Optimization of Enzymatic Methanolysis of Soybean Oil by Response Surface Methodology

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ABSTRACT: Enzymatic methanolysis of refined soybean oil with methanol was investigated using *Rhizomucor miehei* lipase, Lipozyme RM IM, in *n*-hexane for reaction times of 30 min. Response surface methodology (RSM) based on three-level, three-factor (variable) face-centered cube design was used for the optimization of methanolysis. The independent variables that affect the methanolysis reaction conducted in *n*-hexane are temperature (°C), enzyme/oil weight ratio, and oil/methanol molar ratio. A good quadratic model was obtained for the methyl ester production by multiple regression and backward elimination. A linear relationship was observed between the observed and predicted values ($R^2 = 0.9635$). The effects of temperature and enzyme amount, which affected methyl ester content of the product (response) positively, were significant ($P < 0.01$). The quadratic term of temperature and the interaction term of enzyme amount with temperature affected the response negatively ($P < 0.01$). The interaction term of enzyme amount with substrate mole ratio had a positive effect on the response ($P < 0.05$). Critical conditions for the response at which methyl ester content of the product was 76.9% were determined to be 50°C, 2.37 methanol/oil mole ratio, and 0.09 enzyme/oil weight ratio.

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KEY WORDS: Alcoholysis, biodiesel, lipase, optimization, response surface methodology, *Rhizomucor miehei*, soybean oil.

Biodiesel is produced industrially by alcoholysis of oils or fats using alkali or acid catalysts. However, difficulties in the recovery of catalyst and glycerol, high energy requirements, and the necessity for treating wastes are disadvantages in the chemically catalyzed processes. Lipases, which allow mild reaction conditions and easy recovery of glycerol, largely prevent these drawbacks (1,2). Several reports describe lipase-catalyzed alcoholysis reactions in solvents and solvent-free mediums, especially the reaction parameters affecting the rates of lipase activities in alcoholysis reactions (1–9). A high yield of alkyl esters can be achieved in the presence of nonpolar solvents such as *n*-hexane, but the ester yield is low in the absence of organic solvents (3,4). Glycerol liberated as a by-product in the alcoholysis can inhibit the reaction. By using silica gel or other adsorbents for glycerol adsorption, higher alkyl ester yields were reported (4).

The classical method of optimization is an inefficient way to understand relationships between the process independent variables (reaction time, temperature, molar ratio, and amount of enzyme) and the response (percent yield). Response surface methodology (RSM) is a popular and effective statistical technique for investigation of complex processes. RSM with a five-level-five-factor central composite rotatable design has been used to optimize lipase-catalyzed methanolysis of oils with respect to time, temperature, enzyme amount, molar ratio of methanol to oil, and added water (2,10).

Soumanou and Bornscheuer (4) investigated methanolysis of triolein in hexane using five commercial immobilized lipases and reported that *Rhizomucor miehei* lipase gave the highest conversion (96.3%). In the present work the same lipase was selected for the methanolysis of refined soybean oil. Silica gel was used for the adsorption of glycerol liberated in the methanolysis reaction. The main objectives of the study were to understand relationships between the independent variables (reaction temperature, enzyme amount, and substrate molar ratio) and the response (methyl ester content, wt%) and to identify the optimal conditions for soybean oil methanolysis at a short reaction time of 30 min using a three-level-three-factor face-centered cube design and RSM.

MATERIALS AND METHODS

Materials. Refined soybean oil was purchased from a local market. Commercial immobilized lipase from *R. miehei* (Lipozyme RM IM) was supplied by Novozymes A/S (Bagsværd, Denmark). All of the alcohols and other chemicals, which were of analytical grade, were purchased from Merck Chemical Co. (Darmstadt, Germany). Before the reactions, silica gel (0.2–0.5 mm) was dried for 30 min at 180°C.

