Five-Factor Response Surface Optimization of the Enzymatic Synthesis of Citronellyl Butyrate by Lipase IM77 from *Mucor miehei*

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ABSTRACT: Response surface methodology (RSM) and a five-level-five-factor central composite rotatable design (CCRD) were used to evaluate the effects of synthetic variables, such as reaction time (3 to 27 h), temperature (25 to 65°C), enzyme amount (10 to 50%), substrate molar ratio of citronellol to butyric acid (1:1 to 1:3), and added water amount (0 to 20%) on molar percent yield of citronellyl butyrate by direct esterification, using lipase IM77 from *Mucor miehei*. Reaction time and temperature were the most important variables. Substrate molar ratio had no effect on percent molar conversion. Based on contour plots, optimal synthetic conditions were these: reaction time 24 h, temperature 60°C, enzyme amount 20%, substrate molar ratio 1:1.5, and added water 0%. The predicted molar conversion value was 100%. An actual experimental value of 98% molar conversion was obtained.

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KEY WORDS: Citronellyl butyrate, direct esterification, enzymatic synthesis, optimization.

Terpene esters are essential oils widely used in the food, cosmetic and pharmaceutical industries as flavor and fragrance compounds (1). Among them, the acetates and butyrates of acyclic terpene alcohols (geraniol and citronellol) are the most important (2). The esters of terpene alcohols formerly were chemically synthesized by direct esterification of terpene alcohols with short-chain acids (3). Recently, enzymatic synthesis with lipases as catalysts in anhydrous organic solvents (4) has been used to produce a number of commercially important flavor esters, both by transesterification (5–7) and by direct esterification (1,8,9). An optimized process for high-yield enzymatic synthesis of citronellyl esters would benefit food manufacturers.

The current literature on lipase-catalyzed synthesis of terpene esters has been reviewed and the lipases chosen for many of the cited studies are from *Mucor miehei* and *Candida cylindracea* (10). Response surface methodology (RSM) and central composite rotatable design (CCRD) were employed to evaluate the effects of synthetic variables on the synthesis of geranyl butyrate and real optimum conditions were suggested, based on the analysis of planned contour plots (11). The reaction parameters affecting the direct esterification of citronellol with acetic acid have been investigated (1,12). However, there were neither advanced combined effects of reaction parameters nor any detailed optimal conditions specified.

The current paper focuses on the reaction parameters that affect immobilized *M. miehei* lipase (IM77)-catalyzed direct esterification of citronellyl butyrate using butyric acid as acyl donor. Our objectives were to better understand the relationships between the factors (reaction time, temperature, enzyme amount, substrate molar ratio, and added water amount) and the response (% molar conversion); and to determine optimal synthetic conditions for citronellyl butyrate production by lipase IM77 from *M. miehei* using RSM and CCRD.

MATERIALS AND METHODS

Experimental design. A five-level-five-variable CCRD with six replicates at the center point was adopted in this study, requiring 32 experiments (11,13). The variables assumed important in citronellyl butyrate synthesis were: time (3–27 h); temperature (25–65°C); enzyme amount (10–50% by weight of citronellol); substrate molar ratio (1:1–1:3; citronellol/butyric acid); and amount of added water (0–20% by weight of citronellol). The independent variables (x_i), levels, and experimental design in terms of coded and uncoded variables are presented Table 1.

Materials. Immobilized lipase IM77 (7.7 BAUN/G) from *M. miehei* was obtained from Novo Nordisk Bioindustrial, Inc. (Bagsvaerd, Denmark). Citronellol (95% pure), butyric acid (99% pure) and *n*-hexadecane (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). *n*-Hexane (high-performance liquid chromatography grade) was obtained from Fisher Scientific (Norcross, GA). Molecular sieve 4Å was purchased from Davison Chemical (Baltimore, MD).

