Optimizing Production of Ethyl Esters of Grease Using 95% Ethanol by Response Surface Methodology

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ABSTRACT: Previous research suggested that ethyl esters derived from recycled restaurant grease might be a potential source of biodiesel. Accordingly, response surface methodology (RSM) was employed to optimize reaction parameters—temperature, time, level of lipase, mole ratio of reactants—in the PS-30 lipase-catalyzed transesterification reaction of grease to ethyl esters using 95% ethanol. The regression equation obtained by a modified central composite design of RSM predicted optimal reaction conditions of 38.4°C, 2.47 h, 13.7 wt% lipase (PS-30), and a mole ratio of grease to ethanol of 1:6.6. Under these conditions the predicted optimal percentage ethyl ester yield was 85.4%. Subsequent experiments using the predicted parameter combinations indicated a trend where experimental percentage yields of ethyl ester were consistently lower than predicted values. In an effort to improve the experimental yield of esters, a second portion of PS-30 lipase was added without success; however, the addition of 5% SP435 one hour after the start of the initial reaction increased the yield of esters to >96%. Neither lipase PS-30 nor lipase SP435 alone, however, gave the RSM-predicted yield of ethyl esters.

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KEYWORDS: Biodiesel, lipase-catalyzed transesterification, monoalkyl esters, process optimization, two-lipase esterification.

There is increased interest in alternative fuels derived from renewable resources rather than nonrenewable petroleum. Among renewable alternative fuels, biodiesel—simple alkyl esters (primarily methyl and ethyl) of natural fats and oils—has attracted much research interest because of its additional potential to reduce pollutants emitted by heavy-duty diesel engines (1–6).

Previously, we demonstrated that selected monoalkyl esters might be used as biodiesel and could be produced by lipase-catalyzed transesterification of fats and oils with anhydrous alcohols in a solvent-free environment (7). Compared to the traditional alkali-catalyzed chemical reactions, the use of lipases in production of biodiesel may have certain advantages, such as no by-products, ease of product recovery, milder reaction conditions and catalyst recyclability. In addition, lipase use can overcome difficulty in transesterifying materials such as recycled restaurant grease, which may contain high levels of free fatty acids. Our recent research on low-temperature properties and diesel engine performance of selected monoalkyl esters derived from tallow and restaurant grease strongly suggested that ethyl esters of grease (prepared with absolute ethanol) might be an excellent biodiesel candidate (8). We found that ethyl esters of grease (ethyl greasate) had low-temperature properties—such as cloud point, pour point, cold filter plugging point and low-temperature flow test—which closely resemble methyl soyate, the predominant form of biodiesel currently marketed in the United States. Moreover, short-term diesel engine performance and emission tests in a matched dual-cylinder diesel engine indicated that a 20% blend of ethyl greasate in No. 2 diesel fuel had adequate fuel properties (8). Results from this work showed that this fuel promoted good engine performance, less fuel consumption, and contributed no apparent differences in carbon buildup characteristics (CO, CO_2, O_2, and NO_x emissions) compared to No. 2 diesel.

In previous work (7), we screened a series of lipases as catalysts for the synthesis of alkyl esters. That study indicated that of the enzymes studied, the lipase from *Pseudomonas cepacia* (PS-30) was the most effective for transesterification of fats and oils and grease to ethyl esters when using absolute ethanol (>98% yields). In this study, we now have attempted to transesterify recycled restaurant grease using 95% alcohol instead of absolute alcohol, since the latter alcohol is more expensive (9) than 95% alcohol. The approach taken was to determine the optimal reaction parameters for the PS-catalyzed transesterification of grease with 95% alcohol by the combined use of factorial design experiments and response surface methodology. From these results, an alternative approach using sequential addition of a second lipase was attempted to elevate the ethyl ester yields above the value predicted by the model.

EXPERIMENTAL PROCEDURES

Materials. Kaluzny Bros., Inc. (Joliet, IL) supplied the recycled restaurant grease used in this study. The filtered and dehydrated grease (clear and light brown in color) contained 8.5% free fatty acids and had the following fatty acid profile: 0.7% 14:0, 14.4% 16:0, 1.1% 16:1, 9.2% 18:0, 48.6% 18:1, 23.7% 18:2, and 2.3% 18:3. Lipase PS-30 (specific activity 34

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IU/mg solid) from *P. cepacia* was from Amano Enzyme Co. (Troy, VA). Lipase SP435 (specific activity 43 IU/mg solid) from *Candida antarctica* was from Novo Nordisk (Franklinton, NC). Each was used as received. All solvents used were high-performance liquid chromatography (HPLC) grade.

