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Optimization of Lipase-Catalyzed Transesterification of Lard for Biodiesel Production Using Response Surface Methodology

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Abstract Biodiesel, an alternative diesel fuel made from renewable biological resources, has become more and more attractive recently. Combined use of two immobilized lipases with complementary position specificity instead of one lipase is a potential way to significantly reduce cost of lipase-catalyzed biodiesel production. In this study, the process of biodiesel production from lard catalyzed by the combined use of Novozym435 (non-specific) and Lipozyme TLIM (1,3-specific) was optimized by response surface methodology. The optimal reaction conditions were 0.04 of amount of lipase/oil (*w*/*w*), 0.49 of proportion of Novozym435/total lipases (*w*/*w*), 0.55 of quantity of *tert*-butanol/oil (*v*/*v*), 5.12 of quantity of methanol/oil (mol/mol), and 20 h of reaction time, by which 97.2% of methyl ester (ME) yield was attained, very close to the predicted value (97.6%). This optimal reaction condition could be true of other similar reactions with plant and animal oil resources; their ME yield could be higher than 95%. The lipases regenerated by washing with organic solvent after each reaction cycle could be continuously reused for 20 cycles without any loss of activity, exhibiting very high manipulation stability.

Keywords Biodiesel · Lipase · Novozym435 · Lipozyme TLIM · RSM

Introduction

As an ideal alternative fuel synthesized from renewable resources (e.g., vegetable oils, animal fats, and recycled restaurant greases), biodiesel provides many environmental

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advantages. It is renewable, sulfur-free, biodegradable, and non-toxic [1–4]. Utilization of lipase as catalyst for biodiesel production has a higher potential compared to using alkaline as catalyst. This is attributed to its relatively simple operations, little consumption of methanol, easy recovery of glycerol, mild reaction conditions, and no generation of chemical waste. Therefore, many studies have been carried out to find out the most suitable lipase system for biodiesel production. Lipases from various sources have been tested for transesterification of lipid with methanol. Particularly, a number of immobilized lipase systems such as *Candida antarctica* (Novozym435) [4–6], *Thermomyces lanuginosus* (Lipozyme TLIM) [5, 7], *Rhizomucor miehei* (Lipozyme RMIM) [8], and *Pseudomonas cepacia* (PS) [9] have been proven to be effective in biodiesel production. However, all of them require large amount of lipase, which contributed little to resolve the higher production cost of lipase-catalyzed methods. So far, majority of the research only exploited the use of single lipase as the catalyst, and little attention has been paid to the combined use of two or more lipases with complementary region specificities. The synergy of the lipases may offer a potential solution to reduce the total usage of lipases.

Until now, research about the synergy of combined enzymes mainly focuses on the different kinds of enzymes, such as protease, lipase and cellulose used in detergent, glucose oxidase, and lipase used in food processing. Several combined lipases system had been tried for the transesterification reaction. Negishi et al. [10] used Lipase QLM (*Alcaligenes* sp.) and Novozym435 to catalyze structured lipid production, and no synergy or antergy effect has been observed. Li et al. [11] used the mixture of Lipozyme TLIM and Novozym435 to catalyze the methanolysis of rapeseed oil for biodiesel production, and the highest biodiesel yield achieved was 95%. Ibrahim et al. [12] compared the efficiency of inter-esterification of palm stearin with coconut oil by applying a dual lipase system and an individual lipase system, and it revealed a synergistic effect in combined use of various lipases. This demonstrated the possibility of increasing the reaction rate by employing a dual lipase system as a biocatalyst. However, these experiments have been conducted by changing one single factor at one time (COST) under the uniform transesterification condition. Actually, the transesterification reaction is affected simultaneously by many factors such as methanol quantity, reaction temperature, and reaction time in addition to the use of lipases. In this regard, we employed the response surface methodology (RSM) to obtain the optimal reaction conditions for biodiesel synthesis catalyzed by the combined lipases. RSM is a powerful tool for the optimization of chemical reactions and industrial processes [13]. RSM explores the relationships between several undependable variables and one or more response variables. A contour plot is frequently used to find the responses of two variables to find these coefficients by including a large number of trials in each and combination of them and using some sort of interpolation to find potentially better intermediate values between them. RSM was also reported to optimize the process of biodiesel production [11, 14], but this was the first time that RSM was adopted to optimize the process of the combined use of immobilized lipases to catalyze biodiesel production.

