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Optimization of whole cell-catalyzed methanolysis of soybean oil for biodiesel production using response surface methodology

Wei Li, Wei Du *, Dehua Liu *

Department of Chemical Engineering, Tsinghua University, Beijing 100084, PR China

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Abstract

Utilizing whole cell biocatalyst instead of free or immobilized enzyme is a potential way to reduce the cost of catalyst in lipase-catalyzed biodiesel production. *Rhizopus oryzae* (*R. oryzae*) IFO4697 whole cell immobilized within biomass support particles (BSPs) was used for the methanolysis of soybean oil for biodiesel production in this paper. *tert*-Butanol was demonstrated to be an ideal reaction medium, in which the negative effects caused by substrate methanol could be eliminated effectively. A central composite design was adopted to study the effect of *tert*-butanol quantity, methanol quantity, water content and dry biomass of the immobilized cell on biodiesel (methyl ester) yield. Each factor was studied in five levels. Using response surface methodology, a quadratic polynomial equation was obtained for methyl ester yield by multiple regression analysis. Biodiesel yield of 72% could be obtained under the optimal conditions and further verification experiments confirmed the validity of the predicted model.

Keywords: Biodiesel; Whole cell; Lipase; Methanolysis

1. Introduction

Biodiesel is a source of renewable energy that is made from biological sources and is an attractive alternative to fossil diesel fuel because of its environmental benefits. It is biodegradable, non-toxic, and has a low emission profile [1,2]. With an increasing focus on renewable sources of energy in terms of marketing, usage, distribution and general acceptance, the opportunities for producers of biodiesel are prominent. Recently, more and more attentions have been paid to enzymatic production of biodiesel, since no complex operations are needed for the recovery of glycerol and also for eliminating the catalyst and salt in comparison with chemical methods using alkaline catalyst. However, the cost of lipase is the main hurdle of the industrialization of lipase-catalyzed biodiesel production [3,4].

Utilizing whole cell biocatalyst for biodiesel fuel production is one way to reduce the cost of lipase since it can avoid the complicated processes of isolation, purification and immobilization of extracellular lipase which account for a large part in the lipase cost [3,4]. It has been demonstrated that *R. oryzae* whole cell could efficiently catalyze the methanolysis of vegetable oils for biodiesel production in solvent-free system [3–5]. Stepwise addition of methanol was recommended to minimize the negative effect of methanol on the activity of *R. oryzae* whole cell [3]. However, stability of the whole cell during repeated uses was poor.

Studies have showed that the stability of lipase in biodiesel production could be significantly enhanced in *tert*-butanol system [6,7]. Poor activity and stability of the lipase in biodiesel production was due to the negative effects caused by excessive methanol and by-product glycerol [6]. While in *tert*-butanol system, both methanol and by-product glycerol are soluble, so the negative effect caused by methanol and glycerol could be eliminated totally. In this paper, *tert*-butanol system was introduced to whole cell-catalyzed biodiesel production for the first time. Comparative study showed that the *R. oryzae* whole cell exhibited much better operational stability in *tert*-butanol system than in solvent-free system. In this study, the reaction conditions of *R. oryzae* whole cell-catalyzed biodiesel production from soybean oil in *tert*-butanol system were further optimized using response surface methodology. Through this approach, the relationships

* Corresponding authors. Fax: +86 10 62785475.
E-mail addresses: duwei@tsinghua.edu.cn (W. Du), dhliu@tsinghua.edu.cn (D. Liu).

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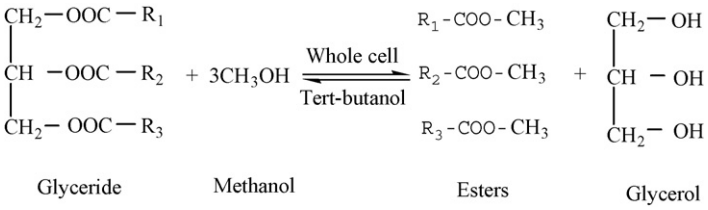

Fig. 1. Whole cell-catalyzed methanolysis of soybean oil for biodiesel production in *tert*-butanol system.

between the variables and their co-influence on methyl ester yield could be analyzed (Fig. 1).

