Transactions C: Chemistry and Chemical Engineering
Vol. 17, No. 1, pp. 97–105

Research Note

Empirical Modeling of the Enzymatic Methanolysis of Canola Oil

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Abstract. *A statistics based design of experiments (Central Composite Design, CCD) was used in the present work to develop an empirical model for describing, quantitatively, the methanolysis reaction of canola oil by the commercially immobilized Candida antarctica lipase, i.e. Novozym 435 in a solvent-free system. The reaction factors under study were the amount of enzyme (x_1), temperature of operation (x_2) and the molar ratio of methanol to canola oil (x_3). The yield of methyl ester was evaluated using a second-order polynomial multiple regression model. An analysis of variance (ANOVA) showed a high coefficient of determination (R^2) value of 0.987, thus, ensuring a satisfactory adjustment of the regression model with the experimental data. The positive sign for the coefficients of the amount of enzyme and the temperature of the reaction indicated that the yield of the methyl ester increased with increased levels of x_1 from 3 to 5 and factor x_2 from 25 to 35°C. The negative effect of the methanol to canola oil ratio on the methyl ester yield indicated that the response level decreased as this factor (x_3) increased. The methyl ester yield, 84.42%, as the highest value obtained through experiment that was in agreement with the predicted yield 89.15%, resulted with use of the optimized operative parameters when enzyme amount, reaction temperature and methanol to canola oil molar ratio were 5%, 38°C and 3, respectively.*

Keywords: *Central composite design; Empirical modeling; Methanolysis; Biodiesel; Immobilized lipase; Canola oil; Response surface methodology.*

INTRODUCTION

Products of the alcoholysis of vegetable oils are esters of fatty acids, such as methyl esters, also known as biodiesel, which are currently of interest because of their environmental benefits, as compared to those of fossil diesel fuel [1]. These esters are biodegradable, non-toxic and give a low emission profile [2]. Extensive research has been carried out on the enzymatic production of biodiesel because of the simplicity of the operation and the recovery of glycerol, while the need for a catalyst and salt, usually used in chemical methods, is eliminated. The cost of lipase enzyme is, however, the major barrier for the commercialization of lipase-catalyzed biodiesel production [3].

The Response Surface Methodology (RSM), as a collection of statistical and mathematical techniques, would be used to develop, improve and optimize a particular process which is under the influence of several different operating factors [4]. RSM uses a design of experiments, such as the Central Composite Design (CCD), to fit a mathematical model by least squares techniques, in order to better define, quantitatively, the relationships that exist between several different factors [5-10].

In the present work, by use of RSM in the form of CCD, a relationship between three controllable factors and the methyl ester yield of the lipase-catalyzed reaction of canola oil was developed. The regression equation completely specified the relationship, and a mathematical model was provided to summarize the relations between the tested variables.

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Received 15 July 2008; received in revised form 3 June 2009; accepted 17 August 2009

MATERIALS AND METHODS

Materials

Refined canola oil was purchased from Behshahr Industrial Co. (Tehran, Iran). Commercial immobilized lipase from *Candida antarctica*, namely Novozym 435, was provided as a gift from Novo Nordisk (A.S., Denmark-Tehran office). According to the commercial product manual, the enzyme catalytic activity was 10000 PLU/g (Propyl Laurate units/g). Methanol and other chemicals used were of analytical grade and purchased from the local market. The saponification value of canola oil was 191 mg KOH/g. The fatty acid composition of canola oil, as determined by Gas Chromatography analysis (GC), was palmitic acid, 4.8%; stearic acid, 2.3%; oleic acid, 60.9%; linoleic acid, 22.6%; arachidic acid, 0.6%; linolenic acid, 7.3%; gadoleic acid, 1.2% and behenic acid, 0.3%. From this composition, an average molecular weight of 881.6 for the canola oil was determined.

Methanolysis Reaction

The methanolysis reaction of canola oil was performed in a 30 mL screw-capped bottle with shaking at 130 rpm at some specified temperature for 72 h. The initial reaction mixture consisted of 10g oil, methanol and immobilized lipase. Methanol was added in three-steps to the reaction mixture: the first portion at the beginning of the reaction, the second and third portions after 24 and 48 hours of the reaction, respectively [11]. At the end of the reaction, the lipase enzyme was removed from the reaction mixture by decantation followed by centrifugation at 5000 rpm for 10 min. The methyl ester at upper phase was separated from the glycerol in lower phase, the upper phase of which was then used for measurement of the methanolysis yield [12].

