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Liquid Lipase-Catalyzed Esterification of Oleic Acid with Methanol for Biodiesel Production in the Presence of Superabsorbent Polymer: Optimization by Using Response Surface Methodology

Hoang Chinh Nguyen ^{1,†} , Dinh Thi My Huong ^{2,3,†}, Horng-Yi Juan ^{3,†}, Chia-Hung Su ^{3,*} and Chien-Chung Chien ³

¹ Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City 700000, Vietnam; nguyenhoangchinh@tdt.edu.vn

² Faculty of Chemical Engineering, University of Technology and Education—The University of Danang, Danang City 550000, Vietnam; myhuongdinh@gmail.com

³ Department of Chemical Engineering, Ming Chi University of Technology, New Taipei City 24301, Taiwan; hyjuan@mail.mcut.edu.tw (H.-Y.J.); m06138117@o365.mcut.edu.tw (C.-C.C.)

* Correspondence: chsu@mail.mcut.edu.tw; Tel.: +88-622-908-9899 (ext. 4665)

† These authors contributed equally to this work.

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Abstract: Liquid lipase-catalyzed esterification of fatty acids with methanol is a promising process for biodiesel production. However, water by-product from this process favors the reverse reaction, thus reducing the reaction yield. To address this, superabsorbent polymer (SAP) was used as a water-removal agent in the esterification in this study. SAP significantly enhanced the conversion yield compared with the reaction without SAP. The lipase-catalyzed esterification in the presence of SAP was then optimized by response surface methodology to maximize the reaction conversion. A maximum conversion of 96.73% was obtained at a temperature of 35.25 °C, methanol to oleic acid molar ratio of 3.44:1, SAP loading of 10.55%, and enzyme loading of 11.98%. Under these conditions, the Eversa Transform lipase could only be reused once. This study suggests that the liquid lipase-catalyzed esterification of fatty acids using SAP as a water-removal agent is an efficient process for producing biodiesel.

Keywords: biodiesel; esterification; liquid lipase; superabsorbent polymer; response surface methodology

1. Introduction

Extensive energy consumption and environmental pollution have stimulated the development of renewable energy sources. Biodiesel, a renewable fuel derived from vegetable oil, is increasingly considered a promising alternative to petrodiesel because of its superior combustion properties, compatibility with diesel engines, and environmental benefits [1–4]. Therefore, biodiesel is being produced globally to reduce the consumption of petrodiesel.

Biodiesel is commonly produced from edible feedstocks such as soybean, sunflower, and rapeseed oils [5–8]; however, the use of these feedstocks for biodiesel production is restricted because of their high cost (which accounts for 75% of the production cost) and competition with demand for the food supply [9–11]. Therefore, inedible and waste oils have been developed as potential feedstocks for biodiesel production [12–15]. These inedible and waste oils usually contain a high level of free fatty acids, which must be esterified into biodiesel before the transesterification [16,17]. In recent years, the esterification of fatty acids for biodiesel production has been widely investigated [18–20].

The common method is acid-catalyzed esterification [16,17,21]. Although biodiesel is successfully produced from fatty acids through acid catalysis, this process retains several drawbacks such as equipment corrosion and negative environmental effects [10,22,23]. Esterification using lipase as a biocatalyst is considered a promising alternative for biodiesel production, because this method is ecofriendly and proceeds at mild reaction conditions, thus reducing the energy consumption and adverse environmental effects [10,24,25]. To improve the stability and reusability of lipase, immobilized lipases have been developed and used for the reaction [10,14,24]. However, the rate of the reaction catalyzed by immobilized lipase is relatively low because of the mass transfer limitation between the enzyme and substrate [26,27]. Moreover, the high cost of immobilized lipase is the main drawback that limits its industrial application [28].

Liquid lipase formulations have increasingly attracted attention as a promising alternative to immobilized lipase for industrial applications because of their low cost (30- to 50-fold lower than that of immobilized lipase) and high catalytic activity [29–31]. Recent studies have shown that liquid lipases can be used for biodiesel production with high yield [26,29,32,33]. However, high water content (from the feedstock and produced from the esterification of fatty acid and methanol) favors the reverse reaction, thus lowering the reaction rate and production yield [32,34]. Efforts have been made to remove the water from the reaction mixture, including the use of a molecular sieve, alumina, or silica gel as adsorbents [35–37]. Although these adsorbents efficiently remove water from the reaction solution, they cannot prevent the inactivation of lipase caused by water [37]. Superabsorbent polymer (SAP) has been widely used for soil water conservation, sewage treatment, mineral dewatering, and drug drying [37,38]. SAP demonstrates rapid water absorption and high water retention capacity [37] and has been employed to remove water formed during the transesterification of corn oil and dimethyl carbonate [37]. However, no report has mentioned the use of SAP as a water-removal agent for the esterification process; this is an attractive research direction.