2. Materials and methods

2.1. Materials

Refined soybean oil was obtained locally. The methyl esters of palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and heptadecanoic acid were purchased from Sigma and were chromatographically pure. Other chemicals were obtained commercially and were of analytical grade. *R. oryzae* IFO 4697 whole cell immobilized within biomass support particles was cultivated and prepared in our laboratory as described below.

2.2. R. oryzae whole cell biocatalyst preparation

The culture medium contains in 1l tap water: 30 g soybean oil, 70 g peptone, 1.2 g $NaNO_3$, 1.2 g KH_2PO_4 and 0.5 g $MgSO_4{\cdot}7H_2O$.

Flasks (500 ml) containing 100 ml of the basal medium were inoculated by aseptically transferring spores (about 10^6 spores) from an agar slant, and incubated for about 72 h at 35 °C on a reciprocal shaker (130 oscillations/min) with 80 BSPs subjected to prior sterilization. The BSPs used for immobilization were 5-mm cubes of reticulated polyurethane foam with a particle voidage beyond 97% and a pore size of 50 pores per linear inch.

The BSPs were separated from the culture broth by filtration. After being washed with tap water, the BSPs were frozen in a −70 °C refrigerator, and dried in a freeze dryer for about 6 h.

2.3. General procedure for methanolysis of soybean oil

The methanolysis reactions were carried out in a 50 ml screw-cap bottle at 35 °C on a reciprocal shaker (130 rpm). The compositions of the reaction mixtures were as follows: oils 5 g, *tert*-butanol, methanol, 0.1 M phosphate buffer (pH 6.8, used to adjust water content) and BSPs with dry biomass of *R. oryzae* whole cell. For solvent-free system, methanol was added into the reaction mixture at 0, 24, 48 h, respectively. Fifty microlitre samples were taken from the reaction mixture at 24 h for *tert*-butanol system and 72 h for solvent-free system, centrifuged to obtain the upper layer and analyzed by capillary gas chromatography.

After one batch, the whole cell was filtrated and added directly into a new batch of reaction mixture.

2.4. Analytical procedure

The methyl esters contained in the reaction mixture was analyzed using a GC-14B gas chromatography (Shimadzu, Kyoto, Japan) equipped with a CP-FFAP CB capillary column (25 m × 0.32 mm × 0.30 μm) supplied by Agilent. Five microlitre of the aforementioned mixture and 600 μl of 0.7 mmol/l heptadecanoic acid methyl ester (served as the internal standard) were precisely measured and mixed thoroughly. The column temperature was kept at 180 °C for 0.5 min, heated to 250 °C at 10 °C/min, and then maintained for 6 min. The temperatures of the injector and detector were set at 245 and 250 °C, respectively.

2.5. Experimental design [8–12]

COST experiments including *tert*-butanol quantity, methanol quantity, water content, dry biomass of the immobilized cell, buffer pH, temperature, and rotate speed of the shaker were first conducted using *R. oryzae* whole cell as catalyst for biodiesel production. The first four parameters were further optimized using RSM. A five-level-four-factor central composite design with 30 runs, including six replications at the centre point, was employed for fitting a second-order response surface. The levels of each independent variable were chosen based on the COST experiments. The coded and corresponding uncoded values were given in Table 1. The corresponding central composite experimental design and their values were shown in Table 2. The experiments were carried out in random order. All the design points except the center point (0, 0, 0, 0) were carried out in two replications. The central point was replicated in six times to study the reproducibility.

2.6. Statistical analysis

The experimental data obtained from CCD were analyzed by response surface methodology. A mathematical model, fol-

Table 1
Independent variables and levels used for response surface design

Independent variables	Symbols	Levels				
		−2	−1	0	1	2
tert-Butanol to oil ratio (v/v)	X_1	1.00	1.25	1.50	1.75	2.00
Methanol to oil ratio (mol/mol)	X_2	3.00	4.00	5.00	6.00	7.00
Water content (%)	X_3	1.00	2.00	3.00	4.00	5.00
Dry biomass amount (%)	X_4	6.00	8.00	10.00	12.00	14.00