Selecting the Reaction Conditions

Before applying the RSM, approximate reaction conditions for the methanolysis of canola oil, namely, amount of enzyme, reaction temperature and molar ratio of the methanol to oil were determined by varying one factor at a time while keeping the other two independent variables constant at the some defined value. Thereafter, an appropriate range for each independent variable was selected for RSM experiments. For instance, to study the influence of methanol to canola oil ratio on the methyl ester yield, different molar ratios were tested from 3-6, while temperature and enzyme weight percentage were kept constant at 35°C and 4%, respectively. Continuation of the study was to find the effect of the reaction temperature in the range of 25 to 55°C, while enzyme loading and methanol to oil molar were selected as 4% and 3. Finally, variation of the enzyme percentage within 1-6% was studied, whereas the methanol to oil molar ratio and temperature were kept at 3 and 35°C.

Methanolysis Yield Measurement

In the reaction mixture, the yield of methyl esters was determined by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy (Avance DRX-500, Bruker), according to the method described by Gelbard et al. [12]. ^{1}H-NMR spectra were obtained at a frequency of 500 MHz with $CDCl_3$ as solvent. The conversion yield of the oil to the corresponding methyl ester was calculated using Equation 1:

$$Y = 100 \times (2A_{ME}/3A_{\mathrm{CH_2}}), \quad (1)$$

where Y is the methyl ester yield, and A_{ME} and $A_{\mathrm{CH_2}}$ are the areas of the methoxy and the methylene protons, respectively.

Experimental Design and Data Analysis

A CCD in the form of three factors, each one at the three levels, was arranged: 17 experimental runs, including three replicates at the center point, and three independent variables were converted to dimensionless ones (x_1, x_2, x_3) with coded values at 3 levels: -1, 0, +1 (Table 1). The arrangement of CCD as shown in Table 2 was in such a way that allows the development of the appropriate empirical equations (second order polynomial multiple regression equations) [4,13]:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3. \quad (2)$$

The predicted response (y) was, therefore, correlated to the set of regression coefficients (β): the intercept

Table 1. Independent variables and their levels for the central composite design used in the present study.

Independent Variables	Symbol	Coded Variable Levels[a]		
		−1	0	1
Enzyme amount (%)	x_1	3	4	5
Temperature (°C)	x_2	25	35	45
Methanol to Canola oil molar ratio	x_3	3	4	5

a: For passage from coded variable level to actual variable level these equations were used: x_1 = Enzyme amount-4, x_2 = (temperature-35)/10, and x_3 = Methanol/Canola oil-4.

Table 2. Arrangement of the central composite design for three independent variables used in the present study, along with the actual and predicted values for the response variable (methyl ester yield).

Exp.	Coded Independent Variable Levels			Methyl Ester Yield (%)	
No.	x_1	x_2	x_3	Actual	Predicted
1	−1	−1	−1	49.69	48.74
2	+1	−1	−1	67.54	69.82
3	−1	+1	−1	60.68	62.10
4	+1	+1	−1	82.68	83.18
5	−1	−1	+1	28.82	26.73
6	+1	−1	+1	38.52	38.60
7	−1	+1	+1	31.51	30.73
8	+1	+1	+1	43.24	42.60
9	−1	0	0	55.29	57.69
10	+1	0	0	76.37	74.16
11	0	−1	0	52.73	53.41
12	0	+1	0	62.58	62.09
13	0	0	−1	84.42	81.18
14	0	0	+1	46.45	49.88
15	0	0	0	67.43	69.44
16	0	0	0	72.12	69.44
17	0	0	0	69.16	69.44

(β_0), linear (β_1, β_2, β_3), interaction (β_{12}, β_{13}, β_{23}) and quadratic (β_{11}, β_{22}, β_{33}) coefficients. The "Design Expert" software version 7 (Stat Ease) was used for regression and graphical analyses of the obtained data.

RESULTS AND DISCUSSION

Characterization of Methyl Ester with NMR Spectroscopy

The typical ^{1}H-NMR spectra of the canola oil and its methyl esters are shown in Figure 1 (Exp. 13). The quantification by ^{1}H-NMR of the methyl esters requires that a target peak be chosen for the methanolysis yield measurement. The proton signals at 4.1-4.4 ppm were assigned to the protons attached to the glycerol moiety of mono-, di- or triacylglycerols. Compared to the spectrum of canola oil before methanolysis, a strong singlet peak at 3.7 ppm appeared, which was attributed to methoxy groups (-CO_2CH_3) in the methyl ester formation. The signals at 2.3 ppm resulted from the protons on the α-carbonyl methylene groups (-CH_2-) presents in all fatty ester derivatives. The areas of methoxy and methylene protons were used for measuring the yield of the methanolysis reaction as suggested elsewhere [12].

