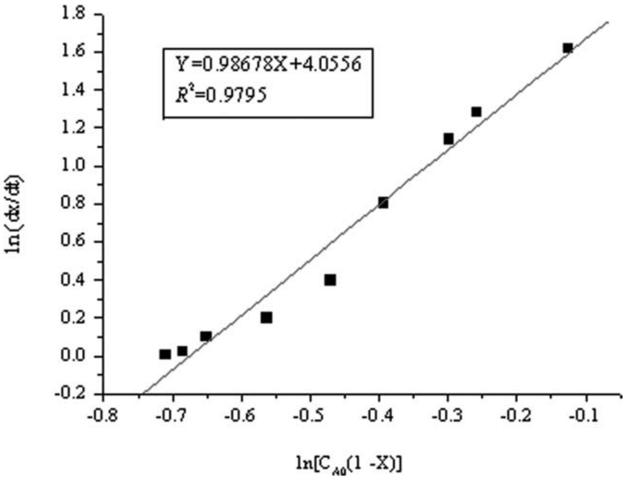

Figure 4. The linear relationship between $\ln\frac{dx}{dt}$ and $\ln[C_{A0}\ (1 - x)]$ at 50°C.

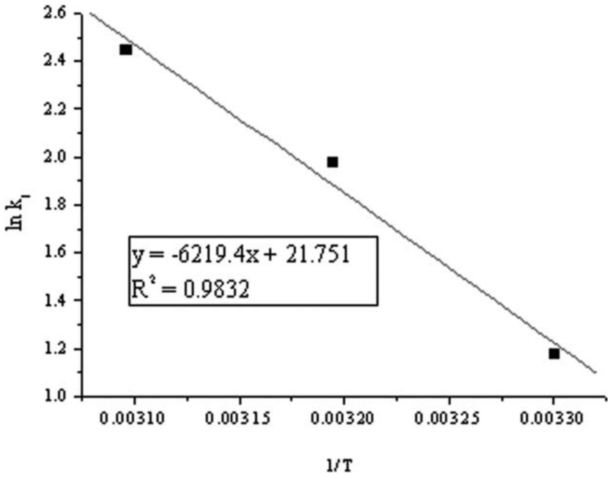

Figure 5. The linear relationship between lnk_1 and 1/T at 30°C, 40 and 50°C.

By comparing Eqs. 16 and 17, activation energy E_α and pre-exponential factor A were estimated to be 51.71 kJ/mol and 2.8×10^9, respectively.

Therefore, the reaction kinetic equation was obtained as Eq. 18 for biodiesel production from WCO catalyzed by the combined lipases of Novozym 435 and Lipozyme TLIM in *tert*-butanol medium

$$r_A = 2.8 \times 10^9\, e^{-\frac{51.71}{RT}} C_A \qquad (18)$$

where r_A is the reaction rate of WCO, mol/(L · min), R is the gas law constant, J/(mol · K), T is temperature K, C_A is the concentration of WCO, mol/L.

Conclusions

Biodiesel obtained catalyzed by combined lipases from WCO has been considered a promising option. WCO is much cheaper for producing biodiesel in comparison with fresh vegetable oil. In this study, the crucial variables affecting biodiesel production catalyzed by the combined lipases of Novozym 435 and Lipozyme TLIM in *tert*-butanol were optimized by RSM. The combined biocatalysts exhibit high-positive synergic effect and excellent operation stability, and its activity maintains 85% of initial activity after repeated 30 cycles (300 h). The kinetic model has been proposed by the decrease of substrate concentration, and the values of the reaction constants in the kinetic model equation are determined from experimental data.

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Notation

r_A = the reaction rate of WCO
k = reaction rate constant
C_{A0} = initial concentration of WCO, mol/L
C_A = concentration of WCO at specific reaction time, mol/L
α = the reaction order of WCO
C_B = the concentration of methanol, mol/L
β = the reaction order of methanol
x = biodiesel yield, %
$W_{s \tan dard}$ = weight of the internal standard, g
A_{FAME} = peak area of FAMEs
f_0 = response factor
$A_{s \tan dard}$ = peak area of the internal standard
W_{sample} = weight of the sample, g
t = reaction time, min
A = pre-exponential factor
E_α = activation energy, kJ/mol
R = gas-law constant, J/(mol · K)
T = temperature, K

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