Table 2
Central composite design arrangement and responses

Design point	Coded independent variable levels				Yield (%)	
	X_1	X_2	X_3	X_4	Predicted	Experimental
1	1	−1	1	1	63.3	62.1
2	0	0	0	0	70.0	71.6
3	1	−1	−1	1	61.4	57.6
4	−1	1	−1	1	56.2	51.8
5	2	0	0	0	57.8	58.5
6	1	−1	−1	−1	56.9	57.2
7	1	1	−1	−1	61.4	62.8
8	0	0	−2	0	50.8	51.3
9	0	0	0	0	70.0	67.4
10	−1	−1	−1	−1	54.2	54.1
11	1	1	1	−1	61.5	58.2
12	0	2	0	0	64.1	70.3
13	1	1	−1	1	64.3	63.5
14	−2	0	0	0	47.0	50.1
15	0	0	0	0	70.0	72.1
16	−1	1	1	−1	53.4	51.3
17	−1	−1	1	−1	54.7	55.8
18	1	−1	−1	1	61.4	61.5
19	0	0	0	0	70.0	68.3
20	0	0	0	0	70.0	68.9
21	0	0	0	0	70.0	71.4
22	1	1	1	1	65.8	63.9
23	−1	−1	−1	1	58.7	60.2
24	−1	1	1	1	63.4	55.6
25	0	0	0	−2	72.3	64.1
26	0	0	0	2	72.3	75.4
27	−1	−1	1	1	60.7	59.6
28	0	−2	0	0	62.5	60.1
29	−1	1	−1	−1	53.3	50.4
30	0	0	2	0	52.8	56.1

lowing a second-order polynomial equation, was developed to describe the relationships between the predicted response variable (methyl ester yield) and the independent variables of methanolysis conditions. It was described as follows:

$$Y_{\text{yield}} = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ij} X_i X_j \qquad (1)$$

where Y_{yield} is the predicted response variable; β_0, β_i, β_{ii}, β_{ij} the intercept, linear, quadratic and interaction constant coefficients of the model, respectively; X_i, X_j ($i = 1, 4$; $j = 1, 4$; $i \neq j$) represent the coded independent variables (methanolysis conditions). Contour plots were developed using the fitted quadratic polynomial equation obtained from regression analysis, holding two of the independent variables at constant values corresponding to the stationary point and changing the other two variables. 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. Confirmatory experiments were carried out to validate the equation, using combinations of independent variables which were not part of the original experimental design but within the experimental region.

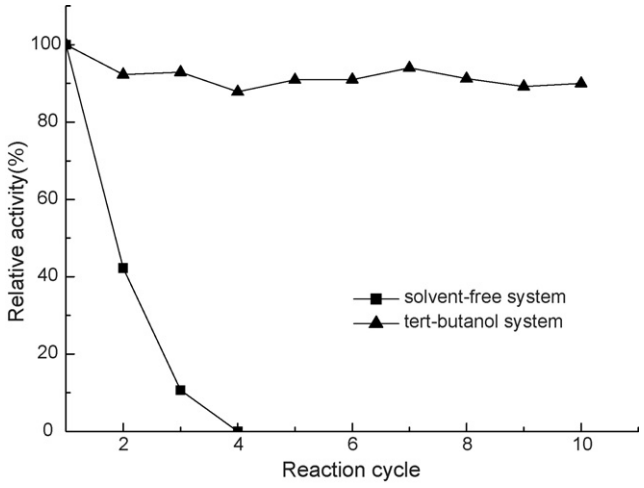

Fig. 2. Comparative study on stability of *R. oryzae* whole cell in solvent-free system and *tert*-butanol system. Solvent-free system: 5% water content (based on oil weight), methanol/oil (mol/mol) 1:1 was added at 0, 24, 48 h, 5% dry biomass (based on oil weight). *tert*-Butanol system: 3% water content (based on oil weight), methanol/oil (mol/mol) 4:1, *tert*-butanol/oil (v/v) 1.5:1, 10% dry biomass (based on oil weight).

3. Results and discussion

3.1. Comparative study on stability of R. oryzae whole cell in solvent-free system and tert-butanol system

The stability of *R. oryzae* whole cell in solvent-free and *tert*-butanol system was compared in Fig. 2. In solvent-free system, the relative activity of *R. oryzae* whole cell dropped quickly during repeated uses. Almost no methyl esters could be detected in the fourth batch. In contract, *R. oryzae* whole cell exhibited good stability in *tert*-butanol system. After ten batches of reaction, the methyl esters yield still remained about 90% of the initial yield.

