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Optimized synthesis of lipase-catalyzed biodiesel by Novozym 435

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Abstract: The ability of immobilized lipase from *Candida antarctica* (Novozym 435) to catalyze the alcoholysis of canola oil and methanol was investigated. Response surface methodology (RSM) and five–level–five–factor central composite rotatable design (CCRD) were employed to evaluate the effects of synthesis parameters, such as reaction time, temperature, enzyme concentration, substrate molar ratio of methanol to canola oil, and added water content on percentage weight conversion of canola oil methyl ester by alcoholysis. Reaction temperature and enzyme concentration were the most important variables. High temperature and superabundant methanol inhibited the ability of Novozym 435 to catalyze the synthesis of biodiesel. Based on the analysis of ridge max, the optimum synthesis conditions were as follows: reaction time 12.4 h, temperature 38.0 °C, enzyme concentration 42.3%, substrate molar ratio 3.5:1, and added water 7.2%. The predicted value was 99.4% weight conversion, and the actual experimental value was 97.9% weight conversion.

Keywords: alcoholysis; enzymatic biodiesel; immobilized lipase; optimization; response surface methodology

INTRODUCTION

Fatty acid methyl esters (FAME), also known as biodiesel, are currently of interest as an alternative fuel resource.[1] At present, biodiesel is commercially made by alkali-catalyzed transesterification of an oil or fat with an alcohol, usually methanol, a process that shifts the glyceride fatty acids from glycerol to methanol, producing FAME and glycerol.[2] Although efficient in terms of reaction time, the chemical approach to synthesis of biodiesel from triacylglycerols has some disadvantages, such as difficulty in the recovery of glycerol, the need for removal of salt residues, and the energy-intensive nature of the process. In contrast, biocatalysts (lipases) allow for synthesis of specific alkyl esters, easy recovery of glycerol, and transesterification of glycerides with high free fatty acid (FFA) content.[3] Therefore, the production of enzymatic biodiesels by lipase-catalyzed chemical reactions under mild conditions has gained current commercial interest. An optimized enzymatic process for biodiesel manufacture could improve the conversion yield and reduce the cost of production, which would benefit the manufacturers and be attractive to the consumer.

The importance of lipase (triacylglycerol hydrolase, EC 3.1.1.3)-catalyzed synthesis of enzymatic biodiesel by alcoholysis reactions in solvent or solvent-free systems has been reviewed.[4] The lipase-catalyzed esterification reactions for esters have also been reviewed, in which the parameters affecting the rates of lipase activities on esterification reaction include: reaction time, temperature, added water content, pH memory, acyl donors, etc.[5]

Nelson *et al* reported that the lipase of *Candida antarctica* was efficient for the transesterification of triacylglycerols with secondary alcohols to give branched alkyl esters and methanolysis of oils.[3] Shimada *et al* found that immobilized *C antarctica* lipase was inactivated in mixtures containing greater than 1.5 molar equivalents of methanol in oil.[4] Kose *et al* investigated alcoholysis of refined cotton seed oil in the presence of immobilized *C antarctica* lipase and suggested that conditions with a maximum methyl esters content of 91.5% were optimum.[6] Wu *et al* conducted biodiesel synthesis using recycled restaurant grease and 95% ethanol using a lipase from *Pseudomonas cepacia*; response surface analysis showed that time and temperature had significant effects on the yield of ethyl ester, and lipase level had a modest effect.[7] Likewise, a study was conducted using response surface methodology (RSM) in combination with principal-component analysis methods for optimizing the enzymatic transesterification of rapeseed oil methyl esters.[8]

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The present work focuses on the reaction parameters that affect immobilized lipase from *C antarctica* (Novozym 435)-catalyzed alcoholysis of canola oil with methanol in *n*–hexane. The main objectives of this work were to better understand the relationships between the reaction variables (time, temperature, enzyme concentration, substrate molar ratio, and added water content) and the response (percent weight conversion); and to obtain the optimum conditions for biodiesel synthesis using central composite rotatable design (CCRD) and RSM analysis.

EXPERIMENTAL

Materials

A refined and edible grade of canola oil was obtained from Wei-Chuan Company (Taipei, Taiwan). Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym 435) from *C antarctica* was a gift of Novo Nordisk Industry A/S (Bagsvaerd, Denmark). According to the commercial product manual, its catalytic activity was 7000 PLU g^{-1} (propyl laurate units per gram) with 1–2% water (w/w). Methanol (99.5% pure) and tributyrin (99% pure) were purchased from Sigma Chemical Co (St Louis, MO, USA). Molecular sieve 4Å was purchased from Davison Chemical (Baltimore, MD, USA) and *n*-hexane was obtained from Merck Chemical Co (Darmstadt, Germany). All other chemicals were of analytical reagent grade.