In this paper, the combined use of immobilized lipases Novozym435 and Lipozyme TLIM were used to catalyze the transesterification of lard with methanol as the acyl acceptor and *tert*-butanol as the reaction medium. Response surface methodology was used to monitor the transesterification reaction efficiency. The main objective was to try to explore an economic technology for industrial purpose that is especially suitable for lipid feedstock from both plants and animals, which presents a promising prospect for the large-scale biodiesel production.

Materials and Methods

Materials

Novozym435 (Nov435, immobilized *C. antarctica* lipase) and Lipozyme TLIM (TLIM, immobilized *T. lanuginosus* lipase) were purchased from Novo Nordisk (Bagsvaerd, Denmark). The specific activities of the lipases for hydrolysis of tributyrin were 3,000 U/g for Novozym435 and 20,000 U/g for Lipozyme TLIM. Lard was obtained at the local market; the compositions of the fatty acid of lard (wt.%) are:$C_{14:0}$, 1.6%; $C_{16:0}$, 26.6%; $C_{16:1}$, 2.2%; $C_{18:0}$, 16.7%; $C_{18:1}$, 41.3%; $C_{18:2}$, 9.3%; and $C_{18:3}$, 1.3%. Methyl esters of myristic acid, palmitic acid, palmtoletic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid were of chromatographic grade and were purchased from Sigma Chemical Co., Beijing. All other chemicals were of analytical reagents and were obtained commercially.

General Procedure for Enzymatic Transesterification of Oils

The transesterification reactions were carried out in covered shaking flasks and heated to the reaction temperature 50 °C on a reciprocal shaker. A standard reaction mixture consisted of oil, *tert*-butanol, methanol, and immobilized lipases. At various time points, an aliquot of 100 μl was collected from the reaction mixture and centrifuged. The upper layer of the sample was harvested and from which 5 μl was mixed with 95 μl of hexane and 300 μl of internal standard solution (heptadecanoic acid methyl ester hexane solution). Gas chromatography analysis was used for methyl ester measurement.

Analytical Procedure

The methyl ester contents were quantified by a gas chromatograph (Fuli, Wenlin, China) equipped with a HP-INNOWax capillary column (0.25 mm×30 m, Agilent, Waldbronn, Germany). The column temperature was kept at 180 °C for 2 min, increased to 230 °C at 3 °C/min, and maintained at this temperature for 1 min. The temperature of the injector and detector was set at 230 °C and 280 °C, respectively. The methyl ester (ME) yield (wt/wt.%) was defined as ME amount produced by the lipases divided by the amount of the oils.

Fuel Properties

The fuel properties, namely, density at 15 °C, viscosity at 40 °C, flash point, cold filter plug point, water content, ash content, carbon residue, distillation temperature (90%, Vol Rec), acid value, and calorific value of the produced biodiesel and conventional diesel were determined as per the prescribed methods and compared with the latest American and European standards (ASTM 2003; DINEN 14214, 2003).

COST Experimental Design of Combined Use of Lipases

Two lipases were mixed to use as catalyst. The summed amount of lipases was as follows:

$$M = M_a \times p + M_b \times (1 - p)$$

where M was the summed amount of lipases; M_a was the optimal amount of Novozym435; M_b was the optimal amount of Lipozyme TLIM; and p was the combined proportion of lipases, between 0 and 1.

RSM Experimental Design

Software program Modde, version 8.0, from Umetris AB, Umeå, Sweden, was used in the statistical experimental design. A five-level five-factor D-Optimal design and RSM were employed in this study, totally requiring 29 experiments. According to the results of the prophase research [15], the parameters of five undependable variables, such as amount of lipase to oil (*w*/*w*), proportion of the two lipases (amount of Novozym435/ total amount of combined lipases; *w*/*w*), *tert*-butanol to oil ratio (*v*/*v*), methanol to oil ratio (mol/mol), and reaction time (h) were proven to have significant effects on methyl ester yield. Levels of each parameter were selected according to the results of COST experiments. The amount of lipase to oil (*w*/*w*) was 2–6%; the proportion of the two lipases (*w*/*w*) was 0.2–0.8; *tert*-butanol to oil ratio (*v*/*v*) was 0.2–0.8; methanol to oil ratio (mol/mol) was 3:1–7:1; reaction time (h) was 4–24. The response measured was the methyl ester yield (ME yield, %). The independent factors (x_i), levels, and experimental design were presented in Tables 1 and 2. Twenty-nine experiments were performed in triplicates.

