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Optimization of Biodiesel Production Catalyzed by Fungus Cells Immobilized in Fibrous Supports

Jyh-Ping Chen · Gen-Hsu Lin

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Abstract A circulating packed-bed bioreactor system using fibrous nonwoven fabric as the immobilization matrix was suitable for simultaneous cell growth and immobilization of *Rhizopus oryzae* fungus cells, which could be used for lipase-mediated production of biodiesel by methanolysis of soybean oil. Response surface methodology and 5-level-5-factor central composite rotatable design was proved to be a powerful tool for the optimization of methanolysis conditions catalyzed by immobilized *R. oryzae* whole cell biocatalyst. A quadratic polynomial regression model was used to analyze the relationship between the yield and the significant reaction parameters. The analysis confirmed that water content, molar ratio of methanol to oil, cell weight, and reaction time were the significant factors affecting the yield at a 95% confidence level ($p<0.05$). Under the optimum condition at 10.97% (*w*/*w*) water content, 0.64 molar ratio of methanol to oil, 2.25% (*w*/*w*) cell weight, and 23.3 h reaction time, the predicted value of yield was 72.6%. Validation experiments with yields of 70.77±2.46% verified the availability and the accuracy of the model.

Keywords Biodiesel · Immobilized cells · Lipase · Response surface methodology · Nonwoven · Whole cell biocatalyst · Transesterification · Fungus cells

Introduction

Production of biodiesel by lipase-catalyzed enzymatic reaction has several advantages over alkaline-catalyzed chemical process such as glycerol recovery, wastewater treatment, and alleviating interference from free fatty acids and water [1]. Both extracellular and intracellular microbial lipases could effectively catalyze transesterification (methanolysis) of oils by short-chain alcohols to produce biodiesel. However, the cost of using lipase as a biocatalyst is significantly higher than that of using chemical catalyst such as sodium

J.-P. Chen (✉) · G.-H. Lin
Department of Chemical and Materials Engineering, Chang Gung University, Kwei-San, Taoyuan 333 Taiwan, Republic of China
e-mail: jpchen@mail.cgu.edu.tw

hydroxide [2, 3]. Effective methanolysis of oils using extracellular microbial lipase have been studied by several researchers [4–12]. Although lipase-catalyzed methanolysis reaction for biodiesel production could be carried out by using immobilized extracellular lipase with an aim to reuse the enzyme and cut down enzyme cost, extracellular enzymes may require purification procedures that are too complex and costly for industrial applications [13]. Furthermore, enzymes recovered through such operations are generally unstable. Utilizing whole cell biocatalyst with intracellular lipase activity for biodiesel production is an alternative 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 of the enzyme cost [14, 15].

Rhizopus oryzae fungus cells have been demonstrated to efficiently catalyze the methanolysis of vegetable oils for biodiesel production in solvent-free systems [14, 16, 17]. To efficiently reuse the whole cell biocatalyst, the cells were subsequently immobilized within biomass support particles made out of reticulated polyurethane foam [18, 19]. Immobilizing cells to biomass support particles allows for easy separation of the whole cell biocatalyst from the reaction mixture and facilitates its reuse during repeated batch reaction. Acyl migration was also observed during whole cell-mediated methanolysis for biodiesel production, and it was demonstrated that acyl migration was promoted with the increase of water content in the reaction mixture [20]. When methanolysis was carried out with the immobilized cells in the presence of water, the level of methyl ester (ME) production is almost the same as that achievable with extracellular lipase but with substantial reduction in the cost of immobilized biocatalyst preparation [21]. These findings suggested that the use of whole cell biocatalysts immobilized within a suitable matrix may offer a promising means of biodiesel production for industrial application with the simplicity of the biocatalyst production process.

Nonwoven fabric is a suitable macroporous fibrous matrix for immobilization of fungus cells. In contrast to extracellular lipase, no enzyme purification and immobilization steps are required when preparing the immobilized whole cell biocatalysts since cell immobilization could be achieved spontaneously during cell growth [22]. In this study, for industrial biodiesel production, immobilized whole cell biocatalyst was prepared from *R. oryzae* cells in a circulating packed-bed bioreactor system using low cost porous nonwoven fabric as the support matrix for cell immobilization. The reaction conditions of the whole cell-catalyzed biodiesel production from soybean oil were optimized using response surface methodology (RSM).

Materials and Methods

Microorganism and Culture Media

R. oryzae (ATCC24563, CCRC31861) was used as the microorganism in this study. The solid culture medium used for culture contains 300 g diced potatoes, 20 g glucose, and 15 g agar in 1 l of distilled water. The liquid culture medium used for culture is 5% corn-steep liquor, 2% olive oil, 0.2% KH_2PO_4, 0.05% KCl, 0.05% $NaNO_3$, and 0.05% $MgSO_4 \cdot 7H_2O$ (all in *w*/*v*), pH 4.6.