Esterification method. Citronellyl butyrate synthesis was carried out in screw-capped test tubes (16 × 125 mm). Citronellol (100 mg) and different molar ratios of butyric acid were added to 3 mL *n*-hexane, followed by different amounts of water and enzyme. The mixture was stirred in an orbital

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TABLE 1
Central Composite Rotatable Second-Order Design and Experimental Data for Five-Factor, Five-Level Response Surface Analysis

Treatment #	Time (h) x_1	Temperature (°C) x_2	Enzyme (% by wt of citronellol) x_3	Substrate molar ratio (citronellol/ butyric acid) x_4	H_2O (% by wt of citronellol) x_5	Yield (% molar conversion) Y
1	–1(9)[a]	–1(35)	–1(20)	–1(1:1.5)	1(15)	5.139
2	1(21)	–1(35)	–1(20)	–1(1:1.5)	–1(5)	34.000
3	–1(9)	1(55)	–1(20)	–1(1:1.5)	–1(5)	21.902
4	1(21)	1(55)	–1(20)	–1(1:1.5)	1(15)	10.728
5	–1(9)	–1(35)	1(40)	–1(1:1.5)	–1(5)	38.651
6	1(21)	–1(35)	1(40)	–1(1:1.5)	1(15)	67.029
7	–1(9)	1(55)	1(40)	–1(1:1.5)	1(15)	21.974
8	1(21)	1(55)	1(40)	–1(1:1.5)	–1(5)	98.043
9	–1(9)	–1(35)	–1(20)	1(1:2.5)	–1(5)	9.830
10	1(21)	–1(35)	–1(20)	1(1:2.5)	1(15)	10.071
11	–1(9)	1(55)	–1(20)	1(1:2.5)	1(15)	1.940
12	1(21)	1(55)	–1(20)	1(1:2.5)	–1(5)	70.735
13	–1(9)	–1(35)	1(40)	1(1:2.5)	1(15)	17.621
14	1(21)	–1(35)	1(40)	1(1:2.5)	–1(5)	59.938
15	–1(9)	1(55)	1(40)	1(1:2.5)	–1(5)	66.094
16	1(21)	1(55)	1(40)	1(1:2.5)	1(15)	46.372
17	–2(3)	0(45)	0(30)	0(1:2)	0(10)	4.551
18	2(27)	0(45)	0(30)	0(1:2)	0(10)	70.005
19	0(15)	–2(25)	0(30)	0(1:2)	0(10)	20.166
20	0(15)	2(65)	0(30)	0(1:2)	0(10)	15.609
21	0(15)	0(45)	–2(10)	0(1:2)	0(10)	6.963
22	0(15)	0(45)	2(50)	0(1:2)	0(10)	71.121
23	0(15)	0(45)	0(30)	–2(1:1)	0(10)	63.494
24	0(15)	0(45)	0(30)	2(1:3)	0(10)	38.133
25	0(15)	0(45)	0(30)	0(1:2)	–2(0)	72.714
26	0(15)	0(45)	0(30)	0(1:2)	2(20)	9.336
27	0(15)	0(45)	0(30)	0(1:2)	0(10)	35.452
28	0(15)	0(45)	0(30)	0(1:2)	0(10)	33.837
29	0(15)	0(45)	0(30)	0(1:2)	0(10)	34.258
30	0(15)	0(45)	0(30)	0(1:2)	0(10)	34.965
31	0(15)	0(45)	0(30)	0(1:2)	0(10)	35.662
32	0(15)	0(45)	0(30)	0(1:2)	0(10)	35.304

[a]Numbers in parentheses represent actual experimental amounts.

shaking water bath (200 rpm) at different temperatures and reaction times (Table 1).

Extraction and analysis. The enzyme was removed by passing reaction media through an anhydrous sodium sulfate column. *n*-Hexadecane (25.7 g/L) was added to each sample as an internal standard before sample analysis. Analysis was done by injecting a 1-µL aliquot in a splitless mode into a Hewlett-Packard 4890 gas chromatograph (Avondale, PA) equipped with a flame-ionization detector. A DB-5 fused-silica capillary column (30 m × 0.32 mm i.d.; film thickness 1 µm; J&W Scientific, Folsom, CA) was used. Injector and detector temperatures were set at 250 and 26°C, respectively. Oven temperature was held at 100°C for 5 min before being elevated to 200°C at 50°C/min, and then held for 3 min. The carrier gas was nitrogen. The percent yield (molar conversion) was defined as [(mmole citronellyl butyrate ÷ mmole initial citronellol) × 100%] (11) and was estimated using peak area integrated by an on-line software Hewlett-Packard 3365 Series II ChemStation.

Data analysis. Experimental data (Table 1) were analyzed by the response surface regression (RSREG) procedure with lackfit option to fit the following second-order polynomial equation (14):

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} x_i + \sum_{i=1}^{5} \beta_{kii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{kij} x_i x_j \qquad [1]$$

where Y is response (% molar conversion); β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients and x_i the uncoded independent variables.

Contour plots were created by holding constant three variables of the second order polynomial equation.