Statistical Analysis

The experimental data were fitted to the multiple linear regression (MLR) procedure (Modde software). The following second-order polynomial equation was obtained:

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i x_i + \sum_{i=1}^{5} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{ij} x_i x_j \qquad (1)$$

where Y was the response; x_i and x_j were the uncoded independent variables and β_0, β_i, β_{ii}, and β_{ij} were intercept, linear, quadratic, and interaction coefficients, respectively. Design Expert software package was used for regression analysis and analysis of variance (ANOVA). Four-dimensional contour plots were developed using the fitted quadratic polynomial equation obtained from regression analysis, changing four of the independent variables and holding the remaining one independent variable to the stationary value. The quality of the fit of the polynomial model equation was evaluated by the coefficient of determination, R^2, and its regression coefficient significance was checked with F test. The optimization point for each independent variable at which a percentage conversion was maximized were obtained. Confirmatory experiments were carried out to validate the equations, using combinations of independent variables, which were not part of the original experimental design but were within the experimental region.

Table 1 Independent variables and levels used for response surface design.

Independent variables	Symbols	Levels				
		−2	−1	0	1	2
Lipase to oil (*w*/*w*)	x_1	0.02	0.03	0.04	0.05	0.06
Lipase proportion (*w*/*w*)	x_2	0.2	0.35	0.5	0.65	0.8
Tert-butanol to oil (*v*/*v*)	x_3	0.2	0.35	0.5	0.65	0.8
Methanol to oil (mol/mol)	x_4	3	4	5	6	7
Reaction Time (h)	x_5	4	9	14	19	24

Table 2 D-Optimal design and response.

Design point	Coded independent variable levels					Response
	x_1	x_2	x_3	x_4	x_5	Y (%)
1	−1	0	−2	−2	−2	43.15
2	2	2	−2	−2	−2	48.68
3	2	−2	2	−2	−2	53.36
4	−2	2	2	−2	−2	50.65
5	−2	−2	−2	−1	−2	8.91
6	0	2	−1	1	−2	56.55
7	2	−2	−2	2	−2	7.04
8	−2	2	−2	2	−2	4.59
9	−2	−2	2	2	−2	16.46
10	2	2	2	2	−2	54.01
11	2	1	−2	1	−1	57.51
12	1	−1	−1	2	−1	45.18
13	−1	−2	0	−2	0	73.36
14	−2	2	−2	−2	1	64.41
15	0	1	2	−1	1	82.79
16	2	−1	1	0	1	87.65
17	2	−2	−2	−2	2	62.58
18	1	1	1	−2	2	80.83
19	−2	−2	2	−2	2	62.51
20	2	2	2	−2	2	64.28
21	−1	2	−2	−1	2	79.35
22	−2	1	−1	−1	2	89.82
23	−2	−2	−2	2	2	12.61
24	2	2	−2	2	2	73.35
25	2	−2	2	2	2	84.81
26	−2	2	2	2	2	85.34
27	0	0	0	0	0	90.11
28	0	0	0	0	0	88.41
29	0	0	0	0	0	87.25

Result and Discussion

Effect of Amounts of Single Lipases

Two different kinds of immobilized lipases Novozym435 and Lipozyme TLIM were tested for transesterification of lard using methanol as acyl acceptor. The ratio of methanol/oil (mol/mol) and *tert*-butanol/oil (*v*/*v*) was kept at 5 and 0.4, respectively, and then various aliquots of lipase were added. As indicated in Fig. 1, the ME yield was initially increased as the amount of lipase increased and then gradually ceased to increase as the amount of lipase continued to increase. The highest ME yields of 90.5% and 72.8% was reached at lipase/oil ratio of 0.03 and 0.08 for Novozym435 and Lipozyme TLIM, respectively. Obviously, a high concentration of lipase provided abundant activation sites and sufficient mass contact, which contributed to the initial increase of ME yield. However, there was a saturation point for the yield, after which there was no significant increase even with the presence of even higher amount of enzymes. Thus, the optimal amounts of the lipases were 3% for Novozym435 and 8% for Lipozyme TLIM.