Cell Culture and Cell Immobilization

Fungus mycelia grown at 30 °C were transferred from the surface of solid culture media with culture loop to a slope solid culture medium. Cells grown on the slope for 96 h were

washed with 5 ml distilled water and loosen with culture loop to prepare a spore solution with $2\sim5\times10^9$ spores per liter under aseptic condition. A poly(ethylene terephthalate)/polyethylene (PET/PE) nonwoven fabric (length 12.5 cm × width 3.0 cm × thickness 0.5 mm, supplied by Taiwan Textile Research Institute) and a stainless steel mesh of the same dimensions were layered together and rolled into a cylinder with 3 cm diameter to be used as the fibrous cell immobilization matrix (Fig. 1). Four pieces of immobilization matrices prepared above were placed inside a glass column (3 cm ID × 13 cm length), and culture medium was circulated through the column by a peristaltic pump from a flask filled with the culture medium. All tubings, fittings, and cell immobilization matrices were sterilized by autoclaving at 120 °C for 30 min and cooled to room temperature before use. The bioreactor system set up for cell culture is shown in Fig. 2. The bioreactor was a 500 ml flask filled with 450 ml culture medium and 45 μl spore solution. Cell growth and immobilization was carried out at 4 vvm air flow rate and 30 °C. The medium circulation velocity was set at 80 ml/min with flow direction was changed every 24 h. Immobilized cells were harvested after 60 to 84 h, dried under vacuum, and weighed.

Immobilized cells were immersed in acetone for 90 min for permeabilization, and permeabilized cells were incubated at 30 °C under vacuum for 24 h [23]. Treatment with soybean oil was followed by incubating the permeabilized cells in soybean oil for 72 h [24]. These treatments were found from the experiments to give immobilized whole cell biocatalyst with the highest transesterification activity.

Production of Biodiesel by Immobilized Whole Cell Biocatalyst

For transesterification of soybean oil with methanol, one piece of immobilization matrix containing 1.05 g dried cells as prepared above were placed in a 50 ml screw-capped sample tube, followed by adding a suitable amount of soybean oil (Wako 1st Grade, Wako Pure Chemical Industries, Ltd.), methanol, and water based on the experiment condition to reach a predetermined water content, cell concentration, and substrate molar ratio (Table 1). One molar equivalent of 38.6 g of soybean oil is 1.4 g of methanol [25]. The reaction mixture was incubated in a shaking incubator at 150 rpm at a fixed temperature (Table 1). Two hundred microliter reaction solution was removed at a fixed time (Table 1), centrifuged at 10,000 rpm, and mixed with an equal volume of internal standard (tricaprylin) in hexane. The ME content in the reaction mixture was determined by gas chromatography (Varian Chrompack CP-3380). A DB-5 GC column from J&W Scientific (column length, 15 m; ID,

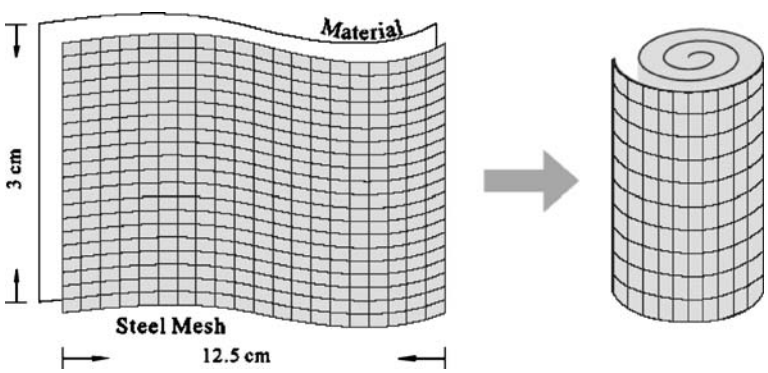

Fig. 1 Configuration of nonwoven material supported by steel mesh as the matrix for cell immobilization