Alcoholysis reactions. In general, the reactions were carried out in a 50-mL round-bottomed flask incubated in a temperature-controlled water bath with magnetic stirrer (Framo-Geratetechnik M22/1 5655; Franz Morad, Eisenbach, Germany). The stirring rate was adjusted to 800 rpm. The reaction time was chosen as 30 min. The mixture of soybean oil (5 g) with different molar ratios of methanol and 25 mL *n*-hexane was heated to the desired temperature with stirring. The reaction was started by adding the proper amount of enzyme and silica gel (60% wt of oil) to the reaction mixture. After 30 min, samples (1 mL) were drawn from reaction mixtures and placed in a water bath kept at 90°C for 15 min to inactivate the enzyme, as

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described previously (11). The effectiveness of enzyme inactivation by heat treatment at 90°C was confirmed by comparing heat-treated samples with a sample that was immediately analyzed after a reaction carried out at 60°C. After heat treatment, samples were cooled to room temperature, 5 mL *n*-hexane and 2 mL of water were added to the samples, and then the hexane phases were drawn into vials containing anhydrous sodium sulfate. The dried hexane phases were mixed with 3 mL *n*-hexane and analyzed by a TLC-FID system.

Analysis of alcoholysis product. The samples of the alcoholysis reactions were analyzed by a combined TLC-FID system using an Iatroscan TH-10 analyzer with SIII rods (Iatron Lab Inc., Tokyo, Japan). Samples (1 μL) taken from the dried hexane phases were fed onto SIII (silica-quartz) rods. Separation of the products mixture, containing TAG, FAME, MAG, DAG, and FFA, was achieved using two different mobile phases: solvent mixture I, 50 mL benzene, 20 mL chloroform, and 0.7 mL acetic acid; and solvent mixture II, 35 mL benzene and 35 mL *n*-hexane. The rods were dried for 6 min at 60°C in a rod dryer (Rod Dryer TK 5; Iatron Lab. Inc.) after being taken out of each tank. During analysis, hydrogen and air were fed at rates of 160 and 2200 mL/min, respectively. The scanning speed was set as 30 s/scan.

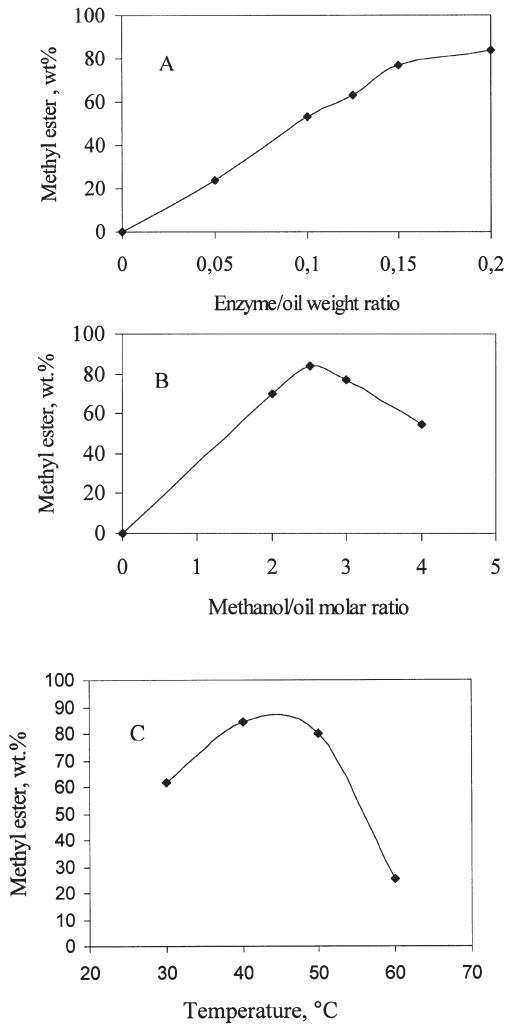

FIG. 1. Effect of the amount of enzyme, methanol/oil molar ratio, and temperature on the methyl ester content of product (A: 40°C, 1:3 oil/methanol molar ratio, 0.6 silica gel/oil ratio, 30 min.; B: 40°C, 0.15 enzyme/oil weight ratio, 0.6 silica gel/oil ratio, 30 min.; C: 0.15 enzyme/oil weight ratio, 1:2.5 oil/methanol molar ratio, 0.6 silica gel/oil ratio, 30 min.

Experimental design. Before RSM was applied, approximate conditions for soybean oil methanolysis, namely, the enzyme load, substrate molar ratio, and the reaction temperature, were determined by varying one independent variable at a time while keeping the others constant (Fig. 1). An appropriate range for each independent variable was determined for RSM.

A three-factor and three-level face-centered cube design requiring a total of 17 experiments with three center points (0,0,0) was adopted in this study (12). The independent variables studied were reaction temperature (X_1,°C), methanol/oil molar ratio (X_2), and enzyme amount (X_3, enzyme/oil weight ratio). The response (dependent variable) was the methyl ester content of the product (wt%).