Experimental design. A modified experimental central composite design (CCD) (10) was used to study the effects of predetermined independent variables, including reaction temperature, reaction time, lipase level (PS-30) and mole ratio of grease to alcohol, on the yield of ethyl esters produced by transesterification of grease using 95% ethanol. The water content of each reaction preparation was measured using a Karl Fischer coulometer (Mettler Toledo, Hightstown, NJ) and was treated as a covariable since water content was presumably correlated with the amount of 95% ethanol used in the reaction mixture. The settings of the independent variables for a total of 36 reactions are listed in Table 1. The design was modified from a CCD by changing one of the so-called "star" points. The "textbook" CCD called for a run at a temperature of 15°C [with the other factors at their center points (time: 1.75 h, lipase level: 4.7%, and molar ratio: 46.7)]. Rather than performing a run at this low temperature, the design was modified by setting the temperature for this run at 25°C. The settings for the molar excess of ethanol to grease also depart slightly from the "textbook" values of 0, 25, 50, 75, and 100%.

Enzymatic transesterification. An aliquot of 2 mmol (1.738 g) grease was accurately weighed into a 25-mL glass-stoppered Erlenmeyer flask. An appropriate amount of 95% ethanol was added according to the experimental design. Lipase PS-30 was added immediately before incubation was started in an orbital water-bath shaker at 25, 35, 45, or 55°C at 200 rpm. At the water levels used in this study, the lipase particles remained dispersed in the medium throughout the course of the reactions. The sealed flasks were placed in an ice bath to terminate the reaction, and 10 μL of the reaction mixture was pipetted into a 16 × 125 mm screw-capped culture tube containing 1 mL of hexane/diethyl ether (1:1, v/v) and 1 mL of 10% aqueous NaCl solution. The organic phase was filtered through a column of anhydrous Na_2SO_4 to remove residual moisture and lipase after vortexing and centrifugation. Solvents (hexane and ether) were removed under nitrogen, and the reaction mixture was redissolved in hexane (0.5 mg/mL) for compositional analysis by HPLC. For the study on sequential addition of a second lipase, the reaction mixture was initially catalyzed with lipase PS-30 (5% by wt of grease). After the 1-h sample was removed, lipase SP435 (5% by wt of grease) was added and the reaction mixture was incubated and sampled for an additional 23 h.

HPLC determination of reaction products. Percentages of ethyl esters, free fatty acids, monoacylglycerols, diacylglycerols, and triacylglycerols in the reaction mixture were determined by an HPLC method developed in our research group (11). Lipid mixtures were separated on a cyanopropyl (CN) column (Phenomenex, Torrance, CA) using a binary mobile phase of hexane and methyl *t*-butyl ether, both containing acetic acid (0.4% by volume), with detection by an evaporative light-scattering detector (Varex, Burtonsville, MD).

RESULTS AND DISCUSSION

Response surface methodology (RSM), a statistical method (10) that has been used by various researchers for the optimization of chemical (12) and lipase-catalyzed reactions (13–15), was employed to investigate the effects of selected reaction parameters, such as reaction temperature (T), time (t), lipase level (L), and mole ratio of substrates (MR), to predict the optimal reaction conditions for ethyl ester synthesis. The quadratic response surface model is of the form shown in Equation 1:

$$\begin{aligned} Y = {} & b_0 + (b_1 \times L) + (b_2 \times T) + (b_3 \times t) + (b_4 \times \mathrm{MR}) + \\ & (b_{11} \times L^2) + (b_{22} \times T^2) + (b_{33} \times t^2) + (b_{44} \times \mathrm{MR}^2) + \\ & (b_{12} \times L \times T) + (b_{13} \times L \times t) + \cdots + (b_{34} \times t \times \mathrm{MR}) \end{aligned} \quad [1]$$

where Y is the response variable (% ethyl esters of grease) and b_0, b_i, b_{ij} (where $i, j = 1, \ldots, 4$) are the regression coefficients.

The percentages of ethyl esters produced in the transesterification of recycled restaurant grease (8.5% free fatty acid) with 95% ethanol under different reaction parameter combinations are shown in Table 1. The factorial combination representing the center point had a temperature of 35°C at 1.75 h using a 4.4:1 mole ratio of ethanol to grease (46.7% molar excess of ethanol) in the presence of 6.25% (w/w grease) lipase (PS-30).