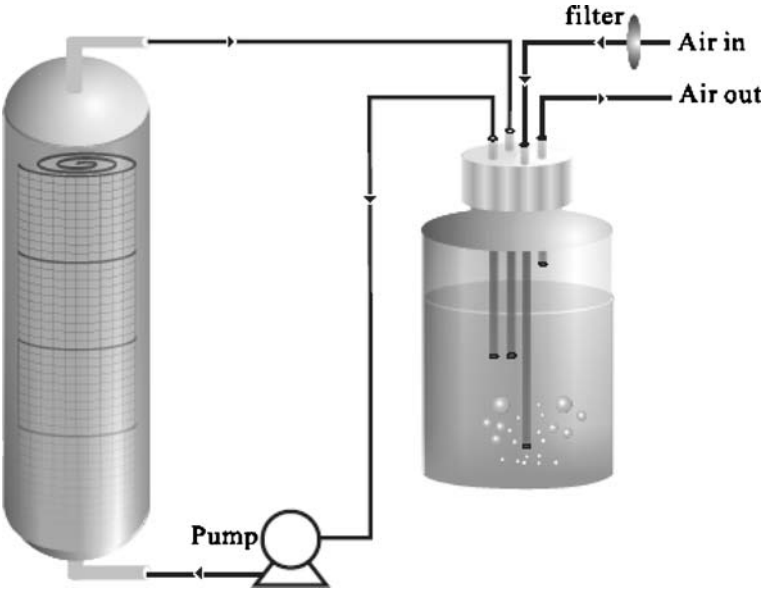

Fig. 2 Bioreactor system for culture of immobilized cell

0.32 mm; and film thickness, 0.25 μm) was used for analysis. The injector temperature was at 270 °C. The column temperature was at 150 °C for 0.5 min and increased at 10 °C/min to 300 °C and held for additional 2 min. The FID detector temperature was 300 °C, and the flow rate of carrier gas (N_2) was 30 ml/min. Three ME peaks (palmitic acid ME, oleic acid ME, and linoleic acid ME) were identified by comparison of their retention times with those of standard solutions, and concentration of ME was calculated base on a standard curve constructed for each ME. The conversion yield (%) was calculated based on the limiting reactant and defined as the molar ratio (%) of ME to the limiting reactant.

Experiments studying the influence of a single parameter on yield (24 h) were first conducted. The parameters under study include reaction time, reaction temperature, substrate molar ratio, water content, and cell concentration. All five parameters were found to be significant and were further optimized. A five-level-five-factor central composite

Table 1 Independent variables and their levels used in experimental design.

	X_1	X_2	X_3	X_4	X_5
Level	Time (h)	Temperature (°C)	Water content (% (*w*/*w*) of oil)	Substrate molar ratio (methanol/oil)	Cell concentration (% (*w*/*w*) of oil)
−2	12	20	0	0.5	1.0
−1	16	25	5	1.0	1.5
0	20	30	10	1.5	2.0
1	24	35	15	2.0	2.5
2	28	40	20	2.5	3.0

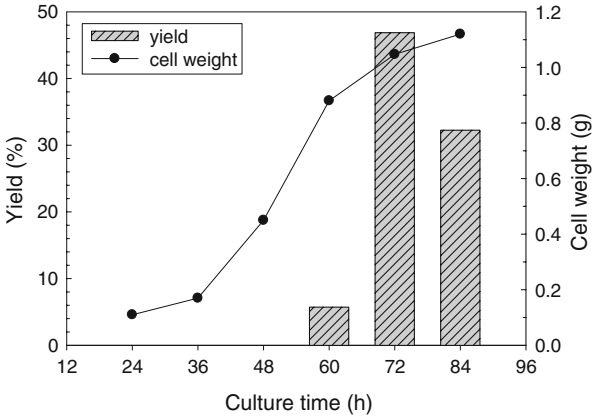

Fig. 3 Influence of cell culture time on the weight (*line with a dot*) of immobilized cells and methyl ester yield (*filled bar with slant lines*) when the immobilized whole cell biocatalyst was used for methanolysis of soybean oil. Transesterification reaction was carried out at 30 °C, substrate molar ratio 1 (methanol/oil), cell concentration 2.5% (*w*/*w*), water content 5% (*w*/*w*), and reaction time 24 h

rotatable design (CCRD) with 32 runs, including six replications at the center point, was employed for fitting a second-order response surface. The levels of each independent variable were chosen based on single parameter experiments. The coded and corresponding uncoded values were given in Table 1.

The experimental data obtained from CCRD were analyzed by RSM. A mathematical model, following a second-order polynomial equation, was developed to describe the