This study examined the potential use of SAP as a water-removal agent in the esterification of fatty acid with methanol when using liquid lipase for biodiesel production. Oleic acid was used as a model substrate, because it is one of the most common fatty acids in plant oils and animal fats [34]. Response surface methodology (RSM) was employed to analyze the effects of reaction conditions (temperature, reaction time, SAP loading, and enzyme loading) on the reaction conversion. Liquid lipase was also studied for its reusability.

2. Materials and Methods

2.1. Materials

Eversa Transform lipase (liquid lipase produced by *Thermomyces lanuginosus*) with activity of 100,000 PLU/g was obtained from Novozymes A/S (Bagsvaerd, Denmark). The SAP was provided by Formosa Plastic Corp. (Kaohsiung, Taiwan). The SAP used in this study is mainly produced from sodium polyacrylate and its properties are absorption capacity (0.9% NaCl) of 60 g/g, centrifuge retention capacity (0.9% NaCl) of 38 g/g, and particle size distribution of 470 μm . Oleic acid (99%) was provided by Showa Chemical Industry Co., Ltd. (Tokyo, Japan). Methanol, ethanol, and other reagents were analytical grade and obtained from Echo Chemical Co. Ltd. (Miaoli, Taiwan).

2.2. Effect of SAP on the Esterification

A comparative study conducted lipase-catalyzed esterification of oleic acid with methanol with and without the presence of SAP (5%, *w/w*) to investigate the effects of SAP on reaction conversion. The reaction was initiated by adding 10% Eversa Transform lipase into reaction mixtures containing methanol and oleic acid at a molar ratio of 3:1 and various amounts of water (0–30%, *w/w*). The reaction was subsequently kept at 35 °C with stirring for 150 min. The sample was regularly withdrawn for determination of the reaction conversion.

The amount of oleic acid during the esterification was determined using a previously reported procedure [39]. A sample was withdrawn from the reaction mixture, weighed, and dissolved in a 20-mL ethanol–diethyl ether solution (1:1, *v/v*). The sample was subsequently titrated against 0.1 M KOH using phenolphthalein as the indicator to determine the acid value (AV). The reaction conversion was then calculated as follows [40]:

$$\text{Reaction conversion (\%)} = \frac{AV_1 - AV_2}{AV_1} \times 100 \quad (1)$$

where AV_1 is the initial acid value, and AV_2 is the acid value after esterification.

2.3. Optimization of Esterification Using RSM

A four-level and four-factorial central composite design was used to study the effects of reaction factors on the reaction conversion. Esterifications with different reaction temperatures (30–50 °C), methanol:oleic acid molar ratios (1:1–9:1), SAP loadings (5–15%), and enzyme loadings (5–15%) were performed in 100-mL screw-cap glass bottles with stirring for 150 min. After the reaction, a sample was withdrawn from the reaction mixture to determine the reaction conversion. A quadratic equation was then used to establish the relationship between the determined reaction conversion and reaction factors:

$$\begin{aligned} Y = & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 \\ & + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 \\ & + \beta_{34} X_3 X_4 \end{aligned} \quad (2)$$

where Y is the reaction conversion; X_1 is the reaction temperature; X_2 is the molar ratio of methanol to oleic acid; X_3 is the SAP loading; X_4 is the enzyme loading; β_0 is the regression coefficient for the intercept term; β_1 – β_4 are linear parameters; β_{12} , β_{13} , β_{14} , β_{23} , β_{24} , and β_{34} are interaction parameters; and β_{11} , β_{22} , β_{33} , and β_{44} are quadratic parameters. These parameters were determined using the least-squares method [41], and an empirical model was subsequently employed to determine the optimal reaction conditions for maximizing reaction conversion [41]. Minitab 16 (Minitab Inc., State College, PA, USA) was employed to develop the empirical model, perform analysis of variance (ANOVA), and determine the optimal reaction conditions.