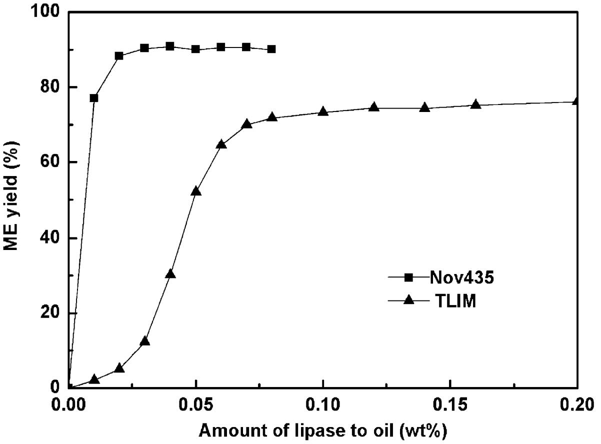

Fig. 1 Effect of lipase amount on transesterification with methanol/oil ratio (mol/mol) of 5 and *tert*-butanol/oil ratio (*v*/*v*) of 0.4 at 24 h

Effect of Combined Use of Two Lipases of COST

To explore the possibility of synergistic effect, two lipases with their optimal amounts, 3% for Novozym435 and 8% for Lipozyme TLIM, were mixed in different combined proportion and used as catalyst for the transesterification. The ME yield of the reaction was presented in Fig. 2. The highest ME yield of 93.5% was achieved when the combined proportion of lipases was 0.6, which was higher than those by single lipase. It suggested that when the usage amount of Novozym435 and Lipozyme TLIM was, respectively, 0.6×3% and 0.4×8%, the combined use of Novozym435 and Lipozyme TLIM generated a markedly positive synergistic action. From this COST experiment, the summed amount of two lipases was 5%, which was composed of 1.8% Novozym435 and 3.2% Lipozyme TLIM.

Model Fitting of RSM

The experimental D-optimal design matrix was presented in Tables 1 and 2. There were three replicates at the center-point level for experimental error estimation in full 29

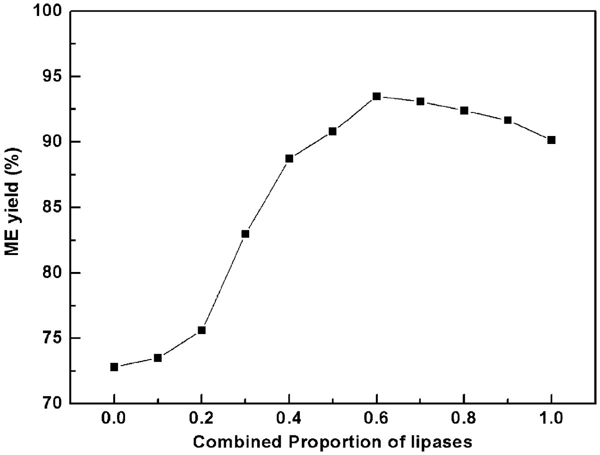

Fig. 2 Effect of different combined proportion of lipases in COST experiment of transesterification with methanol/oil ratio (mol/mol) of 5, *tert*-butanol/oil ratio (*v*/*v*) of 0.4 at 24 h

experiments. The methyl ester yield ranged from 4.59% to 90.11%, and the design points of no. 18 and no. 27 gave the minimum and maximum yields, respectively.

The experimental data were fitted to the second-order polynomial Eq. 1 by use of the MLR procedure of model. A statistical analysis was performed on these experimental values, and the main effects and interaction for variables were calculated. Table 3 showed the analysis of variance (*F* test) and the *p* value with a confidence level of 95% for this experiment.