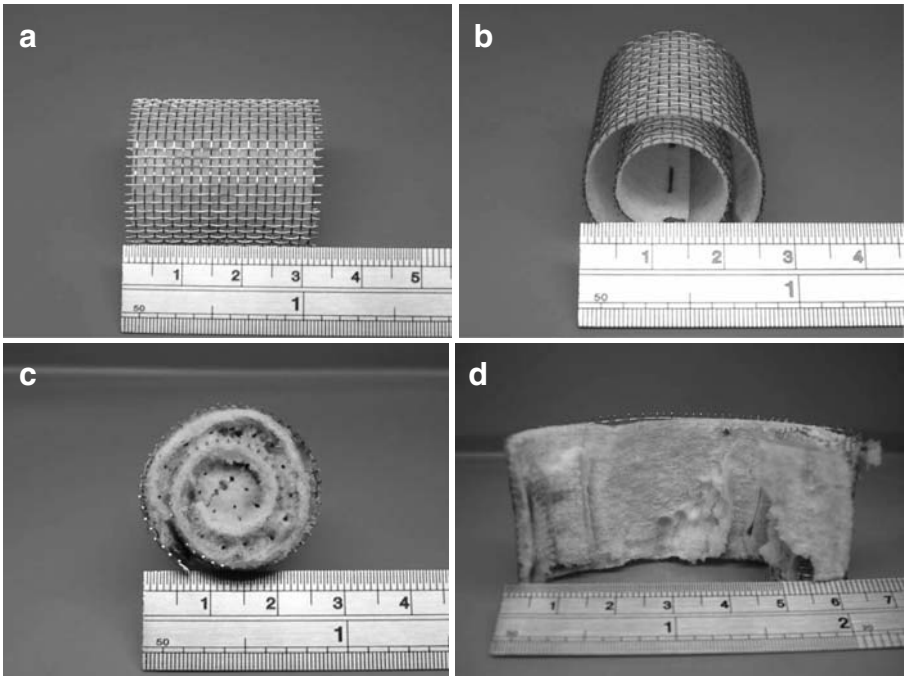

Fig. 4 Pictures of fibrous matrix before (**a** and **b**) and after (**c** and **d**) cell immobilization

relationships between the predicted response variable (yield) and the independent variables (reaction conditions). Response surface plots were developed using the fitted quadratic polynomial equation obtained from regression analysis, holding two of the independent variables at constant value. The quality of the fit of the polynomial model equation was evaluated

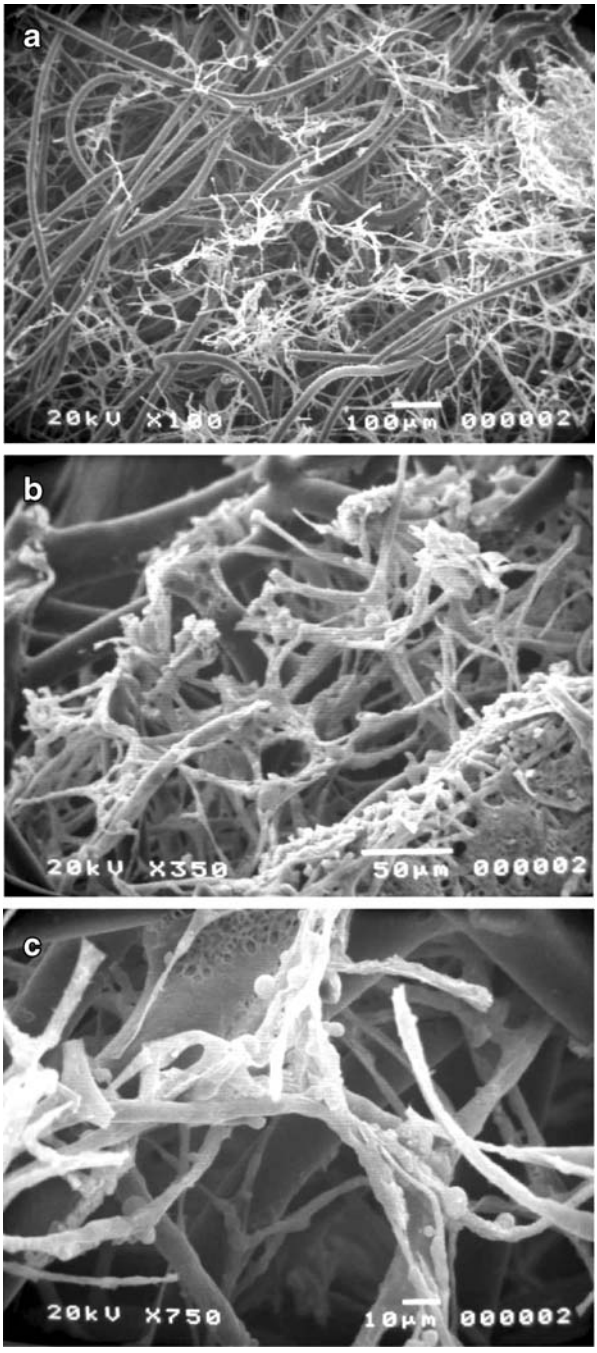

Fig. 5 SEM micrographs of cells immobilized in fibrous matrix. Magnification is **a** 100×, **b** 350×, and **c** 750×

by the coefficient of determination, 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.