More studies were carried out to explore the mechanism why lipases lose its activity quickly in solvent-free system. It was found interestingly that much glycerol and methyl ester was detected inside the whole cell in solvent-free system during the repeated uses, while in *tert*-butanol system, almost no glycerol and methyl ester was detected inside the whole cell. The loss in lipase activity in solvent-free system might be caused by the accumulation of glycerol and methyl ester inside cell. But more studies needs to be conducted further to explore the exact mechanism.

3.2. Optimazition of methanolysis conditions

From the result of COST experiments, the parameters of *tert*-butanol quantity, methanol quantity, water content and dry biomass of the immobilized cell were proved to have significant effect on methyl ester yield. Thus, the effects of these four variables were further studied using RSM. Levels of each parameter were selected according to the results of COST experiments.

The experimental central composite design matrix was presented in Tables 1 and 2. Thirty experiments were performed in duplicate except the central point. The methyl ester yield ranged

Table 3
Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of transesterification conditions

Source	Sum of squares	d.f.	Mean square	*F*-value	Probability (*p*)
Model	1352.18	14	96.58	9.90	<0.0001**
Residual	146.29	15	9.75	–	–
Lack of fit	126.51	10	12.65	3.20	0.1057
Pure error	19.77	5	3.95	–	–
Cor total	1498.47	29	–	–	–

$R^2 = 0.9024$
Adj. $R^2 = 0.8113$

** Significant at 1% level.

from 50 to 75%, and the design points of #14 and #26 gave the minimum and maximum yields, respectively.

Table 3 showed the analysis of variance (*F*-test) and the *p*-value for this experiment. The *p*-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength of each parameter. The smaller the *p*-values are, the bigger the significance of the corresponding coefficient. Here, the *p*-value of the model was smaller than 0.0001, which indicated that the model was suitable for use in this experiment. And the "lack of fit *F*-value" of 3.20 implied the "lack of fit" was not significant relative to the pure error. The *p*-value of "lack of fit" was 0.1057 ($p > 0.1$), which indicated that "lack of fit" was insignificant. The coefficient of determination (R^2) and adjust coefficient of determination (Adj. R^2) were also shown in Table 3. The values indicated that, the accuracy and general availability of the polynomial model were adequate.

The regression coefficients and the corresponding *p*-values were presented in Table 4. From the *p*-values of each model term, it could be concluded that the regression coefficients of the linear term X_1, X_4, the interaction term of X_1X_2 and all the quadratic terms except X_4^2 had significant effect on the methyl ester yield. Among them, X_1, X_4, X_1^2, X_3^2 were significant at 1% level, while X_2^2 and the interaction X_1X_2 was significant at 5% level.

Table 4
Results of regression analysis of a full second-order polynomial model for optimization of methanolysis conditions

Term	Coefficients estimated	*p*-Value
Intercept	69.95	<0.0001**
β_1	2.70	0.0007**
β_2	0.41	0.5315
β_3	0.51	0.4376
β_4	2.22	0.0033**
$\beta_1\beta_2$	1.91	0.0271*
$\beta_1\beta_3$	−0.56	0.4823
$\beta_1\beta_4$	−0.025	0.9749
$\beta_2\beta_3$	−0.100	0.8998
$\beta_2\beta_4$	−0.41	0.6050
$\beta_3\beta_4$	0.36	0.6491
β_1^2	−4.39	<0.0001**
β_2^2	−1.67	0.0135*
β_3^2	−4.54	<0.0001**
β_4^2	−0.53	0.3870

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

Using the designed experimental data (Table 2), the polynomial model for the yield of fatty methyl ester was regressed and shown as below (in terms of coded factors):

$$Y_{\text{yield}} = 69.95 + 2.70X_1 + 0.41X_2 + 0.51X_3 + 2.22X_4 + 1.91X_1X_2 - 0.56X_1X_3 - 0.025X_1X_4 - 0.100X_2X_3 - 0.41X_2X_4 + 0.36X_3X_4 - 4.39X_1^2 - 1.67X_2^2 - 4.54X_3^2 - 0.53X_4^2 \quad (2)$$