Statistical analysis. The experimental data were analyzed by the response surface (MINITAB 8.21; Minitab, Coventry, United Kingdom; and MATLAB R12; MathWorks, Bursa, Turkey) procedure to fit the following second-order polynomial model predicted for optimization of soybean oil alcoholysis:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \qquad [1]$$

where Y is the response (methyl ester content of the product); β_0, β_i, β_{ii}, and β_{ij} are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and X_i and X_j are independent variables. Contour plots were obtained using the fitted model by keeping the least effective independent variable at a constant value while changing the other two variables.

RESULTS AND DISCUSSION

Selection of factor levels of independent variables and experimental design. The effect of the three independent variables on the methyl ester content of alcoholysis product is shown in Figure 1. As the amount of enzyme increased, the methyl ester content increased. Design points selected for optimization of methanolysis were 0.1, 0.15, and 0.2 enzyme/oil weight ratios. Methyl ester content increased with increasing methanol/oil molar ratio up to 2.5:1. A methanol/oil molar ratio higher than 2.5:1 inhibited the reaction. This result is in good agreement with the literature, where it has been reported that the stepwise addition of methanol is more effective than a one-step methanolysis reaction (4,7–9). Therefore, 2:1, 2.5:1, and 3:1 methanol/oil molar ratios were chosen as the lower, middle, and upper points, respectively. The lower, middle, and upper points for the reaction temperature were chosen as 30, 40, and 50°C.

Table 1 shows the independent variables, their levels, and experimental design in terms of coded and uncoded and observed responses. Duplicate experiments were carried out at all

TABLE 1
Face-centered Cube Design, Experimental Data for Three-level-three-factor Response Surface Analysis

Run	Temperature (°C)	Enzyme/oil weight ratio	Substrate molar ratio	Observed methyl ester (wt%)	Predicted methyl ester[a](wt%)	Residual values
1	–1 (30)	–1 (0.1)	–1 (2)	50.65	49.17	1.48
2	–1 (30)	+1 (0.2)	–1 (2)	70.00	71.35	–1.35
3	–1 (30)	–1 (0.1)	+1 (3)	36.06	41.02	–4.96
4	–1 (30)	+1 (0.2)	+1 (3)	82.73	79.50	3.23
5	+1 (50)	–1 (0.1)	–1 (2)	70.05	76.74	–6.69
6	+1 (50)	+1 (0.2)	–1 (2)	77.93	76.43	1.50
7	+1 (50)	–1 (0.1)	+1 (3)	70.84	68.59	2.25
8	+1 (50)	+1 (0.2)	+1 (3)	84.00	84.58	–0.58
9	–1 (30)	0 (0.15)	0 (2.5)	61.84	60.26	–1.58
10	+1 (50)	0 (0.15)	0 (2.5)	80.11	76.59	3.52
11	0 (40)	0 (0.15)	–1 (2)	69.11	77.58	–8.47
12	0 (40)	0 (0.15)	+1 (3)	77.09	77.58	–0.49
13	0 (40)	–1 (0.1)	0 (2.5)	74.97	68.04	6.93
14	0 (40)	+1 (0.2)	0 (2.5)	83.33	87.12	–3.79
15	0 (40)	0 (0.15)	0 (2.5)	76.90	77.58	–0.68
16	0 (40)	0 (0.15)	0 (2.5)	81.68	77.58	4.10
17	0 (40)	0 (0.15)	0 (2.5)	80.00	77.58	2.42

[a]Calculated from the refined model.

TABLE 2
Regression Coefficients of the Second-order Polynomials After Backward Elimination for Response

	Coefficients	SD.	*t*-Ratio	*p*
β_0	77.583	1.851	41.91	0.000
Linear				
β_1 (temperature)	8.165	1.549	5.27	0.000
β_3 (enzyme load)	9.542	1.549	6.16	0.000
Quadratic				
β_{11}	–9.162	2.414	– 3.80	0.003
Interactions				
β_{13}	–5.622	1.732	– 3.25	0.008
β_{23}	4.075	1.732	2.35	0.038
R^2	0.90			
R^2 (adj)	0.85			

design points. The maximum methyl ester content (84%) was obtained at 50°C, 0.2 enzyme/oil weight ratio, and 1:3 oil/methanol molar ratio.