Results and Discussion

Preparation of Immobilized Whole Cell Biocatalyst in a Bioreactor

Lipase from *R. oryzae* ATCC24563 is 1,3-regiospecific lipase. Lipase-mediated acyl migration was observed for intracellular lipase from this strain where isomerization of 1,2-dipalmitin into 1,3-isomer was found in the presence of water [26]. Olive oil was added to

Table 2 Central composite rotatable design arrangement and response.

Design point	X_1	X_2	X_3	X_4	X_5	Yield (%)
1	−1 (16)	−1 (25)	−1 (5)	−1 (1)	1 (2.5)	48.18
2	1 (24)	−1 (25)	−1 (5)	−1 (1)	−1 (1.5)	31.77
3	−1 (16)	1 (35)	−1 (5)	−1 (1)	−1 (1.5)	43.65
4	1 (24)	1 (35)	−1 (5)	−1 (1)	1 (2.5)	60.28
5	−1 (16)	−1 (25)	1 (15)	−1 (1)	−1 (1.5)	35.56
6	1 (24)	−1 (25)	1 (15)	−1 (1)	1 (2.5)	62.86
7	−1 (16)	1 (35)	1 (15)	−1 (1)	1 (2.5)	43.28
8	1 (24)	1 (35)	1 (15)	−1 (1)	−1 (1.5)	58.27
9	−1 (16)	−1 (25)	−1 (5)	1 (2)	−1 (1.5)	22.17
10	1 (24)	−1 (25)	−1 (5)	1 (2)	1 (2.5)	43.77
11	−1 (16)	1 (35)	−1 (5)	1 (2)	1 (2.5)	23.58
12	1 (24)	1 (35)	−1 (5)	1 (2)	−1 (1.5)	4.84
13	−1 (16)	−1 (25)	1 (15)	1 (2)	1 (2.5)	32.40
14	1 (24)	−1 (25)	1 (15)	1 (2)	−1 (1.5)	25.88
15	−1 (16)	1 (35)	1 (15)	1 (2)	−1 (1.5)	20.28
16	1 (24)	1 (35)	1 (15)	1 (2)	1 (2.5)	43.24
17	−2 (12)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	20.43
18	2 (28)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	47.58
19	0 (20)	−2 (20)	0 (10)	0 (1.5)	0 (2.0)	28.77
20	0 (20)	2 (40)	0 (10)	0 (1.5)	0 (2.0)	27.92
21	0 (20)	0 (30)	−2 (0)	0 (1.5)	0 (2.0)	5.62
22	0 (20)	0 (30)	2 (20)	0 (1.5)	0 (2.0)	38.64
23	0 (20)	0 (30)	0 (10)	−2 (0.5)	0 (2.0)	60.96
24	0 (20)	0 (30)	0 (10)	2 (2.5)	0 (2.0)	39.27
25	0 (20)	0 (30)	0 (10)	0 (1.5)	−2 (1.0)	19.01
26	0 (20)	0 (30)	0 (10)	0 (1.5)	2 (3.0)	42.25
27	0 (20)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	42.93
28	0 (20)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	39.41
29	0 (20)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	39.24
30	0 (20)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	38.44
31	0 (20)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	40.55
32	0 (20)	0 (30)	0 (10)	0 (1.5)	0 (2.0)	40.00

Table 3 Analysis of variance for full model and reduced model.

	Full model		Reduced model	
	df	*p* value[a]	*df*	*p* value
Time (X_1)	6	0.0327	5	0.0182
Temperature (X_2)	6	0.3036[b]	–	–
Water content (X_3)	6	0.0249	5	0.0155
Substrate molar ratio (X_4)	6	0.0012	5	0.0004
Cell concentration (X_5)	6	0.0091	5	0.0042

[a] *p* value is the level of significance

[b] Not significant at $p=0.05$

the liquid culture medium since it was reported that the addition of vegetable oil to the culture medium of *R. oryzae* ATCC24563 increased both the lipase activity and cell growth up to three folds compared to medium without oil [27]. To obtain a suitable cell culture time for preparation of the whole cell biocatalyst, the weights of immobilized cells are reported in Fig. 3 as a function of cell culture time, together with ME yield (24 h) when the whole cell biocatalyst was used in methanolysis of soybean oil. A culture time of 72 h was chosen as the most suitable cell culture time due to the highest yield and saturation of cell weight. After permeabilization treatment, the immobilized whole cell biocatalyst showed a specific activity of 1.56 U/g cell (1 unit corresponds to 1 millimole ME produced per hour). Weight of dried cell is 1.05 g per piece of immobilization matrix, corresponding to 1.87 g cells/cm^3 nonwoven. The appearance and SEM photographs of the nonwoven matrices and immobilized cells are shown in Figs. 4 and 5. Cells can be seen to attach to the nonwoven fibrous matrix and fill up the empty space between fibers. Fungus mycelia attached firmly to the fiber surface of the nonwoven fabric, facilitating its use as the whole cell biocatalyst for biodiesel production.