Regression Equation 1 ($R^2 = 0.93$) was fit to the experimental data in Table 1 and yielded the estimated regression coefficients shown in Table 2. For comparative purposes, the predicted Y values (percentage yield ethyl esters) from Equation 1 are listed in Table 1 and agree well with the experimentally obtained values. The mole ratio of ethanol to grease did not have any significant effect on the percentage conversion to ethyl greasate within the range of mole ratios (3:1 to 6:1) tested. These ratios corresponded to a 0 to 100% molar excess of ethanol used in these reactions. The statistical analysis indicated that water content, which was correlated with the amount of 95% ethanol in the reaction system, apparently fell into a range (between 1.2–1.6 wt % of reactants) without significant influence on the ethanolysis reaction. Even though water often is a significant factor in lipase-catalyzed reactions in organic media (16–18), in this study the narrow range of water content apparently had only a modest effect on residual free fatty acids in the ester products (Fig. 1).

The response surface analysis showed that of the variables tested, time and temperature had significant effects (significance level $P < 0.05$) on the response (% alkyl esters) and lipase level modest effects ($P < 0.10$). Optimal synthetic conditions were determined by canonical analysis and resulted in the stationary point (midpoint) given in Table 3. Figure 2 shows the effects of time and temperature on the response at the center point levels of lipase (6.25 wt%) and molar excess of ethanol (46.7%). The observed values are represented in Figure 2 by the spikes with pyramidal heads. Model verification was performed by additional independent trials at the

TABLE 1
Independent Reaction Variables Studied in the Lipase (PS-30)-Catalyzed Transesterification of Grease with 95% Ethyl Alcohol Using Response Surface Methodology (RSM)

Temperature (°C)	Time (h)	Lipase level (wt%)	Molar excess of ethanol (mol%)[a]	Ethyl esters (%)	
				Actual	Predicted
25	1.125	8.125	66.7	25.8	23.9
25	1.125	8.125	33.3	20.7	26.2
25	1.125	4.375	33.3	17.4	17.5
25	2.375	8.125	33.3	61.7	58.8
25	2.375	8.125	33.3	68.8	61.1
25	2.375	6.250	66.7	63.9	60.9
25	1.750	4.375	46.7	25.9	42.2
25	2.375	4.375	66.7	59.7	55.8
25	1.125	4.375	66.7	10.8	12.3
35	0.500	6.250	46.7	53.1	41.9
35	1.750	6.250	46.7	70.5	69.4
35	1.750	6.250	46.7	70.7	69.4
35	1.750	6.250	46.7	70.5	69.4
35	1.750	6.250	46.7	71.4	69.4
35	1.750	6.250	46.7	69.3	69.4
35	1.750	6.250	100	62.2	64.1
35	1.750	6.250	46.7	70.9	69.4
35	1.750	6.250	46.7	70.9	69.4
35	1.750	6.250	46.7	70.4	69.4
35	1.750	10.000	46.7	75.0	76.1
35	1.750	6.250	46.7	70.1	69.4
35	1.750	2.500	46.7	57.8	57.0
35	1.750	6.250	46.7	70.6	69.4
35	1.750	6.250	0	70.0	69.4
35	1.750	6.250	46.7	71.0	69.4
35	3.000	6.250	46.7	74.1	85.6
35	1.750	6.250	46.7	70.0	69.4
45	1.125	4.375	33.3	53.1	59.6
45	2.375	4.375	66.7	66.0	63.3
45	2.375	4.375	33.3	67.6	66.5
45	1.125	8.125	66.7	66.7	71.4
45	2.375	8.125	33.3	73.5	74.2
45	1.125	4.375	66.7	50.4	54.4
45	1.125	8.125	33.3	71.9	73.8
45	2.375	8.125	66.7	76.6	74.0
55	1.750	6.250	46.7	47.0	43.2

[a]Percent molar excess of ethanol corresponds to the following molar ratios of ethanol to grease (E/G): 0%, 3:1; 33.3%, 4.0:1; 46.7%, 4.4:1; 66.7%, 5.0:1; 100%, 6.0:1. PS-30, lipase from *Pseudomonas cepacia* (Amano Enzyme Co., Troy, VA).

suggested optimal reaction conditions and generally gave experimental values approximately 10 to 15% lower than the predicted values. There was indication also that the predicted response surface of the stationary point was shaped like a saddle without a unique optimum. The saddle-shaped response surface indicates that there is significant interaction between some factors and implies that the behavior of the response with respect to temperature depends on the values of the time factor. The response surface itself is just an approximation model to describe the trends in yield over an experimental region. The fact that the surface turns out to be saddle-shaped has no dire implications, but only tells us that we do not have a situation where there is either a linear increase in yield with all variables independent of the others or a curved surface with a unique maximum.