Selecting the Reaction Conditions

The low and high levels for the amount of enzyme x_1 were found based on the results obtained from the preliminary experiments in which the reaction temperature and methanol to canola oil molar ratio were kept constant at 35°C and 3, respectively, while the amount of enzyme changed from 1 to 6%. No change in the yield of methyl ester was observed as the amount of enzyme increased above 5%. A similar procedure was followed to determine the levels of the other two controllable factors, namely, the reaction temperature and methanol to canola oil molar ratio (for more information, please see the previous sections). Selection of the levels of the factors was from the results presented in Figure 2.

Central Composite Design and Fitted Regression Model

In the present work, the relationships between the criteria of the ester formation from the canola oil, i.e. yield of methyl ester, and three controllable factors were studied. The CCD shown in Table 2 allows the development of a mathematical equation where the response variable as methyl ester yield (y) is assessed as a function of enzyme amount (x_1), temperature (x_2) and methanol to the oil molar ratio (x_3) and calculated as the sum of a constant, three first-order effects (terms in x_1, x_2 and x_3) three interaction effects (term in x_1x_2, x_1x_3 and x_2x_3) and three second-order effects (terms in x_1^2, x_2^2 and x_3^2) according to Equation 2. The application of CCD in the present study with three factors and three center runs was advantageous, mainly because the center run itself is considered as a proper replication and the cost and availability of the materials limit a repetition of each one of the tested treatments. Also, center runs give a kind of checking reality, since information and selecting a choice of researcher is focused on settings that are around the best region of the model estimation. Moreover, by using a quadratic regression model, a researcher finds the curvature in the response (minimum, maximum and saddle points) [14]. The results obtained were then analyzed by ANOVA to assess the "goodness of fit". Only terms that were found to be statistically significant were included in the model. The β_{12} coefficient was non-significant, therefore, this coefficient was dropped from the model and then a new ANOVA was performed for the reduced model. The model for the yield of methyl ester was significant by the F-test at the 5% significance level (Prob$<$ 0.05). The following fitted regression model