From Table 4 and Eq. (2), it could be concluded that the linear effect of X_1, X_4 and the quadric effect of X_1^2, X_3^2 were the primary determining factors of the responses on Y_{yield} as they had the largest coefficient. Meanwhile, the quadric effect of X_2^2 and the interaction effect of X_1X_2 were the secondary determining factors with medium coefficient. Other terms of the model had no significant effect on Y_{yield}. Positive coefficient of X_1, X_4 and interaction term X_1X_2 indicated an enhancement on Y_{yield}. However, all the quadratic terms had negative effects on Y_{yield}. The relationships between independent and dependent variables of the developed model were shown in Figs. 3–8 in the form of con-

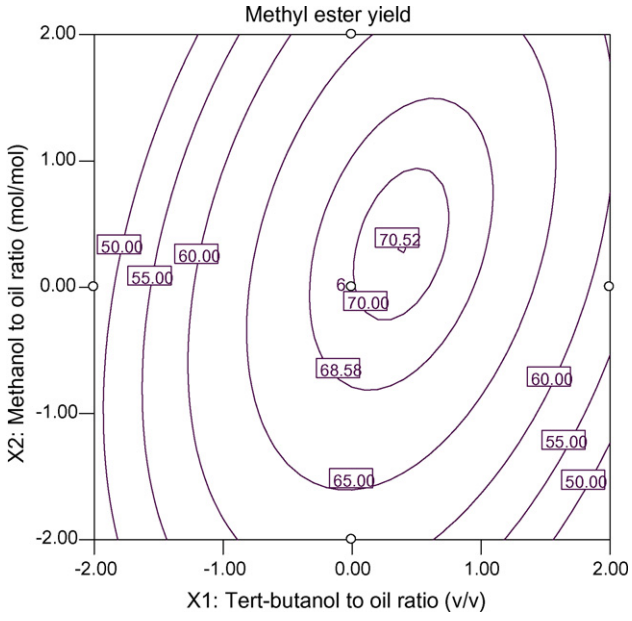

Fig. 3. Contour plots of methyl ester yield predicted from the model at 3% water content and 10% dry biomass amount.

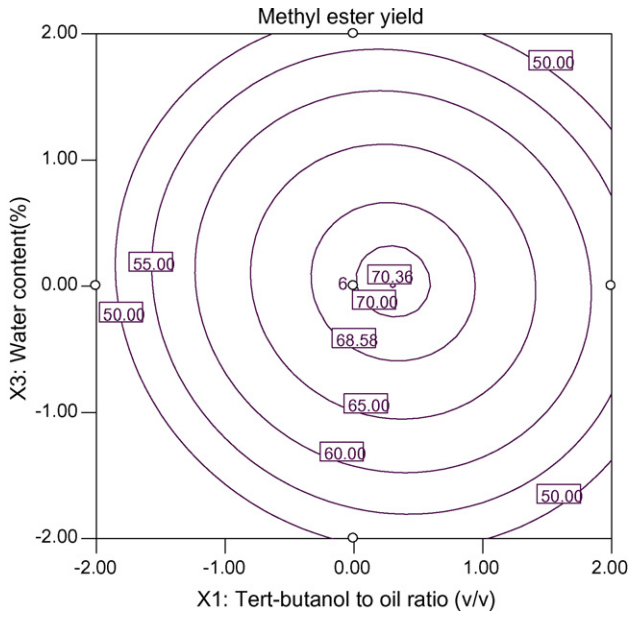

Fig. 4. Contour plots of methyl ester yield predicted from the model at 10% dry biomass amount and 5 molar ratio of methanol to oil.

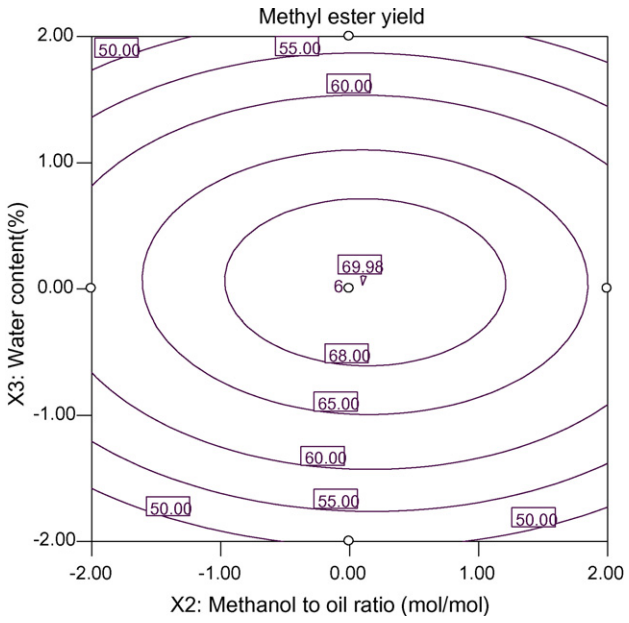

Fig. 6. Contour plots of methyl ester yield predicted from the model at 1.5 volume ratio of *tert*-butanol to oil and 3% water content.

tour plots. From the shape of contour plots, the significance of the mutual interactions between the independent variables could be estimated. An elliptical profile of the contour plots indicates remarkable interaction between the independent variables.