Production of Biodiesel by Immobilized Whole Cell Biocatalyst

From the results of single parameter experiments, reaction time, reaction temperature, substrate molar ratio, water content, and cell concentration were found to have significant effect on yield. Thus, the effects of these variables were further studied using RSM. The

Table 4 Analysis of variance for the fitted quadratic polynomial model.

Source	*df*	Sum of squares	Mean square	*F* value	*p* value
Model	14	5,428.21		6.95	0.0002
Linear	4	4,003.29		17.94	< 0.0001
Quadratic	4	835.68		3.75	0.0231
Cross product	6	589.24		1.76	0.1675
Lack of fit	10	782.55	72.85	2.32	0.1380
Pure error	7	219.65	31.38		
Total error	17	948.19	55.77		
R^2					0.8513

R^2 is the coefficient of determination

experimental CCRD matrix was presented in Table 2. Thirty-two experiments were performed in duplicate except the central point. The yield ranged from 5 to 63%, and the design points of number 12 and number 6 gave the minimum and maximum yields, respectively. Table 3 shows the analysis of variance for the parameters used in the experiment. Since p value for X_2 (reaction temperature) is higher than 0.05, it was dropped out from the full model and only four parameters were used as a reduced model to obtain the regression equation. Table 4 showed the analysis of variance and the p values 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 0.0002, which indicated that the model was suitable for use in this experiment (significant at 1% level). The F value of "lack of fit" was 2.32, which implied that the "lack of fit" was not significant relative to the pure error. The p value of "lack of fit" was 0.138 ($p>$ 0.1), which indicated that "lack of fit" was insignificant. The coefficient of determination (R^2) indicated that the accuracy and general availability of the polynomial model were adequate. The regression coefficients were presented in Table 5.

Using the designed experimental data (Table 1), a polynomial model for the ME yield was regressed and it is shown below as (in terms of coded factors):

$$\begin{aligned} Y_{\text{yield}} = {} & 37.16 + 4.83\ X_1 + 4.63\ X_3 - 8.78\ X_4 + 6.70\ X_5 - 0.23\ X_1^2 - 3.25\ X_3^2 + 3.8\ X_4^2 \\ & - 1.08\ X_5^2 + 3.42\ X_1X_3 - 1.47\ X_1X_4 + 0.77\ X_3X_4 + 4.03\ X_1X_5 - 1.99\ X_3X_5 \\ & + 1.47\ X_4X_5 \end{aligned}$$

From Table 5 and the correlation equation, it could be concluded that the linear effects of X_1, X_3, X_4, and X_5, the quadric effects of X_3 and X_4, and the interaction effects of X_1X_3, and X_1X_5 were the primary determining factors of the response Y_{yield} as they had the largest coefficients. Meanwhile, the quadric effect of X_5 and the interaction effects of X_1X_4, X_3X_5,

Table 5 Results of regression analysis of a full second-order polynomial model.

Parameter	Coefficient	Standard errors	t value
Intercept	37.16	2.64	14.07
X_1	4.83	1.52	3.17
X_3	4.63	1.52	3.04
X_4	−8.78	1.52	−5.76
X_5	6.70	1.52	4.39
X_1^2	−0.23	1.37	−0.17
X_3^2	−3.25	1.37	−2.36
X_4^2	3.80	1.37	2.76
X_5^2	−1.08	1.37	−0.78
X_1X_3	3.42	1.87	1.83
X_1X_4	−1.47	1.87	−0.78
X_1X_5	4.03	1.87	2.16
X_3X_4	0.77	1.87	0.41
X_3X_5	−1.99	1.87	−1.07
X_4X_5	1.47	1.87	−0.79

and X_4X_5 were the secondary determining factors with medium coefficients. Other terms of the model had no significant effect on Y_{yield}. Positive coefficient indicated an enhancement effect on Y_{yield} and all the linear terms had positive effects on Y_{yield} except X_4 (substrate molar ratio). Therefore, adding more methanol to the reaction system above a substrate molar ratio 0.5 will decrease yield, presumably, originating from the inactivation effect of methanol on lipase during the reaction.