ANOVA indicated that the model was significant (see Table 3). Herein, the *p* value of the model was smaller than 0.0001, indicating that the model was significantly suitable. The *F* value of "lack of fit" of 11.28 implied that the "lack of fit" was not significantly relative to the pure error. The *p* value of "lack of fit" was 0.08 ($p>0.05$), indicating that "lack of fit" was insignificant. The coefficient of determination (R^2) value was 0.993 for ME yield. The R^2 value provided a measure of how much variability in the observed response values could be explained by the experimental factors and their interactions [13]. The value 0.993 implied that the sample variation of 0.993 for ME yield production was attributed to the independent variables, and only 0.007 of the total variation was not explained by the model. The adjust coefficient of determination (adj. R^2) value was 0.975, very close to the R^2 value, which corrects the R^2 values for the sample size and for the number of terms in the model. The values indicated that the accuracy and general availability of the polynomial model was adequate.

The best fitting response surface by the MLR procedure could be expressed as follows:

$$\begin{aligned} Y = 88.09 &+ 7.53x_1 + 8.54x_2 + 10.41x_3 - 5.61x_4 + 16.24x_5 - 5.07x_1^2 - 4.71x_2^2 \\ &-17.08x_3^2 - 9.74x_4^2 - 3.60x_5^2 - 4.15x_1x_2 - 3.34x_1x_3 + 4.83x_1x_4 - 2.02x_1x_5 \\ &-4.01x_2x_3 + 4.07x_2x_4 - 0.19x_2x_5 + 7.00x_3x_4 - 1.51x_3x_5 + 5.39x_4x_5 \end{aligned} \quad (2)$$

Effect of Parameters

The regression coefficients and the corresponding *p* values of coefficients were presented in Table 4. From the *p* values of each model term, most of the coefficients were significant at 1% level, including of all the linear terms, the quadratic terms of x_3^2,x_4^2, and the interaction terms of x_1x_2, x_1x_4, x_2x_3, x_2x_4, x_3x_4, and x_4x_5. The interaction terms of x_1x_3 were significant at the 5% level. Other terms of the model had no significant effects on ME yield. Moreover, from Table 4, it could be concluded that the regression coefficients of the linear term x_3, x_5, and the quadratic term of x_3^2,x_4^2 were the primary determining factors of the responses on ME yields, as they had highest coefficients, followed by the linear term x_1, x_2, and the interaction terms of x_3x_4. All of the quadratic terms had negative coefficients.

Table 3 ANOVA and fit statistics for *Y.*

Source	*df*	SS	MS	*F* value	Probe>*F*
Total	28	20,527.20	733.11		
Model	20	20,383.00	1,019.154	56.55	<0.0001
Residual	8	144.18	18.02		
Lack of fit	6	140.04	23.34	11.28	0.08
Pure error	2	4.14	2.07		
R^2=0.993					
Adj. R^2=0.975					

Table 4 Regression coefficient of predicted quadratic polynomial model.

Term	Coefficients estimated	Standard error	Probe>F
Intercept	88.09	1.93	<0.0001[a]
β_1	7.53	0.97	<0.0001[a]
β_2	8.54	0.99	<0.0001[a]
β_3	10.41	0.96	<0.0001[a]
β_4	−5.61	0.97	0.0004[a]
β_5	16.24	0.95	<0.0001[a]
$\beta_1{}^2$	−5.07	2.77	0.1043
$\beta_2{}^2$	−4.71	2.93	0.1460
$\beta_3{}^2$	−17.08	3.19	0.0007[a]
$\beta_4{}^2$	−9.74	2.65	0.0062[a]
$\beta_5{}^2$	−3.60	2.86	0.2443
$\beta_1\beta_2$	−4.15	1.03	0.0038[a]
$\beta_1\beta_3$	−3.34	1.02	0.0113[b]
$\beta_1\beta_4$	4.83	1.07	0.0020[a]
$\beta_1\beta_5$	−2.02	1.03	0.0848
$\beta_2\beta_3$	−4.01	1.02	0.0044[a]
$\beta_2\beta_4$	4.07	1.05	0.0046[a]
$\beta_2\ \beta_5$	0.19	1.03	0.8546
$\beta_3\beta_4$	7.00	1.03	0.0001[a]
$\beta_3\beta_5$	−1.51	1.02	0.1747
$\beta_4\beta_5$	5.39	1.04	0.0008[a]

[a] Significant at 1% level

[b] Significant at 5% level

The nine contour plots (see Fig. 3a–i) were drawn from the predicted model (Eq. 2) at constant value of 19-h reaction time. From Table 4, *tert*-butanol quantity (x_3) and methanol quantity (x_4) were important variables for ME yield by lipase-catalyzed transesterification and considered as indicators of effectiveness. So, Fig. 3 represented the ME yields with the variable of *tert*-butanol quantity and methanol quantity. Figure 3a–c represented ME yield by keeping the amount of lipase constant as 0.02, 0.04, and 0.06, respectively; Fig. 3a,d,g represented ME yield by keeping the proportion of two lipases constant as 0.8, 0.5, and 0.2, respectively.