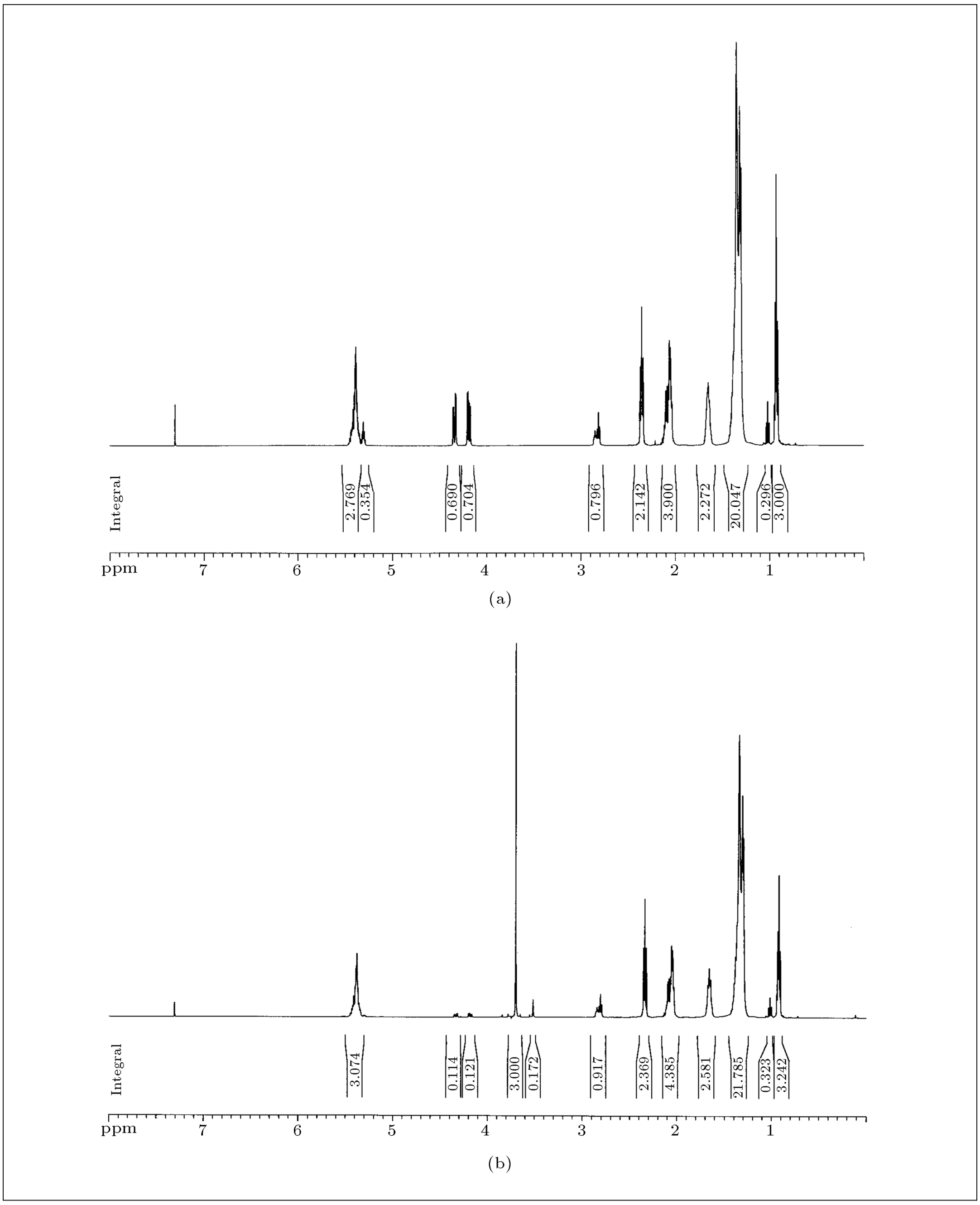

Figure 1. ^{1}H-NMR spectra of (a) canola oil and (b) methyl esters of canola oil. The result of Exp. 13 shown in Table 2, is shown.

(equation in terms of coded values for the predictor) was used to describe quantitatively the effects of three controllable factors in this study (the result of ANOVA is shown in Table 3).
Methyl ester yield:

$$y = 69.44 + 8.24x_1 + 4.34x_2 - 15.65x_3 - 2.3x_1x_3 - 2.34x_2x_3 - 3.52x_1^2 - 11.7x_2^2 - 3.92x_3^2. \quad (3)$$

Statistical parameters obtained from the ANOVA for the reduced model of ester formation are given in Table 4. The coefficient of multiple determinations (R^2) was 0.987. This coefficient gives the proportion of the total variation in the response variable described by the predictors included in the model. Because R^2 always decreases when a regressor variable is dropped from a regression model, statistical suggestions are for the use of the adjusted R^2, which takes the number of regressor variables into account [15]. In the present study, the adjusted R^2 was 0.974. The R^2 coefficient ensured a satisfactory adjustment of the

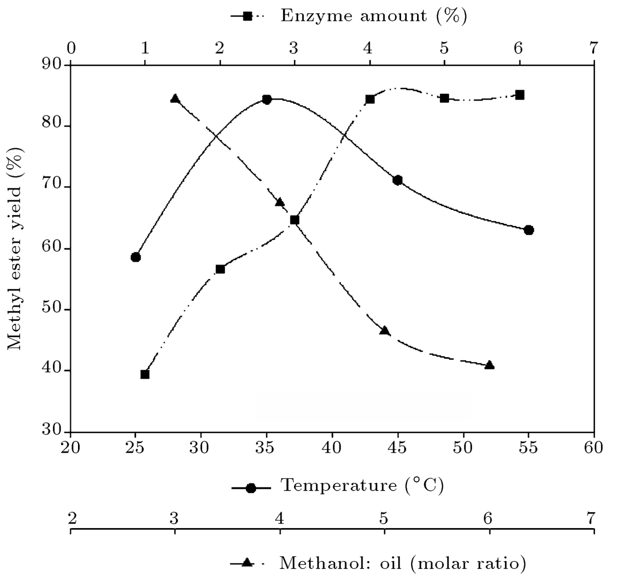

Figure 2. Effect of the reaction parameters on the methyl ester yield during the three sets of the experiment: enzyme amount (%), temperature (°C), and methanol to oil molar ratio. Study of each of the reaction parameters during these three sets of the experiment was carried out when the other two parameters were kept constant. See the text for explanation.