2.4. Enzyme Reuse

Eversa Transform lipase was reused in the esterification in the presence of SAP. The reaction was conducted under the optimal conditions determined through RSM. After the reaction was completed, the reaction mixture was centrifuged for phase separation. The oil phase was collected for the reaction conversion determination, and the water phase containing liquid lipase was subsequently remixed with fresh reactants and SAP to start a new reaction.

3. Results and Discussion

3.1. Effect of SAP on the Reaction Conversion

This study compared liquid lipase-catalyzed esterification of oleic acid with methanol in various water contents with and without the presence of SAP. As shown in Figure 1, the conversion of the reaction without SAP increased when the amount of water increased. This indicated that a certain amount of water is required for the activity of liquid lipase. Nevertheless, a further increase in water content caused a significant decrease in the conversion of the reaction without SAP. This is attributed to high water content driving the reaction equilibrium to the reverse reaction [35,36,42] and decreasing the activity of liquid lipase [32,35,37]. This result agrees with those of other studies [29,32,37]. Studies have reported that a minimal amount of water is required for optimal enzyme activity, but excess water adversely affects enzyme activity and stability, thus reducing the reaction conversion [32,35,37].

To overcome this obstacle, SAP was used as a water-removal agent for the reaction. The results showed that the conversion of the reaction with SAP increased and reached the highest conversion when increasing the water content from 0% to 5%. Remarkably, higher water contents resulted in no loss in reaction conversion, indicating that excess water had no negative effect on the conversion of the reaction with SAP. This is attributed to the efficient water absorption of the SAP that reduced the negative effects caused by water [37]. In biodiesel production, the feedstock always contains various amount of water; the presence of water in feedstock is the main concern, because it can reduce the conversion yield [32,43,44]. The feedstock is thus treated to remove water before being used for the reaction [44–46]. However, based on the results of this study, the water-removal can be eliminated by adding SAP directly into the reaction solution. Therefore, the liquid lipase-catalyzed esterification using SAP as a water-removal agent is a promising process for biodiesel synthesis from feedstock containing high water content.

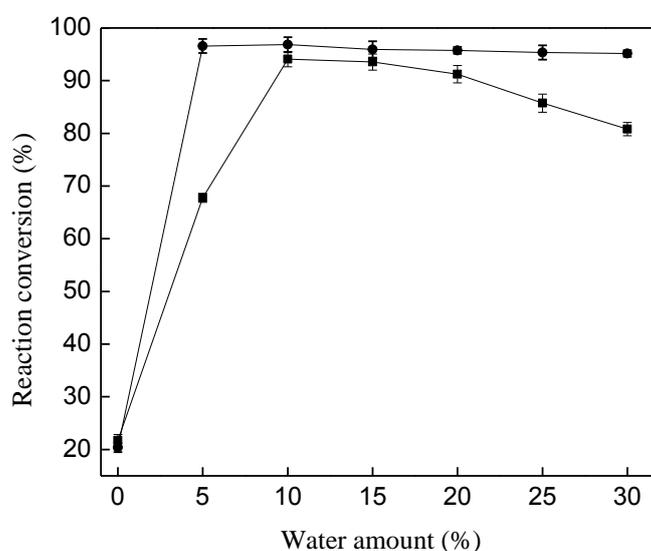


Figure 1. Effects of SAP on the esterification with varied water content. The reaction was conducted under the following conditions: molar ratio of methanol to oleic acid of 3:1, a temperature of 35 °C, enzyme loading of 10%, in the presence of 5% SAP (●), or without SAP (■).

3.2. The RSM Model Development

A central composite RSM model was employed to establish the relationships between reaction conversion (measured response) and reaction factors (input variables)—temperature, molar ratio of methanol to oleic acid, SAP loading, and enzyme loading. Table 1 shows the input variables with their coded and un-coded values. Table 2 illustrates the experimental design for obtaining the optimal reaction conditions. Based on the results shown in Table 2, the measured responses and input variables in term of code values were input into an empirical model as the following quadratic polynomial equation:

$$\begin{aligned}
 Y = & 89.5 - 6.6X_1 - 6.39X_2 + 1.26X_3 + 5.9X_4 - 3.77X_1^2 - 4.53X_2^2 - 3.45X_3^2 - 3.75X_4^2 \\
 & + 0.86X_1X_2 + 1.21X_1X_3 - 0.26X_1X_4 - 1.99X_2X_3 + 0.15X_2X_4 \\
 & - 0.21X_3X_4
 \end{aligned} \quad (3)$$

where X_3 , X_4 , X_1X_2 , X_1X_3 , and X_2X_4 have positive effects on the response, and the other parameters have adverse effects.