Model fitting. Regression coefficients, obtained by using a least squares technique to predict a quadratic polynomial model for response (methyl ester content, wt%), are shown in Table 2. Linear ($P < 0.01$) and quadratic ($P < 0.01$) effects of temperature as well as a linear ($P < 0.01$) effect of enzyme amount were statistically significant. Temperature × enzyme amount ($P < 0.01$) and molar ratio × enzyme amount ($P < 0.05$) interactions were also significant.

Figure 2 shows the experimental values of methyl ester content of the alcoholysis product (wt%) vs. those calculated from the Equation 1 (Table 1). The figure indicates that the values of the response predicted from the empirical model are in agreement with the observed values in the range of the operating variables ($R^2 = 0.96$). As can be seen in Figure 2, most of the points are located in the range of 70–85%. Only a few points are located below 70%. Probably this situation arose from the selection of design points.

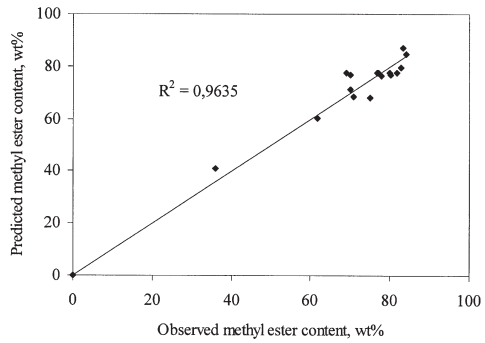

FIG. 2. Correlation of experimental and predicted methyl ester contents (wt%).

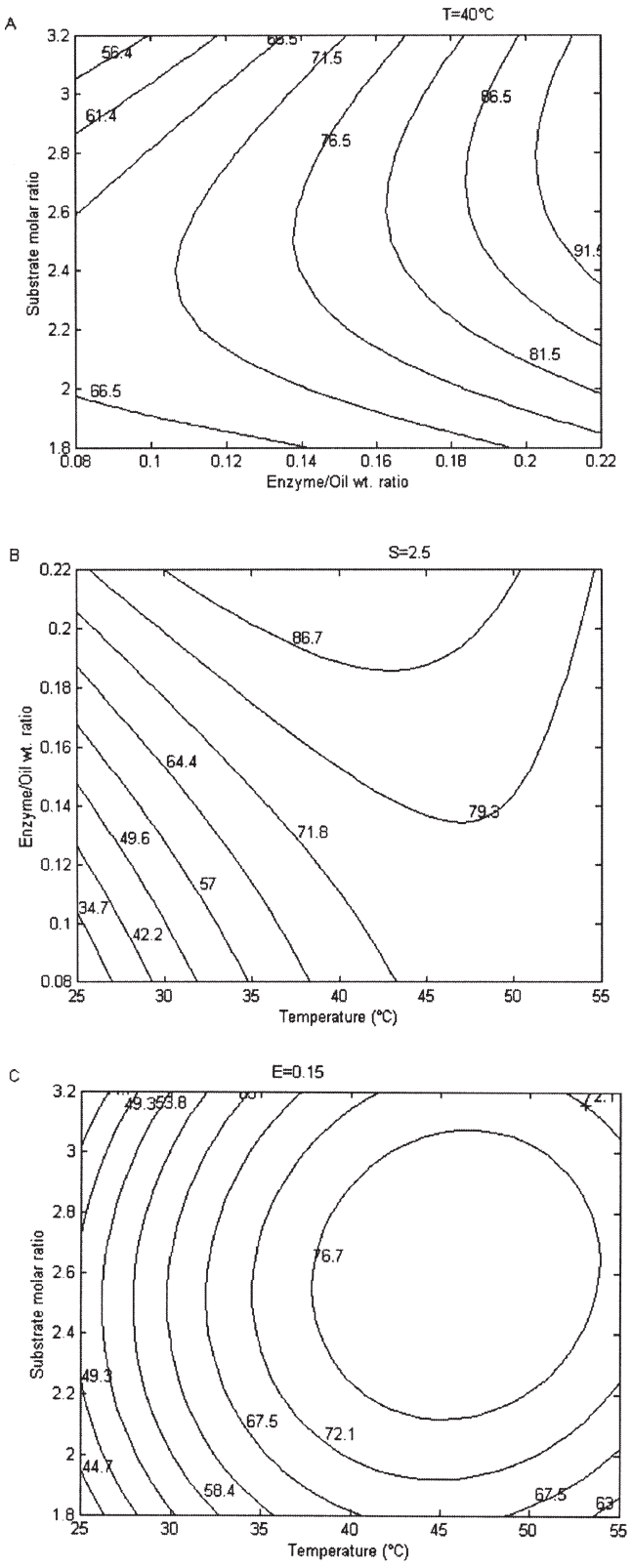

FIG. 3. 2-D contour plot showing the effect of variables on the methyl ester content. A: substrate molar ratio vs. enzyme/oil weight ratio; B: enzyme/oil weight ratio vs. temperature; C: substrate molar ratio vs. temperature.