The contour plots were generally the graphical representation of the regression equation for the optimization of methanolysis conditions. Figs. 3–8 presented the effect of two variables on methyl ester yield, while the other two variables were held at a constant level.

Fig. 3 showed the interaction relationship between the two independent variables (volume ratio of *tert*-butanol/oil, molar ratio of methanol/oil) and their effects on the response variable (methyl ester yield). It was indicated that the methyl ester yield was more sensitive to the volume ratio of *tert*-butanol to oil. An increase in methyl ester yield was observed with the increasing of the volume ratio of *tert*-butanol to oil at first. But the trend was reversed when the ratio reached a certain value. It could be interpreted that, under a certain amount, the solvent *tert*-butanol was used to improve the solubility of methanol and water in soybean oil. With the increasing of *tert*-butanol amount, the reaction mixture would become well mixed, thus the decrease of enzyme activity caused by excessive methanol was reduced. However, when the *tert*-butanol amount surpassed the critical

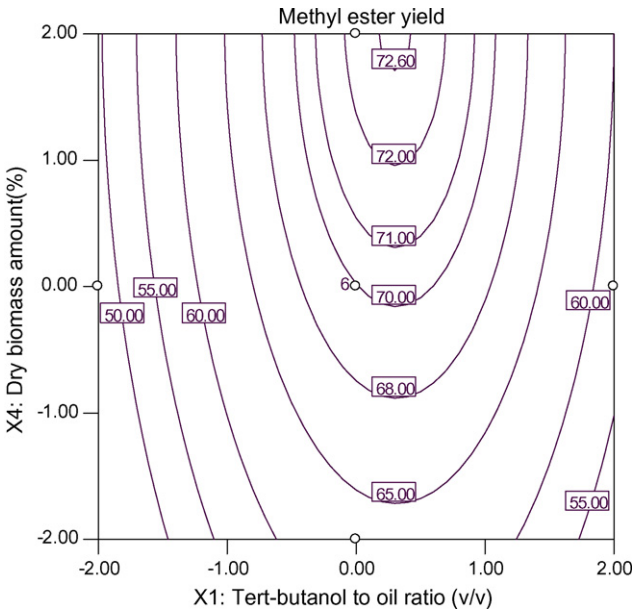

Fig. 5. Contour plots of methyl ester yield predicted from the model at 5 molar ratio of methanol to oil and 3% water content.

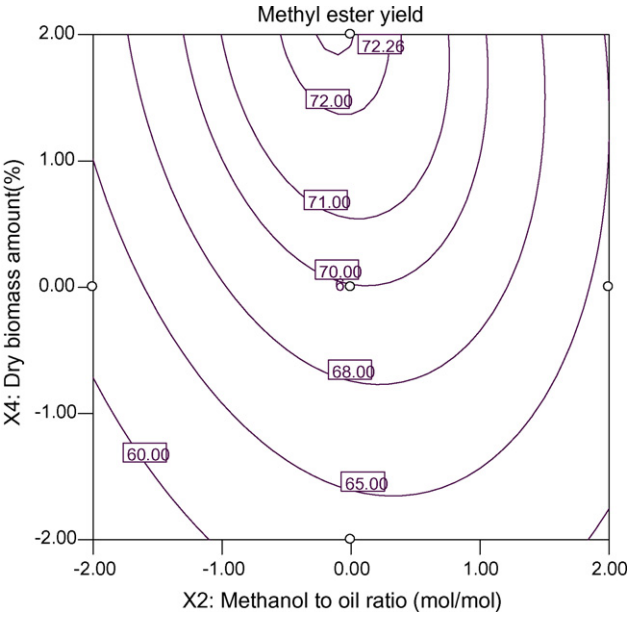

Fig. 7. Contour plots of methyl ester yield predicted from the model at 1.5 volume ratio of *tert*-butanol to oil and 10% dry biomass amount.