Table 1. Coded values of the input variables for the central composite RSM design.

Variables	Symbols	Variable Levels				
		−2	−1	0	1	2
Temperature (°C)	X_1	30	35	40	45	50
Methanol:oleic acid molar ratio	X_2	1	3	5	7	9
SAP loading (%)	X_3	5	7.5	10	12.5	15
Enzyme loading (%)	X_4	5	7.5	10	12.5	15

Table 2. Experimental design for the influences of the four independent variables on the reaction conversion in coded values and experimental results.

Run	Variable				Response, Y
	X_1	X_2	X_3	X_4	
1	1	1	1	1	66.87
2	1	−1	1	1	88.13
3	−2	0	0	0	85.83
4	1	1	−1	1	68.91
5	0	0	2	0	77.68
6	0	2	0	0	60.76
7	−1	−1	1	1	96.20
8	1	−1	−1	1	74.42
9	−1	−1	−1	1	95.88
10	0	0	0	2	81.21
11	1	1	1	−1	55.43
12	1	−1	1	−1	70.65
13	−1	1	−1	−1	65.83
14	−1	−1	−1	−1	80.98
15	−1	−1	1	−1	85.09
16	1	1	−1	−1	55.23
17	1	−1	−1	−1	64.56
18	0	−2	0	0	79.16
19	2	0	0	0	60.15
20	−1	1	1	1	78.20
21	0	0	0	−2	64.98
22	0	0	−2	0	70.88
23	−1	1	−1	1	83.59
24	−1	1	1	−1	65.38
25	0	0	0	0	90.92
26	0	0	0	0	89.90
27	0	0	0	0	88.79
28	0	0	0	0	90.92
29	0	0	0	0	87.92
30	0	0	0	0	88.36
31	0	0	0	0	89.72

Repeated experiments based on the central runs (25–31) showed a low coefficient of variance (1.33%), indicating the high reproducibility and precision of the experiments. The model was evaluated for statistical significance using the F test for ANOVA (Table 3). Results showed a very low p value (<0.0001) of the model in the F test, confirming that the regression was statistically significant at the 95% confidence level. The coefficient of determination (R^2) was determined to evaluate the quality of the developed model. Result showed that a high R^2 value (0.97) was achieved, signifying high reliability of the model for predicting reaction conversion. As shown in Figure 2, the model predictions were in good agreement with experimental values, indicating that the established model provided satisfactory and accurate results. Table 4 presents the overall effects of the input variables on the reaction conversion, which were examined using t tests. Low p values (<0.05) of the intercept term,

three linear terms (X_1 , X_2 , and X_4), all quadratic terms, and an interaction term (X_2X_3) indicated their significant effects on the reaction. The developed model can therefore be used to forecast the optimal reaction conditions for obtaining maximal responses.

Table 3. Analysis of variance for the empirical model.

Source	DF ^b	SS ^b	MS ^b	F Value	Probability (P) > F
Model ^a	14	4319.58	308.54	32.91	<0.0001
Residual (error)	16	150.01	9.38	-	-
Total	30	4469.59	-	-	-

^a Coefficient of determination (R^2) = 0.97; adjusted R^2 = 0.94. ^b DF, degree of freedom; SS, sum of squares; MS, mean square.

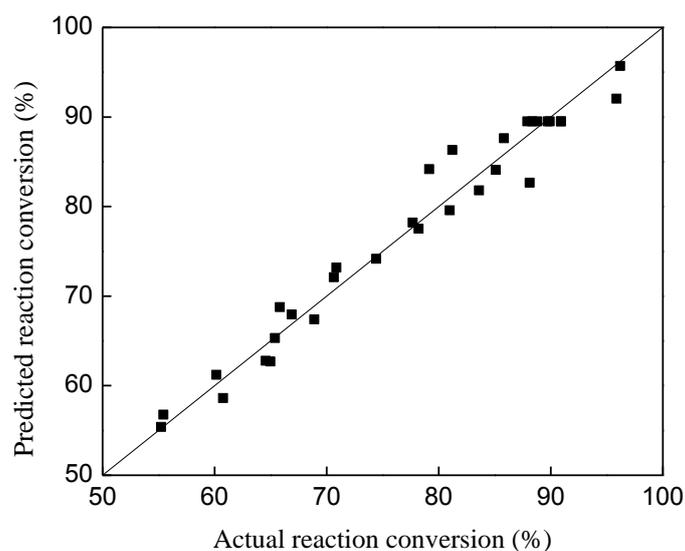


Figure 2. Correlation between experimental and fitted conversions of the reaction.