Table 3. Analysis of variance (ANOVA) for the fitted reduced quadratic model.

Source	Sum of Squares	*df*	Mean Square	*F*-value	*p*-value
Model	4505.4	8	563.17	76.79	< 0.0001
x_1	678.32	1	678.32	92.48	< 0.0001
x_2	188.27	1	188.27	25.67	0.0010
x_3	2448.29	1	2448.29	333.81	< 0.0001
x_1x_3	42.41	1	42.41	5.78	0.0429
x_2x_3	43.8	1	43.80	5.97	0.0403
x_1^2	33.22	1	33.22	4.53	0.0660
x_2^2	366.52	1	366.52	49.97	0.0001
x_3^2	41.09	1	41.09	5.60	0.0455
Residual	58.68	8	7.33		
Lack of fit	47.43	6	7.90	1.41	0.4720
Pure error	11.25	2	5.63		
Total	4564.07	16			

quadratic model to the experimental data. Therefore, the empirical equation is adequate to represent the relationship between the variables. The CV as a ratio of the standard error of estimation to the mean value of the observed response (as a percentage) is a measure of the reproducibility of the model and, as a general rule, a model can be considered reasonably reproducible if its CV is not greater than 10% [16]. As given in Table 4, the Coefficient of Variance (CV) was found to be 4.65%. The Predicted Residual Error Sum of Squares (PRESS), which is a measure of how a particular model fits each point in the design was 286. The adequate precision value gives a measure of the "signal-to-noise ratio" and was found to be 28.6 which indicates an adequate signal. A ratio greater than 4 is desirable [16]. This model can be used to make negative the design space.

Table 4. Statistical parameters obtained from the analysis of variance for the reduced quadratic model.

Variable	
R^2	0.9871
R^2 adjusted	0.9743
Std. Dev.	2.71
Mean	58.19
Coefficient of variation	4.65
PRESS	286.17
Predicted R^2	0.9373
Adequate precision	28.644

The relative contribution of each term of the independent variable to the response, i.e. yield of methyl ester, was directly measured by the respective coefficient in the fitted model. The positive sign for the coefficients of the amount of enzyme and the temperature of the reaction indicated that the yield of methyl ester increased with the increased levels of x_1 from 3 to 5 and factor x_2 from 25 to 35°C. The negative effect of the methanol to canola oil ratio on the methyl ester yield indicated that the response level decreased as this factor (x_3) increased.

The predicted values for the yield of methyl ester in the methanolysis reaction of canola oil in the present study are given in Table 2 (the regression coefficients of the reduced model). The measured data for the response variable are also given in Table 2.

Response Surface Plotting and Regressors Effects

For the graphical interpretation of interactions, the use of three-dimensional and contour plots of the regression model is highly recommended [4,13]. Regressor variables giving quadratic and interaction terms with the largest absolute values of coefficients in the fitted model were chosen for the axes of the response surface plots to account for the curvature of surfaces. The reaction temperature and methanol to canola oil molar ratio were selected when the amount of enzyme held constant at the level of 4% (Figure 3), while the temperature and