From the shape of contour plots, all nine elliptical profile of the contour plots indicated that ME yield was more sensitive to *tert*-butanol quantity than methanol quantity. Meanwhile, all nine contour plots exhibited similar behavior in terms of ME yield. ME yield increased with the increase of *tert*-butanol quantity until the volume ratio for *tert*-butanol to oil reached 0.6. This indicated that *tert*-butanol could improve the mixture of methanol and lard [11]. The proper quantity of *tert*-butanol in the reaction solution could reduce the lipase activity decrease caused by excessive presence of methanol. However, more *tert*-butanol (>0.6) would dilute the substrate concentration. Similarly, for the variable of methanol quantity, ME yield had the same trend with the variable of *tert*-butanol quantity. There, we could get the maximum of ME yield when the molar ration of methanol quantity to oil is between 4 and 5. In Fig. 3g, when the amount of lipase and proportion of lipase were 0.02 and 0.2, ME yield decreased with the increasing amount of methanol. This was due to the fact that methanol quantity was the most significant variable with negative effect. It is well known that excessive methanol might inhibit the lipase activity. So, in contrast with Fig. 3g, the reaction with more amount of lipase in Fig. 3i got higher ME yield at the same methanol quantity. In general, ME yield increased with the increase of the

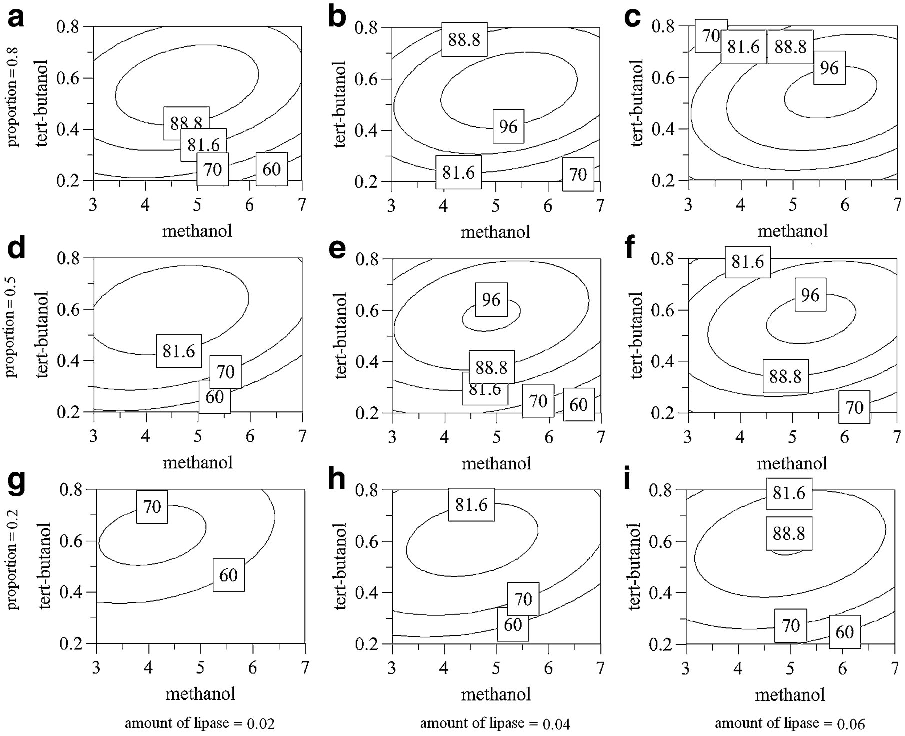

Fig. 3 Contour plots for ME yields of biodiesel production at 19 h. The *numbers inside the contour plots* indicate ME yields at given reaction conditions

amount of lipases and their proportion (ratio of each other or the ratio to the whole reaction) because their effects were positive. But with the value of proportion and amount of lipase >0.5, the ME yield changed insignificantly.