Table 4. Significance of the coefficients in the empirical model.

Model Term	Parameter Estimate	Standard Error	t Value ^a	p Value
β_0	89.50	1.16	77.34	0.000 ^b
β_1	-6.60	0.63	-10.55	0.000 ^b
β_2	-6.39	0.63	-10.22	0.000 ^b
β_3	1.26	0.63	2.01	0.062
β_4	5.90	0.63	9.43	0.000 ^b
β_{11}	-3.77	0.57	-6.59	0.000 ^b
β_{22}	-4.53	0.57	-7.91	0.000 ^b
β_{33}	-3.45	0.57	-6.03	0.000 ^b
β_{44}	-3.75	0.57	-6.54	0.000 ^b
β_{12}	0.86	0.77	1.13	0.275
β_{13}	1.21	0.77	1.58	0.133
β_{14}	-0.26	0.77	-0.34	0.740
β_{23}	-1.99	0.77	-2.61	0.019 ^b
β_{24}	0.15	0.77	0.19	0.850
β_{34}	-0.21	0.77	-0.27	0.788

^a $t_{\alpha/2, n-p} = t_{0.025, 19} = 2.093$. ^b $p < 0.05$ indicates that the model terms are significant.

3.3. Effect of Reaction Factors on Reaction Conversion

Figure 3 shows the effects of the methanol:oleic acid molar ratio and SAP loading on the reaction conversion while maintaining temperature and enzyme loading at their central levels. Results showed a significant interaction between the methanol:oleic acid molar ratio and SAP loading. At high methanol:oleic acid molar ratios, SAP loading slightly affected the reaction conversion. However, at a low methanol:oleic acid molar ratio, increasing the SAP loading significantly enhanced the reaction conversion. This is attributed to the SAP absorbing water from the reaction mixture and enhancing the enzyme activity to create a suitable microenvironment for an efficient reaction [37]. However, a higher SAP loading resulted in a significant decrease in reaction conversion. Because liquid lipase requires a minimal amount of water for optimal activity, high SAP loading caused less water content to be present in the reaction solution [35,37]. Consequently, the enzyme activity was reduced, leading to a reduction in reaction conversion.

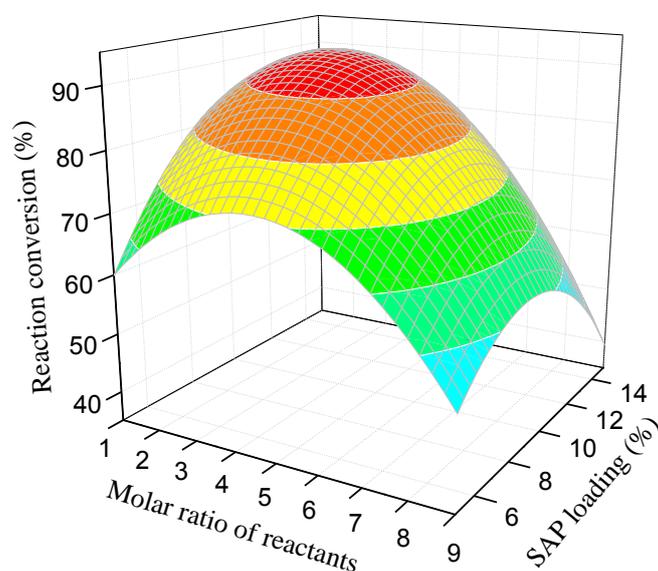


Figure 3. Response surface plot of combined effects of the reactant molar ratio and SAP loading on the conversion of the reaction at a constant temperature (40 °C) and enzyme loading (10%).

Figure 4 presents a response surface curve obtained by plotting the reaction conversion against temperature and molar ratio of methanol to oleic acid while maintaining the other factors at their central levels. At any temperature, the reaction conversion significantly increased when increasing the methanol:oleic acid molar ratio. This was because a high amount of methanol is required for esterification [20,47,48]. However, the reaction conversion decreased with a further increase in the methanol:oleic acid molar ratio. This result is similar to that of other studies [10,14]. Studies have reported that high methanol content in the reaction mixture can inhibit the activity of lipase [10,14]. In this study, the maximal reaction conversion was obtained at a methanol:oleic acid molar ratio of 3.34:1.