The relationships between independent and dependent variables of the developed model were shown in Figs. 6, 7, and 8. From Fig. 6a, the yield is higher for longer reaction time and lower substrate molar ratio, but the increase of yield with reaction time is less pronounced at higher substrate molar ratio due to the inactivation effect of methanol as mentioned above. From Fig. 6b, the inactivation effect of methanol could be alleviated by

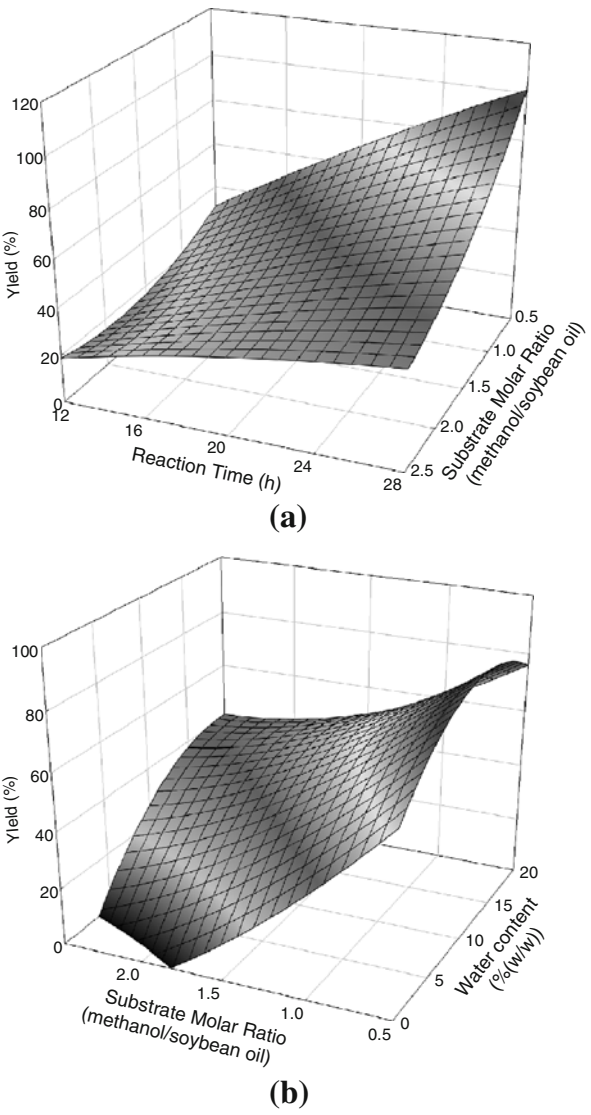

Fig. 6 Response surface plots of yield to **a** reaction time and substrate molar ratio (water content 15%; cell concentration 2.5%) and **b** substrate molar ratio and water content (reaction time 24 h; cell concentration 2.5%)

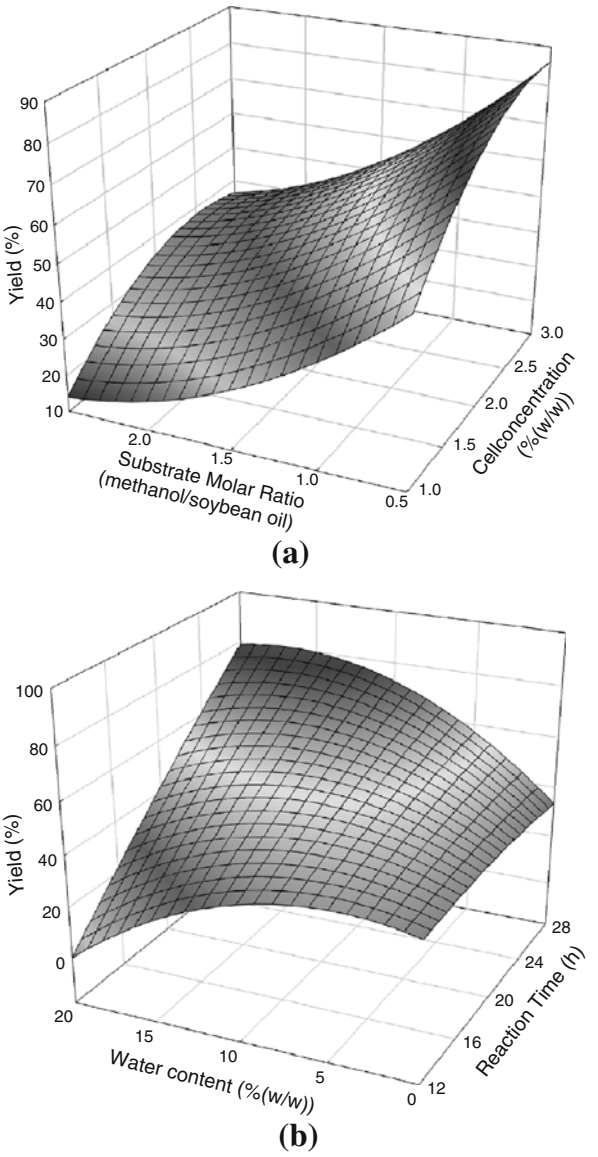

Fig. 7 Response surface plots of yield to **a** substrate molar ratio and cell concentration (water content 15%; reaction time 24 h) and **b** water content and reaction time (substrate molar ratio 1; cell concentration 2.5%)

increasing the water content in the system, and optimum water content could be found for substrate molar ratio of less than one. This effect could be the synergistic effect of polar ethanol and water on the activity of enzyme activity. From Fig. 7a, increasing cell concentration could only partially offset the inactivation effect of methanol and a reasonable yield could only be achieved at a substrate molar ratio below one irrespective of the amount of cells added. The best yield could be obtained by using the highest amount of cells and the lowest substrate molar ratio. From Fig. 7b, water content showed significant influence on yield at longer reaction time. Higher yield could be obtained by using more water. From Fig. 8a, the optimum water content and cell concentration occurred at the midpoint of the experiment conditions. From Fig. 8b, the yield showed strong dependence