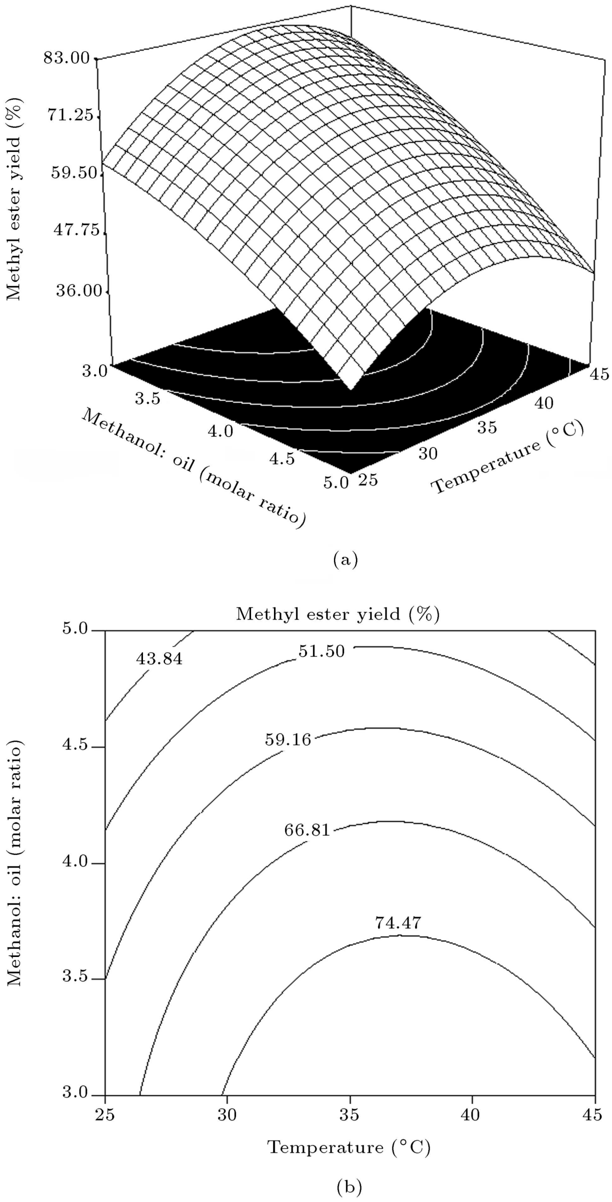

Figure 3. Response surface (a) and contour plot (b) indicating the effect of methanol to oil molar ratio and temperature (°C) on the enzymatic synthesis of methyl ester. The enzyme amount was constant at 4%.

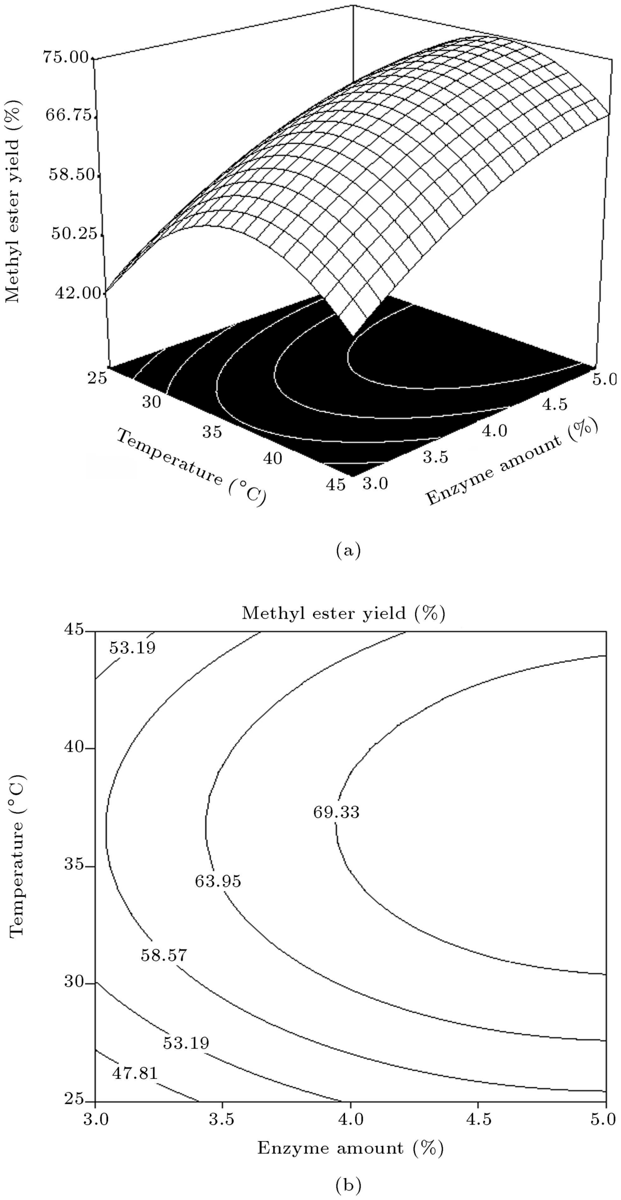

Figure 4. Response surface (a) and contour plot (b) indicating the effect of temperature (°C) and enzyme amount (%) on the enzymatic synthesis of methyl ester. The methanol to oil was constant at 4 (molar ratio).

the amount of enzyme factor were used as axes when the ratio of methanol to canola oil kept constant at a ratio of 4 (Figure 4). The interaction implies that the effect produced by changing the operation temperature as the one regressor variable depends on the level of methanol to canola oil molar ratio as the other regressor variable.