Figure 5 presents the effects of temperature and enzyme loading on the reaction conversion while maintaining methanol:oleic acid molar ratio and SAP loading at their central levels. At any enzyme loading level, the reaction conversion increased when increasing the temperature. Nevertheless, a high temperature resulted in a decrease in the reaction conversion because the enzyme becomes inactive at high temperatures [14,32,49]. This result agrees with that of other studies [32,33]. Studies have demonstrated that lipases are sensitive to temperature, and therefore low or elevated temperatures caused a dramatic decrease in their activity [14,33,49]. Similar to temperature, the enzyme loading also significantly affected the reaction conversion. At a given temperature, increasing enzyme loading led to a significant increase in reaction conversion. Studies have reported that an increase in the amount of the enzyme increased contact between the enzyme active surface area and the reactants,

thus enhancing the reaction [14,50]. However, a further increase in enzyme loading resulted in a slight decrease in reaction conversion. Excess enzyme possibly caused enzyme aggregation, which limited the enzyme flexibility to react with the oleic acid–methanol interface, thus lowering the conversion of the reaction [14,50].

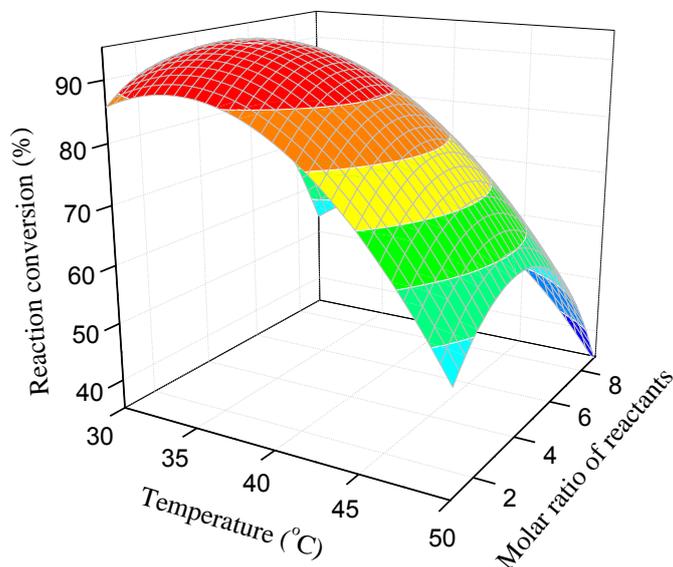


Figure 4. Response surface plot of the combined effects of temperature and reactant molar ratio on the conversion of the reaction at a constant SAP loading (10%) and enzyme loading (10%).

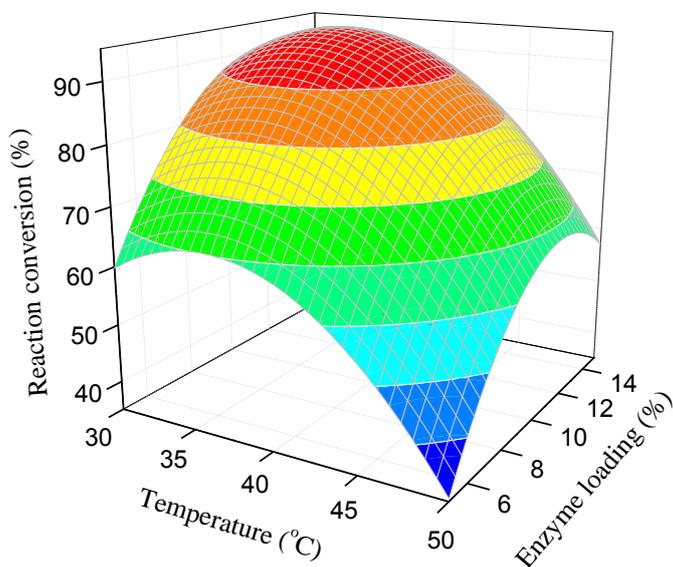


Figure 5. Response surface plot of the combined effects of temperature and enzyme loading on the conversion of the reaction at a constant reactant molar ratio (5:1) and SAP loading (10%).