Experimental design

A five–level–five–factor CCRD was employed in this study, requiring 32 experiments.[9] The fractional factorial design consisted of 16 factorial points, 10 axial points (two axial points on the axis of each design variable at a distance of 2 from the design center), and six center points. The variables and their levels selected for the study of biodiesel synthesis were: reaction time (4–20 h); temperature (25–65 °C); enzyme concentration (10%–50% weight of canola oil, 0.1–0.5 g); substrate molar ratio (2:1–5:1; methanol: canola oil) and amount of added water (0–20%, by weight of canola oil). Table 1 shows the independent factors (x_i), levels and experimental design in terms of coded and uncoded. To avoid bias, 32 runs were performed in a totally random order.

Synthesis and analysis

Molecular sieve 4Å (10% w/w of substrate and *n*-hexane) was added to all chemicals for at least 24 h before reaction in order to remove all water. Canola oil (1 g) and different molar ratios of methanol were added to 3 cm^3 *n*-hexane, followed by different amounts of added water (0–20%, w/w) and enzyme (10%–50%, w/w). The mixtures of canola oil, methanol and Novozym 435 were stirred in an orbital shaking water bath (200 rpm) at different reaction temperatures and reaction times (Table 1). The enzyme and any residual water were removed by passing reaction media through an anhydrous sodium sulfate column. Before sample analysis, the reactant was mixed with an equal volume of an internal standard solution (150 mmol dm^{-3} tributyrin). Then analysis was performed by injecting a 1 mm^3 aliquot in splitless mode into a Hewlett Packard 6890 gas chromatograph (Avondale, PA, USA) equipped with a flame-ionization detector (FID) and a DB-5 fused-silica capillary column (30 m × 0.32 mm id; film thickness 1 μm; J&W Scientific, Folsom, CA, USA). Injector and detector temperatures were set at 300 °C. The oven initiating temperature was at 190 °C, elevated to 215 °C at 6 °C min^{-1}, and then increased up to 300 °C at 32 °C min^{-1}, held for 3 min. Pure nitrogen was used as a carrier gas. The percentage yield (weight conversion) was defined as (mg biodiesel ÷ mg initial canola oil) × 100% and was estimated using peak area integrated by on-line software, Hewlett Packard 3365 Series II ChemStation (Avondale, PA). The US standard of biodiesel is available as ASTM D6584.[2] This test method provides for the quantitative determination of free and total glycerin in B-100 methyl esters by gas chromatography. The range of detection for free glycerin is 0.005 to 0.05 mass %, and total glycerin from 0.05 to 0.5 mass %. Compared with this method, our analysis of biodiesel by gas chromatograph produced similar results.

Statistical analysis

The experimental data (Table 1) were analyzed by the response surface regression (Proc RSREG) procedure to fit the following second-order polynomial equation:[10]

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} x_i + \sum_{i=1}^{5} \beta_{kii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{kij} x_i x_j \quad (1)$$

where Y is response (percent weight conversion); β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients and x_i the uncoded independent variables. The option of ridge max was employed to compute the estimated ridge of maximum response for increasing radii from the center of the original design.

RESULTS AND DISCUSSION

Reaction time

The time course for the alcoholysis of canola oil with methanol by Novozym 435 is presented in Fig 1. The percent weight conversion of biodiesel increased up to ~60% at 16 h and there was no significant increase after 20 h. Therefore, the range of reaction time was chosen as from 4 to 20 h in the CCRD experimental design. The selection of reaction time range needs to be extremely precise in fractional factorial design; otherwise, the optimum condition of synthesis may be located outside the experimental region through the analyses of statistics and contour plots.