The study of Kaieda et al. [17] on the effect of the amount of lipase of *Rhizopus oryzae* on the methanolysis of soybean oil showed that the rate of the reaction increased as the level of the added enzyme to the reaction mixture increased. The researchers found that the interfacial area between the oil and water in the system (i.e. the small area) plays a role in limiting the rate. Moreover, the level of enzyme activity decreased after some specified reaction time, which was found to be due to a low level of water. The denaturing effect of methanol, therefore, caused the enzyme inactivation [17]. The ability of the lipase from the various microbial sources toward methanol inactivation was found to be different [18]. Figure 5 shows the effect of varying the amounts of enzyme and methanol to canola oil molar ratio on the methanolysis. The lowest methyl ester yield was at the lowest enzyme level (i.e. 3%) when methanol to canola oil molar ratio was at its highest level (i.e. 5). This is indicative

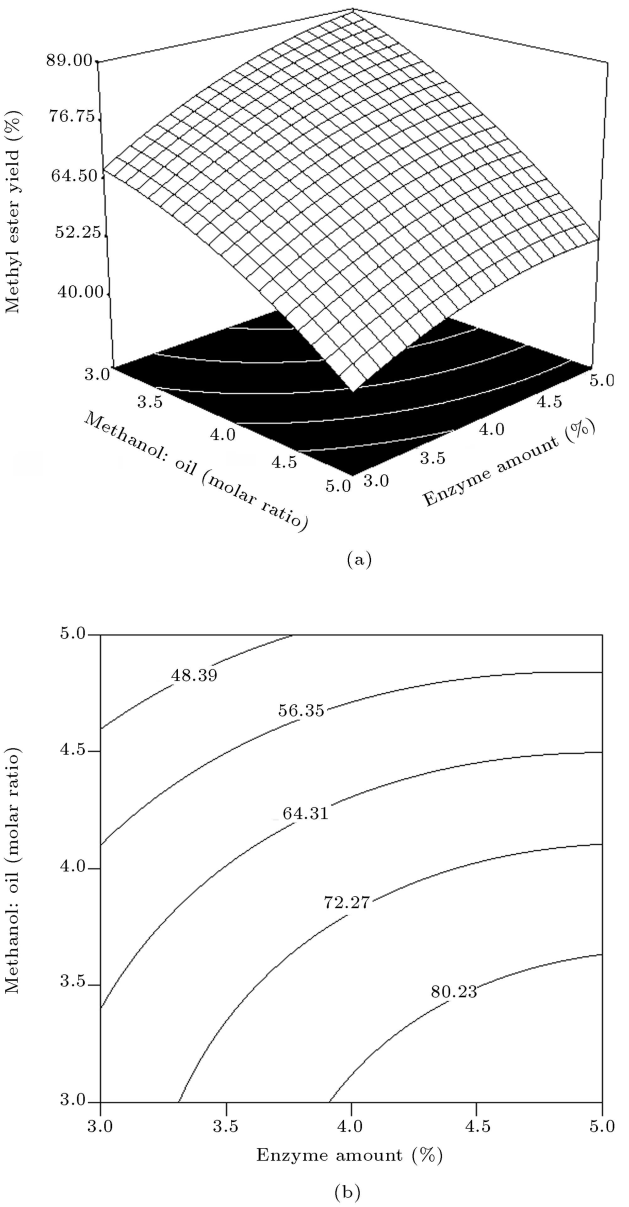

Figure 5. Response surface (a) and contour plot (b) indicating the effect of methanol to oil molar ratio and enzyme amount (%) on the enzymatic synthesis of methyl ester. The temperature was constant at 35°C.

of the inhibitory action of methanol on the enzyme. The highest yield of methyl ester was obtained at the highest enzyme level and at a low level of methanol to canola oil molar ratio.

In the model for the yield of the transesterification of canola oil, x_1 and x_3 were the major regressor variables affecting the response (largest coefficients, β_1 and β_3), while x_3 had significant interactions with either x_1 or x_2. The regression coefficients, β_1 and β_3, were 8.24 and -15.65, respectively. The coefficients for β_{13} and β_{23} were -2.3 and -2.34, respectively. The dependence of the methyl ester yield on methanol to canola oil ratio and temperature is shown in Figure 3 and the yield decreases almost linearly as methanol to canola oil molar ratio) increases to a high level (i.e. 5), while the curvature type relationship exists between the methyl ester yield and temperature. At temperatures above 25°C, the yield increases with increasing temperature toward its midrange region (i.e. 35°C). Thereafter, the yield decreases as the reaction temperature increases toward a high level, i.e. 45°C, and the stronger decreasing influence of temperature occurs when the temperature is at a high level. The contour plot confirmed these results (Figure 3b).