From the results of the regression equation and our model observations, it was apparent that the yield of ethyl esters

TABLE 2
Estimated Coefficients of the Regression Equation for Lipase (PS-30)-Catalyzed Transesterification of Grease Using 95% Ethanol

Parameter	Estimated coefficient[a] b_i, b_{ij}
Intercept	–281.38
Lipase level (*L*)	3.85*
Temperature (*T*)	12.82*
Time (*t*)	83.46*
Mole ratio (MR)	–0.19
$L \times L$	–0.21
$T \times L$	0.07
$T \times T$	–0.14*
$t \times L$	–1.37
$t \times T$	–1.38*
$t \times t$	–3.68
MR × *L*	0.02
MR × *T*	–0.01
MR × *t*	0.08
MR × MR	–0.01

[a]An * indicates coefficient is significantly nonzero ($P < 0.05$).

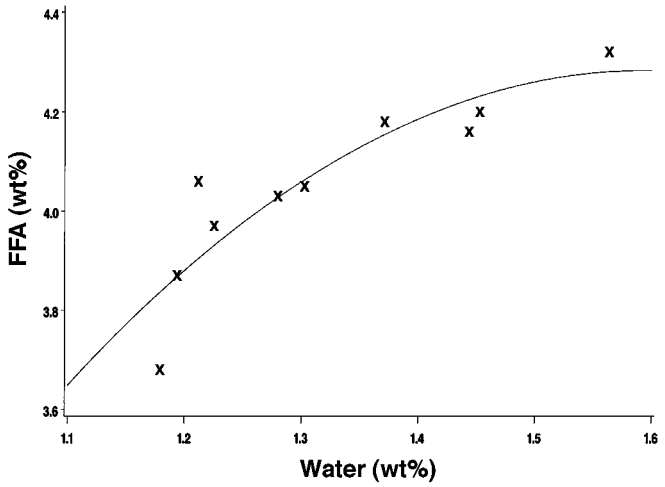

FIG. 1. Effect of water content on residual free fatty acid (FFA) content of ethyl ester products in response surface methodology (RSM) reactions listed in Table 1. The data shown correspond to the center points of the experimental design, namely, 35°C, 1.75 h, lipase level of 6.25%, and a molar excess of ethanol of 46.7%.

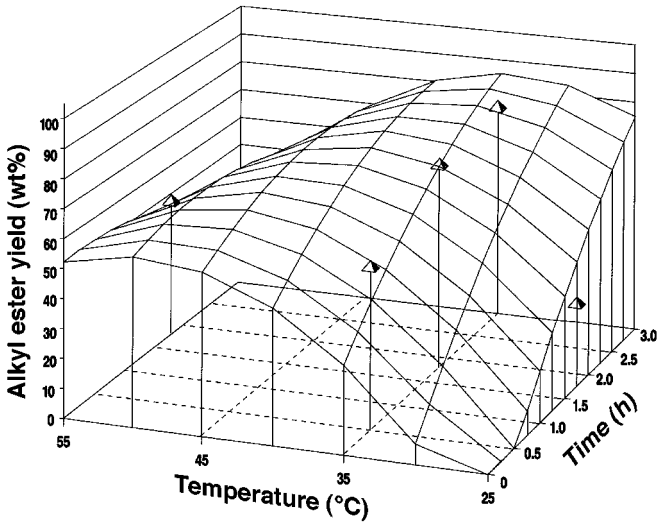

FIG. 2. Contour plot of time and temperature vs. percent alkyl ester at center points of lipase level and mole ratio of reactants listed in Table 1. The spikes represent the observed yields where molar excess of ethanol is 46.7 and lipase level is 6.75 wt%. (Center spike is average of 12 runs.)

would not exceed 85% within the parameter ranges established for transesterification. Accordingly, we took an alternative approach to improve the ethyl ester yields from grease. A high conversion to ethyl esters is essential if the method is to be considered feasible for production of biodiesel. Lipase PS-30 was used at the 5% (w/w grease) level to establish a time course of ethanolysis of grease using 95% ethanol at 35°C (mole ratio of ethanol to grease; 4:1) (Fig. 3). A lipase level of 5% was selected since the data in Table 1 indicated that at 35°C over the range of 4.4 to 8.1% lipase level, the predicted increase in yields of ethyl esters was <5 wt%. Examination of the reaction time course (Fig. 3) suggested that the rate of ethyl ester synthesis was approximately linear within 1 h but started to deteriorate afterward, and the percentage conversion to ethyl esters reached a plateau after approximately 4 h of reaction. Accordingly, in an attempt to improve the ethyl ester yields, a second portion of lipase PS-30 (5%) was added 1 h after the start of the initial reaction. This was done in an attempt to improve the yields and favor the ester products. Unfortunately, the second addition of lipase PS-30 did not significantly improve the conversion of grease to ethyl esters above the value predicted by the model (85%). On the other hand, addition of the supported lipase SP435 (5 wt%) 1 h after the initial PS-30-catalyzed reaction gave significantly improved ester yields (Fig. 4). It appeared that the rates of the lipase PS-30-catalyzed reactions at 35°C and 45°C within the first hour were similar but higher than the rate at 55°C, which