Model fitting

The RSREG procedure for SAS was employed to fit the second-order polynomial equation, eqn (1), to

Table 1. Central composite rotatable second-order design and experimental data for five-factor-five-level response surface analysis

Treatment No	Random No	Time (h) x_1	Temperature (°C) x_2	Enzyme content (%) x_3	Substrate molar ratio (methanol/canola oil) x_4	Added H_2O (% by wt of canola oil) x_5	Observed yield (% weight conversion) Y
1	8	−1(8)[a]	−1(35)	−1(20)	−1(3:1)	1(15)	31.87
2	13	1(16)	−1(35)	−1(20)	−1(3:1)	−1(5)	47.37
3	27	−1(8)	1(55)	−1(20)	−1(3:1)	−1(5)	24.73
4	11	1(16)	1(55)	−1(20)	−1(3:1)	1(15)	23.19
5	24	−1(8)	−1(35)	1(40)	−1(3:1)	−1(5)	91.31
6	23	1(16)	−1(35)	1(40)	−1(3:1)	1(15)	83.74
7	22	−1(8)	1(55)	1(40)	−1(3:1)	1(15)	60.72
8	7	1(16)	1(55)	1(40)	−1(3:1)	−1(5)	68.51
9	1	−1(8)	−1(35)	−1(20)	1(5:1)	−1(5)	23.05
10	14	1(16)	−1(35)	−1(20)	1(5:1)	1(15)	24.28
11	25	−1(8)	1(55)	−1(20)	1(5:1)	1(15)	24.85
12	21	1(16)	1(55)	−1(20)	1(5:1)	−1(5)	22.43
13	16	−1(8)	−1(35)	1(40)	1(5:1)	1(15)	72.77
14	32	1(16)	−1(35)	1(40)	1(5:1)	−1(5)	72.19
15	12	−1(8)	1(55)	1(40)	1(5:1)	−1(5)	35.44
16	28	1(16)	1(55)	1(40)	1(5:1)	1(15)	36.15
17	9	−2(4)	0(45)	0(30)	0(4:1)	0(10)	36.91
18	5	2(20)	0(45)	0(30)	0(4:1)	0(10)	46.09
19	3	0(12)	−2(25)	0(30)	0(4:1)	0(10)	72.19
20	2	0(12)	2(65)	0(30)	0(4:1)	0(10)	23.50
21	18	0(12)	0(45)	−2(10)	0(4:1)	0(10)	22.50
22	6	0(12)	0(45)	2(50)	0(4:1)	0(10)	96.45
23	30	0(12)	0(45)	0(30)	−2(2:1)	0(10)	60.77
24	10	0(12)	0(45)	0(30)	2(6:1)	0(10)	24.58
25	20	0(12)	0(45)	0(30)	0(4:1)	−2(0)	95.71
26	26	0(12)	0(45)	0(30)	0(4:1)	2(20)	50.49
27	15	0(12)	0(45)	0(30)	0(4:1)	0(10)	50.40
28	4	0(12)	0(45)	0(30)	0(4:1)	0(10)	54.50
29	31	0(12)	0(45)	0(30)	0(4:1)	0(10)	67.78
30	29	0(12)	0(45)	0(30)	0(4:1)	0(10)	59.87
31	19	0(12)	0(45)	0(30)	0(4:1)	0(10)	71.48
32	17	0(12)	0(45)	0(30)	0(4:1)	0(10)	51.56

[a] Numbers in parenthesis represent actual experimental amounts.

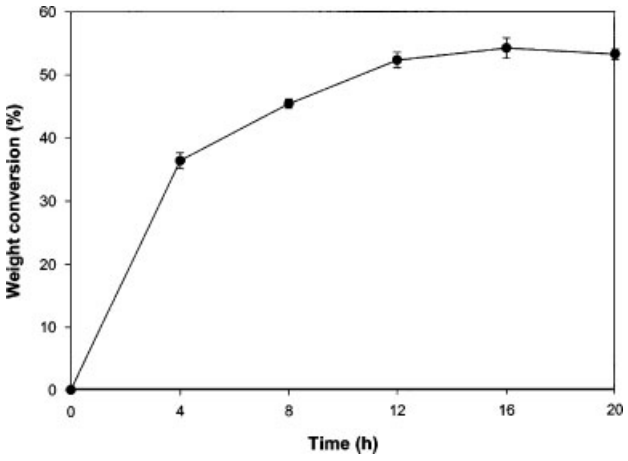

Figure 1. Time course of the alcoholysis of canola oil with methanol by Novozym 435. The reaction was carried out at 45 °C in 3 cm^3 hexane containing 1 g canola oil, substrate molar ratio of 4:1 (methanol:canola oil), 0.1 g (10%, w/w, weight of canola oil) Novozym 435. The activity of 10% Novozym 435 was 700 PLU.