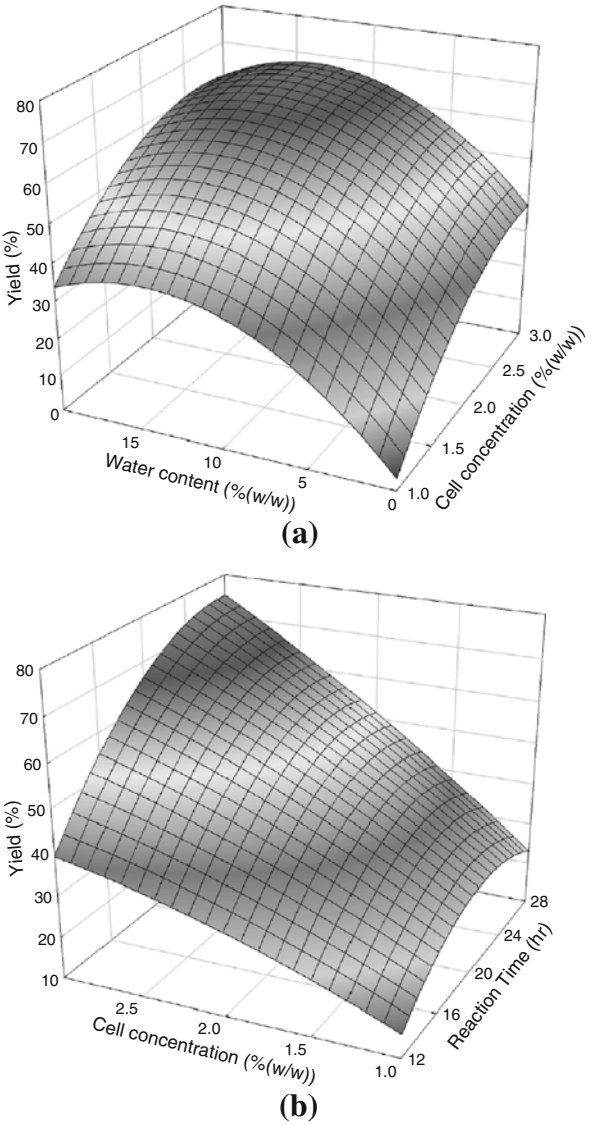

Fig. 8 Response surface plots of yield to **a** water content and cell concentration (reaction time 24 h; substrate molar ratio 1) and **b** cell concentration and reaction time (water content 15%; substrate molar ratio 1)

on cell concentration and reaction time; a higher cell concentration or a longer reaction time could substantially enhance yield.

Optimum reaction conditions were obtained through the regression model according to the limit criterion of maximum response Y_{yield}. The optimum conditions of whole cell-catalyzed methanolysis of soybean oil for biodiesel production system were 10.97% (*w*/*w*) water content (based on oil), 0.64 molar ratio of methanol to oil, 2.25% (*w*/*w*) cell weight (based on oil), and 23.3 h reaction time. The accuracy of the model was validated with repeated experiments (N=4) under the aforementioned optimum conditions. The experiment yields were 70.77±2.46% in contrast with the predicted value of 72.61%. Therefore, verification experiments confirmed the validity of the predicted model, and the model from

CCRD was considered to be accurate and reliable for predicting the yield during biodiesel (ME) production.

In conclusion, a circulating packed-bed bioreactor system using fibrous nonwoven fabric as the immobilization matrix was suitable for simultaneous cell growth and immobilization of *R. oryzae* fungus cells. The optimum culture time considering both cell weight and transesterification activity is 72 h. Response surface methodology was proved to be a powerful tool for the optimization of methanolysis conditions catalyzed by immobilized *R. oryzae* whole cell biocatalyst. A second-order model could be obtained to describe the relationship between the yield and the reaction parameters (water content, substrate molar ratio, cell concentration, and reaction time). Under the optimum condition, the predicted yield was 72.6%, which could be verified from independent validation experiments. This study also provides useful information and reference for optimization of other enzymatic alcoholysis processes.

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