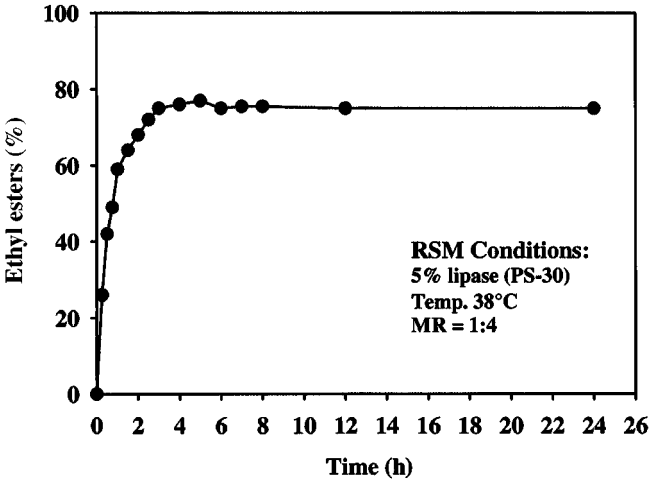

FIG. 3. Time course of percentage yield of ethyl esters in the transesterification of grease with 95% ethyl alcohol at 38°C catalyzed by lipase PS-30 (5% w/w of grease). PS-30, lipase from *Pseudomonas cepacia* (Amano Enzyme Co., Troy, VA). MR, mole ratio; for other abbreviations see Figure 1.

was predicted by the regression model (optimal temperature of 38.4°C). The addition of 5 wt% lipase SP435 1 h after incubation had started apparently improved the percentage conversion of ethyl esters to >96% at 35 and 45°C. These results

TABLE 3
RSM-Predicted Optimal Reaction Conditions and Yield of Ethyl Esters for the Lipase (PS-30)-Catalyzed Transesterification of Triacylglycerols with 95% Ethanol

Temperature (°C)	Time (h)	% Lipase PS-30 (w/w grease)	Mole ratio of E/G	Predicted values of % ethyl esters
38.4	2.47	13.12	6.6:1	85.4

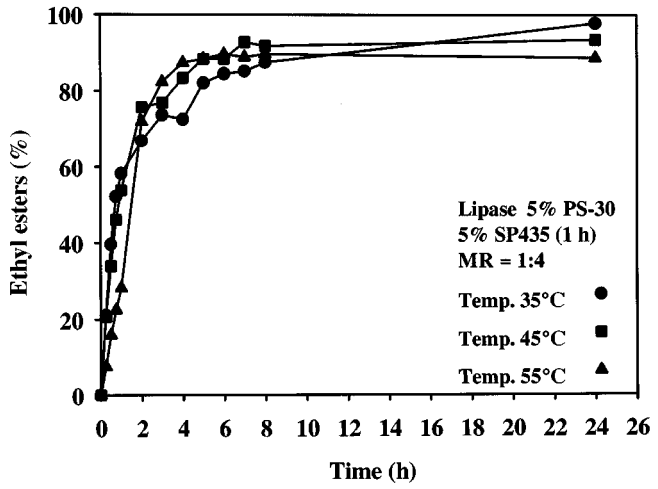

FIG. 4. Time course of percentage yield of ethyl esters in the transesterification of grease with 95% ethanol at 35, 45, or 55°C catalyzed initially by lipase PS-30 (5% w/w of grease) for 1 h with the sequential addition of lipase SP435 (5% w/w of grease). SP435, lipase from *Candida antarctica* (Novo Nordisk, Franklinton, NC). For other abbreviations and supplier see Figure 2.

initially were attributed to the adsorption of water from the reaction medium by the SP435 lipase support since this would shift the reaction equilibrium to favor products. This was not the case, however, since rather poor conversions to esters were observed at 35, 45, and 55°C when lipase SP435 was used alone in this two-step procedure. It is unclear at this time as to why the two-step, two-lipase reaction process gives higher ester yields than the one-step, one-lipase or two-step, one-lipase procedures. Studies are underway in our laboratory to further optimize the lipase-catalyzed production of a potential biodiesel—ethyl esters of recycled restaurant grease—using fuel-grade ethanol based on the RSM model presented in this study.

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