Validation of Model

The optimal condition of ME yield was obtained through the regression model (Eq. 2). The parameters were as follows: amount of lipase (*w*/*w*) was 0.04, proportion of the two lipases (*w*/*w*) was 0.49, *tert*-butanol quantity (*v*/*v*) was 0.55, methanol quantity (mol/mol) was 5.12, and reaction time was 20 h. Under the optimal conditions, the predicted value of ME yield was 97.6%, and the actual experimental value was 97.2±0.6%.

From Fig. 4, the ME yields catalyzed by the same amount of Lipozyme TLIM, Novozym435, and the combined use of the two lipases were 48.5%, 89.2%, 97.2%, respectively. The lipase activity of the combined lipases was 49.8% and 8.2% higher than that of Lipozyme TLIM and Novozym435, respectively. During the first 0.5 h, the reaction rate using combined lipases was the fastest, followed by the one which used Lipozyme TLIM and then Novozym435. This phenomenon might be due to the fact that the joining of the non-specific lipase Novozym435 and 1,3-specific lipase Lipozyme TLIM could accelerate the transesterification reaction rate at 1- and 3- sites and meanwhile eliminate the limitation of the acyl migration at 2- site, which is considered to be the confined step of transesterification reaction. The similar observations were also reported by other research groups [5, 12]. The detailed mechanism needs to be examined in the further work.

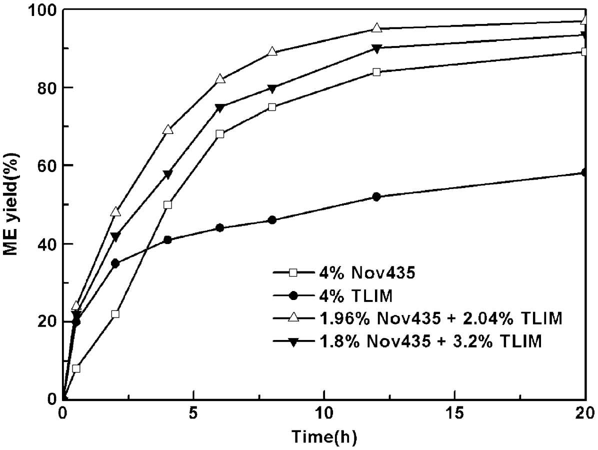

Fig. 4 Time course of the transesterification. Conditions: 50 °C, methanol/oil molar ratio of 5.12:1, *tert*-butanol/oil ratio (*v*/*v*) of 54.8%

From Fig. 4, from RSM model, the best synergistic effect was achieved with 1.96% of Novozym435 and 2.04% of Lipozyme TLIM with the ME yield of 97.2%. It was higher than the ME yield of 93.5% achieved by COST experiments with 1.8% Novozym435 and 3.2% Lipozyme TLIM. Considering the price of Novozym435 (US $1,500/kg) and Lipozyme TLIM (US $350/kg), the new lipase combination used generated from RSM model was not only cost-effective but also more efficient.

In conclusion, verification experiments confirmed the validity of the regression model. Thus, the optimization of transesterification of biodiesel production catalyzed by Lipozyme TLIM and Novozym435 was successfully achieved by D-optimal and RSM.

Repeated Use of the Lipase

The high price of the lipase may be a major bottleneck for the large-scale lipase-catalyzed biodiesel production [1]. Therefore, the operational stability of the lipase is of great importance. In our experiment, the lipases were regenerated by washing with organic solvent *tert*-butanol after each reaction cycle. Little loss of enzymatic activity of the lipases was detected after it was continuously used for 20 cycles (about 400 h) of reactions with