The relationships between the responses and process parameters were examined by 2-D contour plots (Fig. 3). As seen in Figure 3A, when the substrate molar ratio was between 2.5 and 3.0 the methyl ester content could reach values exceeding 80% when the enzyme/oil ratio was higher than 0.16. Contour plots in Figure 3B show the effects of enzyme/oil ratio, temperature, and their interactions on the methyl ester content. At temperatures between 40 and 50°C, increases in enzyme/oil ratios above 0.16 led to higher methyl ester content.

Figure 3C shows contour plots regarding the effect of substrate molar ratio, temperature, and their interactions on the methyl ester content. An increase in temperature produced an increase in methyl ester content. The response value reached the highest levels between 40 and 50°C while the substrate molar ratio was 2.4–2.8. Critical values for the methyl ester formation were found at 50°C, 2.37 methanol/ oil molar ratio, and 0.09 enzyme/ oil ratio.

REFERENCES

1. Nelson, L.A., T.A. Foglia, and W.N. Marmer, Lipase-Catalyzed Production of Biodiesel, *J. Am. Oil Chem. Soc.*, *73*:1191–1195 (1996).
2. Shieh, C.J., H.F. Liao, and C.C. Lee, Optimization of Lipase-Catalyzed Biodiesel by Response Surface Methodology, *Bioresource Technol. 88*:103–106 (2003).
3. Fukuda, H., A. Kondo, and H. Noda, Biodiesel Fuel Production by Transesterification of Oils, *J. Biosci. Bioeng. 92*:405–416 (2001).
4. Soumanou, M.M., and U.T. Bornscheuer, Improvement in Lipase-Catalyzed Synthesis of Fatty Acid Methyl Esters from Sunflower Oil, *Enzyme Microb. Technol. 33*:97–103 (2003).
5. Köse, Ö., M. Tüter, and H.A. Aksoy, Immobilized *Candida antarctica* Lipase-Catalyzed Alcoholysis of Cotton Seed Oil in a Solvent-Free Medium, *Bioresource Technol. 83*:125–129 (2002).
6. Watanabe, Y., Y. Shimada, A. Sugihara, and Y. Tominaga, Conversion of Degummed Soybean Oil to Biodiesel Fuel with Immobilized *Candida antarctica* Lipase, *J. Mol. Catal. B. Enzym. 17*:151–155 (2002).
7. Du, W., Y. Xu, and D. Liu, Lipase-Catalysed Transesterification of Soya Bean Oil for Biodiesel Production During Continuous Batch Operation, *Biotechnol. Appl. Biochem. 38*:103–106 (2003).
8. Noureddini, H., X. Gao, and R.S. Philkana, Immobilized *Pseudomonas cepacia* Lipase for Biodiesel Fuel Production from Soybean Oil, *Bioresource Technol. 96*:769–777 (2005).
9. Deng, L., T. Tan, F. Wang, and X. Xu, Enzymatic Production of Fatty Acid Alkyl Esters with a Lipase Preparation from *Candida* sp. 99-125, *Eur. J. Lipid Sci. Technol. 105*:727–734 (2003).
10. Chang H.M., H.F. Liao, C.C. Lee, and C.J. Shieh, Optimized Synthesis of Lipase-Catalyzed Biodiesel by Novozym 435, *J. Chem. Technol. Biotechnol. 80*:307–312 (2005).
11. Virto, M.D., J.M. Lascaray, R. Solozabal, and M. de Renobales, Enzymic Hydrolysis of Animal Fats in Organic Solvents at Temperatures Below Their Melting Points, *J. Am. Oil Chem. Soc. 68*:324–327 (1991).
12. Senanayake, S.P.J.N., and F. Shahidi, Lipase-Catalyzed Incorporation of Docosahexaenoic Acid (DHA) into Borage Oil: Optimization Using Response Surface Methodology, *Food Chem. 77*:115–123 (2002).

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