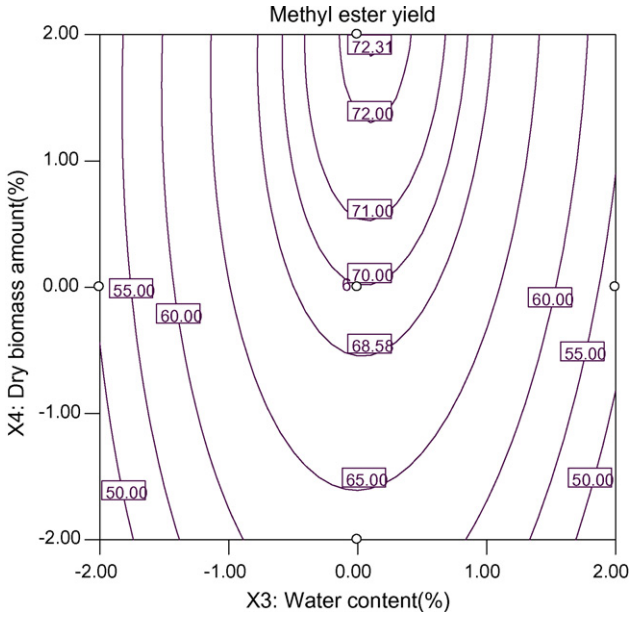

Fig. 8. Contour plots of methyl ester yield predicted from the model at 1.5 volume ratio of *tert*-butanol to oil and 5 molar ratio of methanol to oil.

value, the methyl ester yield decreased due to the dilution of substrate concentration by excessive solvent. The effect of methanol amount on Y_{yield} followed the similar trend. With the increasing of methanol amount, Y_{yield} first increased and then decreased as a result of the decrease of enzyme activity caused by excessive methanol. A maximal contour ($Y_{yield} = 0.705$) could be determined under certain condition. The elliptical profile of the contour plot indicated that the interaction between the amount of *tert*-butanol and the amount of methanol was strong. The slantwise elliptical represented that with the increasing of *tert*-butanol amount, the system could endure more amount of methanol. This was consistent with the physical explanation. The interaction between *tert*-butanol/oil ratio and water content (Fig. 4) as well as the interaction between methanol/oil ratio and water content (Fig. 6) followed nearly the same pattern.

Figs. 5, 7 and 8 showed the interaction between dry biomass of whole cell and other three parameters (*tert*-butanol/oil ratio, methanol/oil ratio and water content). The mutual interactions between dry biomass of whole cell and all the other three parameters were poor. With the increasing of dry biomass amount, the response Y_{yield} was always increasing. However, the slope slowed down gradually.

3.3. Validation of the model

Optimal methanolysis conditions were obtained through the regression model (Eq. (2)) according to the limit criterion of maximum response Y_{yield}. The optimum conditions of *R. oryzae* whole cell-catalyzed methanolysis of soybean oil for biodiesel production in *tert*-butanol system were: 1.6 (v/v) ratio of *tert*-butanol to oil, 5.2 (mol/mol) ratio of methanol to oil, 3.1% water content (based on oil weight) and 12% dry biomass of whole cell. The accuracy of the model was validated with triplicate experiments under the aforementioned optimal methanolysis conditions. Verification experiments confirmed the validity of the predicted model. As a result, the model from CCD was considered to be accurate and reliable for predicting the yield of methyl ester.

4. Conclusion

RSM was proved to be a powerful tool for the optimization of methanolysis conditions catalyzed by *R. oryzae* whole cell in *tert*-butanol system. A second-order model was obtained to describe the relationship between the methyl ester yield and the parameters (*tert*-butanol to oil ratio, methanol to oil ratio, water content and dry biomass of the whole cell). Under optimal condition (1.6 (v/v) ratio of *tert*-butanol to oil, 5.2 (mol/mol) ratio of methanol to oil, 3.1% water content and 12% dry biomass of whole cell), the predicted values of methyl ester yield was about 72%. Validation experiments verified the availability and the accuracy of the model. The predicted value was in agreement with the experimental value. The study also provided useful information and reference for the conditions optimization of other enzymatic alcoholysis processes.

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