the experimental data—percent weight conversions (Table 1). Among the various treatments, the greatest weight conversion (96.5%) was treatment No 22 (12 h, 45 °C, 50% enzyme, substrate molar ratio 4:1, added water 10%), and the smallest conversion (only 22.4%) was treatment No 12 (16 h, 55 °C, 20% enzyme, substrate molar ratio 5:1, added water 5%). From the SAS output of RSREG, the second-order polynomial equation, eqn (1), is given below:

$$\begin{aligned} Y = {} & -185.209 + 10.414x_1 + 2.838x_2 + 5.828x_3 \\ & + 31.402x_4 - 4.357x_5 - 0.268{x_1}^2 \\ & - 0.006x_2x_1 - 0.027{x_2}^2 - 0.019x_3x_1 - 0.055x_3x_2 \\ & + 0.002{x_3}^2 - 0.0238x_4x_1 + 0.023x_4x_2 \\ & - 0.345x_4x_3 - 3.993{x_4}^2 - 0.184x_5x_1 + 0.019x_5x_2 \\ & - 0.001x_5x_3 + 0.467x_5x_4 + 0.145{x_5}^2 \end{aligned} \quad (2)$$

The analysis of variance (ANOVA) indicated that the second-order polynomial model (eqn (2)) was statistically significant and adequate to represent the actual relationship between the response (percent weight conversion) and the significant variables, with

Table 2. Analysis of variance for joint test

Factor	Degrees of freedom	Sum of squares	Prob $>F$[a]
Time (x_1)	6	821.752	0.1450[b]
Temperature (x_2)	6	3273.163	0.0016
Enzyme content (x_3)	6	9006.313	0.0000
Substrate molar ratio (x_4)	6	2307.255	0.0064
Added water (x_5)	6	1279.665	0.0465

[a] prob $> F$ = level of significance.
[b] Not significant at $p = 0.05$.

very small p-value (0.0001) and a satisfactory coefficient of determination ($R^2 = 0.955$). Furthermore, the overall effect of the five synthesis variables on the percent weight conversion of biodiesel was further analyzed by a joint test (Table 2). The results revealed that the reaction temperature (x_2), enzyme concentration (x_3), substrate molar ratio (x_4) and added water content (x_5) were the important factors, exerting a statistically significant overall effect ($p < 0.05$) on the response weight conversion of biodiesel; but reaction time (x_1) had a less significant effect ($p > 0.05$) on the synthesis of biodiesel.

Effect of synthesis parameters

Fig 2 shows the effect of reaction temperature, enzyme concentration, and their mutual interaction on biodiesel synthesis at 12 h, substrate molar ratio of 4:1, and added water amount of 10%. Apparently, increased enzyme concentration increased the weight conversion at lower temperatures. A reaction at medium reaction temperature (35 °C) and the greater concentration of enzyme (50% weight of canola oil) favored maximal conversion of weight (~95%). At temperatures greater than 35 °C, weight conversion was decreased at 20% enzyme (weight of canola oil), probably due to the thermal inhibition of enzyme, indicating that the optimum temperature for Novozym 435-catalyzed biodiesel production was around 35 °C. The effect of varying reaction temperature and substrate molar ratio at constant reaction time (12 h), enzyme concentration (30%), and added water content (10%) is shown in Fig 3. In general, an increase in substrate molar ratio led to lower yields at any temperature. It was concluded that high concentrations of methanol inactivated Novozym 435. Similar results, that an excess of methanol decreased the production of enzymatic biodiesel catalyzed by Lipozyme IM77, were reported in a previous study.[11] A reaction temperature of 35 °C at a lower substrate molar ratio (3:1) favored maximal yield ~70% biodiesel (by weight). The inhibition of Novozym 435 was confirmed (see Fig 3).

Overall effects

The entire relationships between reaction factors and response can be better understood by examining the planned series of contour plots (Fig 4) generated from the predicted model (eqn (2)) by holding constant the enzyme content (20, 30, 40%, weight of canola oil) and substrate molar ratio (3:1, 4:1, 5:1). The ratio of added water was constant (10%, by weight) in the optimization studies. In Fig 4 (A), (B) and (C) represent the same substrate (3:1); and (A), (D) and (G) represent the same enzyme content (20%). The application of contour plots could be employed to study the synthesis variables simultaneously in a five-dimensional space and to observe readily the overall effects of synthesis