The enzymatic methanolysis of a plant oil substrate appears to follow a sequential type of mechanism [17]: Cleavage of the ester bond of triacylglycerol units by lipase, production of free fatty acids during the hydrolysis step and formation of methyl ester in the next esterification step until complete utilization of methanol. The free fatty acid content may remain constant during esterification and this may increase after completion of the transesterification. Thus, the hydrolysis and esterification processes have comparable value for the methanolysis [17]. As the usual practice is to determine the intermediates of transesterification, it would be possible to explain the probable mechanism of the action. The intermediates were not detected in the presented study, although the work of other researchers on the composition of intermediates in the course of the reaction progress confirms the appearance of free fatty acids, mono- and di-glycerides [19].

Figure 4 shows the second-order response surface plot for the methyl ester yield by considering temperature and the amount of enzyme factor as axes. Again, the yield increases almost linearly as the amount of enzyme increases toward its high level, i.e. 5%. The contour plot shows the highest yield (i.e. 69%) at the enzyme level 4% (Figure 4b), while the dependence of the yield on the reaction temperature presents a curvature type relationship. The lowest level of methyl ester yield occurred when the temperature of the reaction and the amount of enzyme were both at their lowest levels. The results obtained in the present study are in agreement with the findings reported by Gunawan et al. [7] on the immobilized lipozyme catalyzed synthesis of palm-based wax ester using oleyl alcohol.

The enzymatic methanolysis of canola oil by use of RSM has been studied by Chang et al. [1]. The optimization results including the predicted level of methyl ester yield were 99% at 42.3% as enzyme concentration, and 38°C as the temperature of the reaction. Methanol in n-hexane has been used in the latter study and the optimum substrate molar ratio was 3.5:1. The findings reported in the present study, thus, are interesting, at least from the point of view of the amount of enzyme and absence of organic solvent.

Optimization of the Reaction Conditions

The meaningful impression from the process optimization is to apply the concept of sequential experimentation, which is the basis of using RSM followed by the CCD equation model prediction (please see two previous sections) [14]. The optimum conditions for the enzymatic methanolysis of canola oil for the three controllable factors tested in the present study were obtained by using the numerical optimization feature of Design Expert 7 software. In order to set the criteria for all variables including factors, the software uses five possibilities for a "goal" to construct a desirability index. The numerical optimization function of the Design Expert combines the individual desirabilities into a single number and then looks for the greatest overall desirability (desirability ranges from zero to one for the response). An "in range" possibility was used in the present study. The goal was set to maximize methyl ester yield. The suggested optimum methyl ester yield was 89.15% with a desirability of 0.915, while methanol to canola oil was at the lowest ratio (i.e. 3), the amount of enzyme for this optimum condition was at its highest level (i.e. 5%). The reaction temperature as the other independent variable was optimized at 38°C (less than 7°C below the highest reaction temperature).

CONCLUSIONS

The methanolysis of canola oil by the immobilized *C. antarctica* lipase, Novozym 435, was studied using CCD and RSM. Optimum conditions for this enzymatic biodiesel production could be achieved by setting the experiment with methanol to canola oil molar ratio at 3 (low level of regressor x_3), while the other two regressors (amount of enzyme and reaction temperature) were studied at high and midrange levels, respectively. Adjustment of the quadratic model with experimental data was satisfactory. The analysis of variance showed a high coefficient of determination value, 0.987. It was possible, therefore, to develop the empirical equation describing and predicting the yield of the methyl ester of canola oil. The amount of enzyme and the reaction temperature showed a joint effect on the yield. The interaction of temperature and methanol to canola oil molar ratio was also pronounced in the studied range in the present work. By using the numerical optimization function of the Design Expert 7 software, optimum conditions for this methanolysis reaction were obtained. The predicted value for the methyl ester yield was 89.15%.

ACKNOWLEDGMENTS

The authors are grateful to Mehrnaz Mehranian, senior researcher in the Food Engineering and Biotechnology Laboratory at the Chemical Engineering Department, for her meticulous laboratory work in this research, and to Alireza Monazzami, director of the Computer Center of the Chemical Engineering Department, for his valuable support.

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