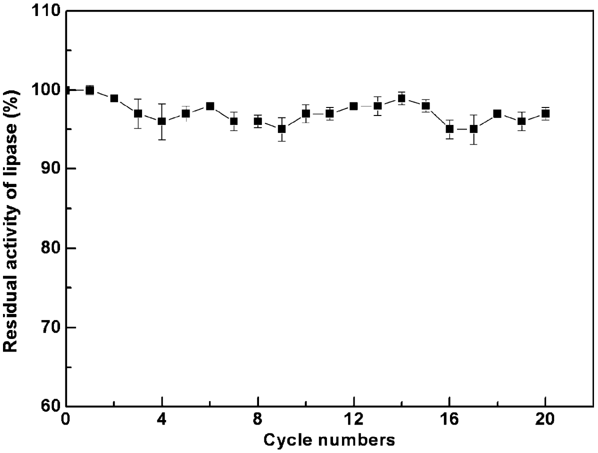

Fig. 5 Operational stability of lipase with methyl acetate as acyl acceptor. Conditions: 50°C, 1.96% Nov435+2.04% TLIM, methanol/oil molar ratio of 5.12:1, *tert*-butanol/oil ratio (*v*/*v*) of 54.8%

lard (see Fig. 5), which indicated that the operational stability of the combined lipases was fairly good. *Tert*-butanol as a good organic solvent could dissolve lard, methanol, and the by-product glycerol which would block the immobilized lipase and lower its activity [4]. Therefore, this technique is promising in the enzymatic transesterification of oils for biodiesel production.

Transesterification and Fuel Properties of Biodiesel

The above study has demonstrated that lard could be used as lipid feedstock by effectively converting into biodiesel in the certain reaction system. Meanwhile, some plant oil feedstocks were also studied herein. The ME yields were still higher than 95%, such as 98.3% for soybean oil and 95.8% for cottonseed oil (data not shown). Though fatty acid profiles between lard and plant oils were different [16], the ME yields were very close. The synergistic effect of the combined lipases might overcome the limitation of the fatty acid and positional specificities of a single lipase.

Table 5 summarized the fuel properties of biodiesel generated by the aforementioned optimized process. It showed that the fuel properties of lard biodiesel were similar to those of plant oil biodiesel, except the cold fitter plug point. Moreover, lard biodiesel had comparable fuel properties with those of biodiesel and coincides with the latest published American and European standards for biodiesel.

Conclusion

In this study, the immobilized lipases Novozym435 and Lipozyme TLIM were used to catalyze the transesterification of lard using methanol as the acyl acceptor and *tert*-butanol as the reaction medium. The combined use of Novozym435 and Lipozyme TLIM was found to generate a markedly positive synergistic action. The best synergistic effects from COST and RSM experiments were compared. Under the optimal conditions of RSM, biodiesel yield of 97.2% could be obtained, and further verification experiments confirmed the validity of the predicted model. Furthermore, the optimized reaction condition could also be used in the reactions with other oils resources, and the biodiesel (methyl ester) yield

Table 5 Fuel properties of lard biodiesel, soybean oil biodiesel, cottonseed oil biodiesel, and diesel.

Property	Unit	Biodiesel			Diesel	Biodiesel standards	
		Lard	Soybean oil	Cottonseed oil		ASTM D6751-02	DIN EN 14214
Density at 15°C	$\mathrm{kg/m^3}$	877.4	881.2	883.7	850	–	860–900
Viscosity at 40°C	$\mathrm{mm^2/s}$	4.84	4.47	4.13	2.6	1.9–6.0	3.5–5.0
Flash point	°C	143.5	178	165	68	>130	>120
Cold fitter plug Point	°C	7	−4	−3	−20	–	–
Water content	%	0.02	0.01	0.01	0.02	<0.03	<0.05
Ash content	%	0.002	0.001	0.001	0.01	<0.02	<0.02
Carbon residue	%	0.21	0.16	0.18	0.17	–	<0.3
Acid value	mg·KOH/g	0.12	0.013	0.015	0.35	<0.8	<0.5
Calorifiic value	MJ/kg	36.5	37.3	38.1	42	–	–
Distillation temp (90% Vol Red)	°C	352.5	332.5	332.5	–	360	–

was still higher than 95%. The lipases were regenerated by washing with organic solvent after each reaction cycle and could be continuously reused for 20 cycles nearly without any loss of the activity.

The combination use of lipases may decrease the catalyst cost and increase the ME yield of biodiesel production through synergistic effect between lipases. The detailed mechanism of synergistic effect will be further investigated in our following work using methods of the reaction kinetics.

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