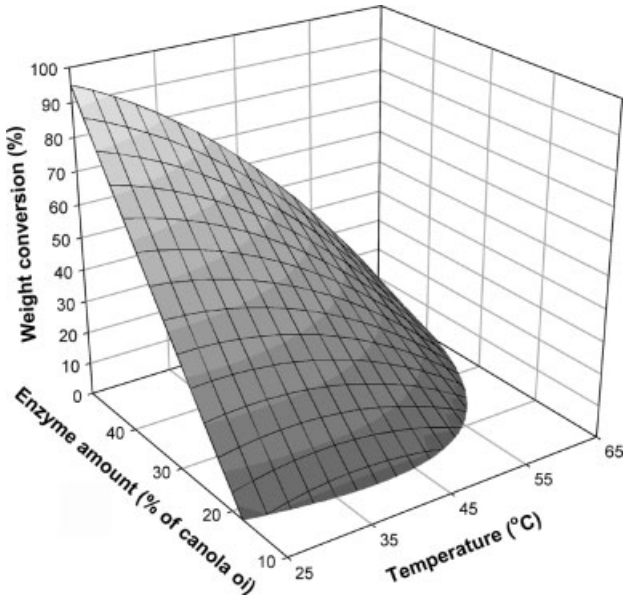

Figure 2. Response surface plot showing the effect of reaction temperature, Enzyme concentration, and their mutual interaction on biodiesel synthesis. Other synthesis parameters (reaction time, substrate molar ratio, and added water amount) are constant at 0 levels.

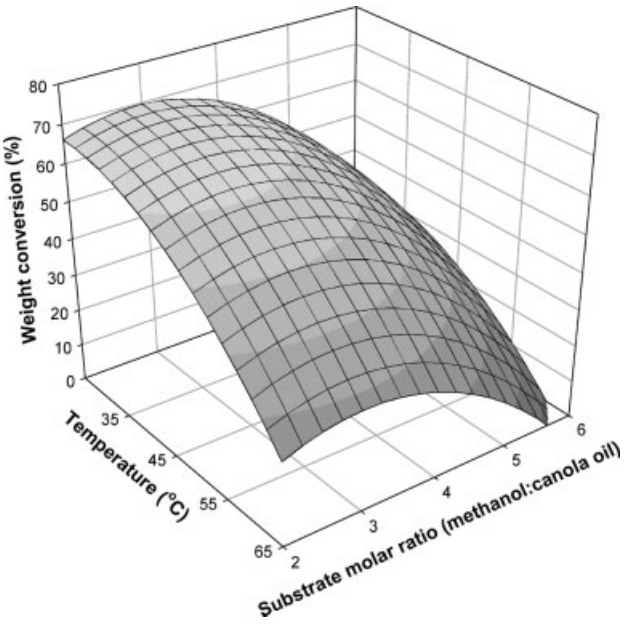

Figure 3. Response surface plot showing the effect of substrate molar ratio, reaction temperature, and their mutual interaction on biodiesel synthesis. Other synthesis parameters (reaction time, substrate molar ratio, and added water content) are constant at 0 levels.