3.4. Obtaining Optimal Reaction Conditions

Based on the empirical model [Equation (3)], the canonical method was used to forecast the optimal reaction conditions for maximizing reaction conversion. The highest conversion was predicted to be 97.83% at 35.25 °C, methanol:oleic acid molar ratio of 3.34:1, SAP loading of 10.55%, and enzyme loading of 11.98%. An experiment was then carried out under optimal conditions to verify the prediction. A conversion of 96.73% \pm 0.15% was obtained, indicating consistent with the empirical

model prediction. The developed RSM model can be therefore used to describe the relationships between the response and the variables in the liquid lipase-catalyzed esterification of oleic acid with methanol. Furthermore, the reaction conversion was comparable with other process but exhibited a shorter reaction time [25,29]. In this study, high reaction conversion (96.73%) was obtained under the reaction time of 2.5 h whereas 24 h was required to yield conversion of 90.8% in the esterification of oleic acid with methanol using lipase without SAP reported by Rosset et al. [25]. This suggests that the liquid lipase-catalyzed esterification of fatty acids with methanol using SAP as a water-removal agent is a promising process for producing biodiesel.

3.5. Reusability of Liquid Lipase

Although immobilized lipase is being used for its stability and reusability, its high cost restricts its industrial application. To solve this concern, liquid lipase was developed as an alternative for the reaction. Liquid lipase can be reused several times without significant loss in activity [29]. This study investigated the reusability of Eversa Transform lipase through the esterification of oleic acid and methanol under the optimal reaction conditions. As indicated in Figure 6, the Eversa Transform lipase could only be reused once to drive the reaction to high conversion. After one cycle, the reaction conversion decreased sharply. This was attributed to the inactivation effect of methanol on the enzyme [10,14] and the low stability of liquid enzyme [51]. Further investigation is required to address this limitation. Although liquid lipase demonstrated low reusability in this study, the enzyme remains a promising alternative to immobilized lipase for industrial application because of its low cost [33,52]. Studies have demonstrated that the cost of liquid lipase is 30- to 50-fold lower than that of immobilized lipase [30]. Additionally, the preparation of the liquid lipase is much simpler. Because of these merits, liquid lipase is suggested as a potential alternative for the reaction to improve economic viability.

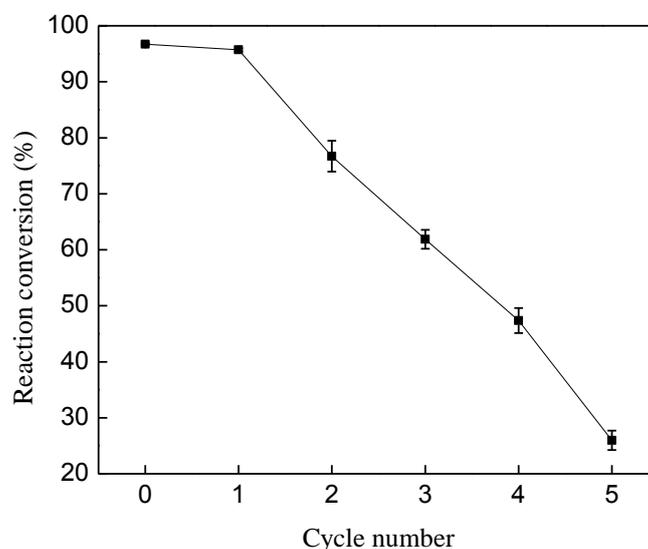


Figure 6. Reusability of liquid lipase for esterification of oleic acid and methanol.

4. Conclusions

This paper reports the liquid lipase-catalyzed esterification of oleic acid with methanol using SAP as a water-removal agent for biodiesel production. The use of SAP significantly enhanced the reaction conversion by suppressing the reverse reaction. The reaction conditions were then optimized to obtain maximal conversion yield using RSM. A maximal conversion of 96.73% was achieved and verified the optimization calculations. Although Eversa Transform lipase was ineffectively reused due to the deactivation of enzyme caused by methanol, liquid lipase-catalyzed esterification in the presence of SAP is promising for biodiesel production from feedstocks containing high water content.

Author Contributions: Hoang Chinh Nguyen and Chia-Hung Su conceived and designed the experiments; Dinh Thi My Huong and Horng-Yi Juan performed the experiments; Hoang Chinh Nguyen and Chien-Chung Chien analyzed the data; Chia-Hung Su contributed reagents and materials; Hoang Chinh Nguyen and Chia-Hung Su wrote the paper.

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