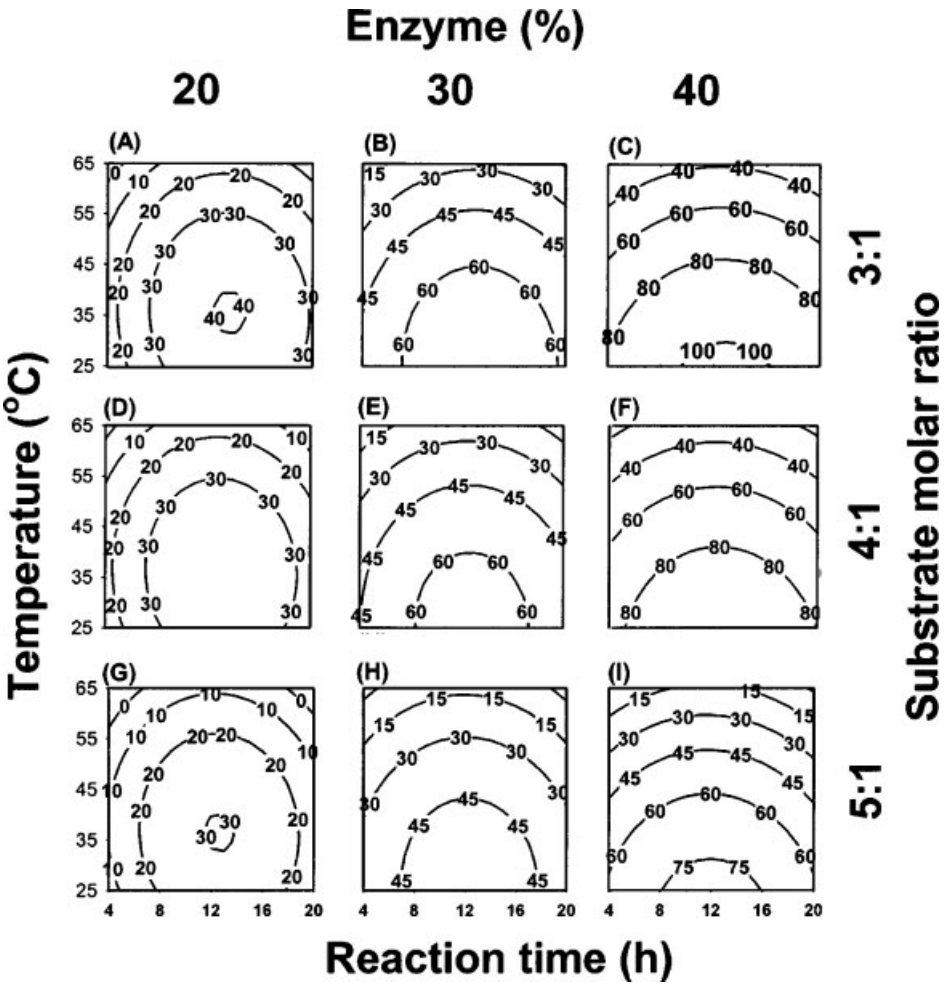

Figure 4. Contour plots of percent weight conversion of biodiesel. Enzyme concentration was by weight of canola oil and substrate molar ratio was methanol to canola oil. The numbers inside the contour plots indicate weight conversions at given reaction conditions.

variables on yield conversions. Reaction time (x_1) and temperature (x_2) were considered to be the important variables for lipase-catalyzed biodiesel as indicators of effectiveness and economical performance. Generally, all nine contour plots in Fig 4 exhibited similar behavior in that predicted weight conversion increased at the start and decreased after 12 h. Therefore, a 12-h synthesis gave the highest percent weight conversion compared with the others in the experimental region. The decreased weight conversion after 12 h was probably a consequence of product inhibition of the alcoholysis reaction. Likewise, an increase in reaction temperature from 25 to 35 °C resulted in higher product yield. However, above 35 °C weight conversion was reduced, indicating that the higher temperatures may denature Novozym 435. Overall, all nine contour plots in Fig 4 indicated that predicted weight conversion increased with increasing enzyme concentration. In practice, increased enzyme concentration gave higher weight conversion. However, conversion was decreased by increased substrate molar ratio because the superabundant methanol inhibited the activity of Novozym 435. Therefore, the optimum substrate molar ratio was very important in the production of lipase-catalyzed biodiesel for the alcoholysis reaction.

Attaining optimum conditions

The optimum synthesis of enzymatic biodiesel was determined by the ridge max analysis.[10] The method of ridge analysis computes the estimated ridge of maximum response for increasing radii from the center of original design. The ridge max analysis (Table 3) indicated that maximum molar conversion was 99.3% ± 4.6% at 12.4 h, 38.0 °C, 42.3% enzyme amount, 3.5:1 substrate molar ratio, and 7.2% added water content at the distance of the coded radius 0.8.

Model verification

The adequacy of the predicted model here was examined by additional independent experiments at the suggested optimum synthesis conditions. The predicted value was 99.4% molar conversion and the actual experimental value was 97.9%. A chi-square test (p-value = 0.96, degrees of freedom = 5) indicated that observed values were significantly the same as the predicted values and the generated model adequately predicted the percent molar conversion.[12] Thus, the optimization of lipase-catalyzed synthesis for biodiesel (canola oil methyl ester) by Novozym 435 was successfully developed by CCRD and RSM.

Table 3. Estimated ridge of maximum response for variable percent weight conversion

Coded radius	Estimated response (corporation)	Standard error	x_1 (h)	x_2 (°C)	x_3 (%)	x_4 (methanol/canola oil)	x_5 (added water) (%)
0	55.26	3.27	12.00	45.00	30.00	4.00	10.00
0.2	65.00	3.23	12.09	43.23	33.18	3.87	9.51
0.4	75.57	3.24	12.18	41.46	36.31	3.74	8.89
0.6	87.01	3.59	12.30	39.73	39.35	3.62	8.12
0.8	99.40	4.60	12.43	38.03	42.27	3.51	7.20

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