RESULTS AND DISCUSSION

The time course of the direct esterification of citronellol with butyric acid by IM77 is shown in Figure 1. The yield of citronellyl butyrate increased up to 60% at 12 h. Therefore, the range of reaction time was investigated from 3 to 27 h. The

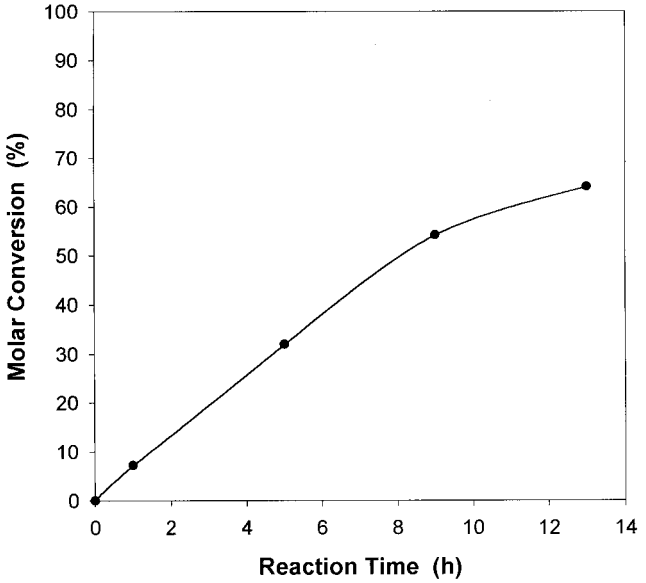

FIG. 1. Time course of the esterification of citronellol with butyric acid by IM77. The reaction was carried out at 45°C in 3 mL hexane containing 100 mg citronellol, 112.7 mg butyric acid (molar ratio of citronellol to butyric acid of 1:2), 30 mg lipase IM77, and without added water.

effect of added water is illustrated in Figure 2. Apparently, lipase IM77 performed best under anhydrous conditions in the direct esterification of citronellol with butyric acid under the conditions employed here.

Equation 1 was fitted to the experimental data (Table 1) using the RSREG with lackfit option of the Statistical Analysis System (SAS) software to obtain a full model. The adequacy and fitness of the full model was tested by analysis of variance (data not shown), with a significant lack of fit, indicating that data variation was not adequately explained (15). Then, the overall effect of the five synthesis variables on percent molar conversion was further analyzed by a joint test (14), which tested the hypothesis that all parameters involving one particular factor are zero. The results (Table 2) showed that time (x_1), temperature (x_2), enzyme % (x_3), and H_2O% (x_5) were the most important factors, exerting a statistically significant overall effect on response (% molar conversion). Substrate molar ratio (x_4) had no significant effect, indicating that synthesis with low substrate molar ratio (1:1.5; citronellol/butyric acid) is possible. Such an application would reduce production cost by reducing the amounts of cosubstrate (butyric acid) required to synthesize citronellyl butyrate. Therefore, substrate molar ratio (x_4) was removed from the full model and a reduced model (four variables) was produced. Joint test (Table 2) revealed that all variables in the reduced model had significant overall effect with *P*-values less than 0.01. Analysis of variance (Table 3) indicated that the reduced model was adequate to represent the actual relationships between response percent molar conversion and the significant variables, with no significant lack-of-fit (*P*-value = 0.127) and acceptable coefficient of determination (R^2 = 0.907). The secondary order polynomial equation (2) is given below:

$$\begin{aligned} Y = {} & -178.294307 - 1.269825x_1 + 6.104070x_2 - 0.621944x_3 \\ & - 5.114317x_5 + 0.000201{x_1}^2 + 0.014761x_2x_1 - 0.048404{x_2}^2 \\ & + 0.041999x_3x_1 + 0.001862x_3x_2 + 0.004357{x_3}^2 \\ & - 0.080652x_5x_1 - 0.166501x_5x_2 - 0.001426x_5x_3 + 0.037759{x_5}^2 \end{aligned} \quad [2]$$

The stationary points were obtained by analytical techniques including canonical analysis and ridge analysis (data not shown). Not only was it found that the stationary point was located inside the experimental region, but also that the predicted degree of conversion at the stationary point was 102.8% (>100%), indicating that this analysis could not be used to identify the optimal conditions. Thus, identification of the optimum required another approach which employed graphical solutions of predicted response model. Reaction time (x_1) and reaction temperature (x_2) were the most important variables for citronellyl butyrate synthesis, with very

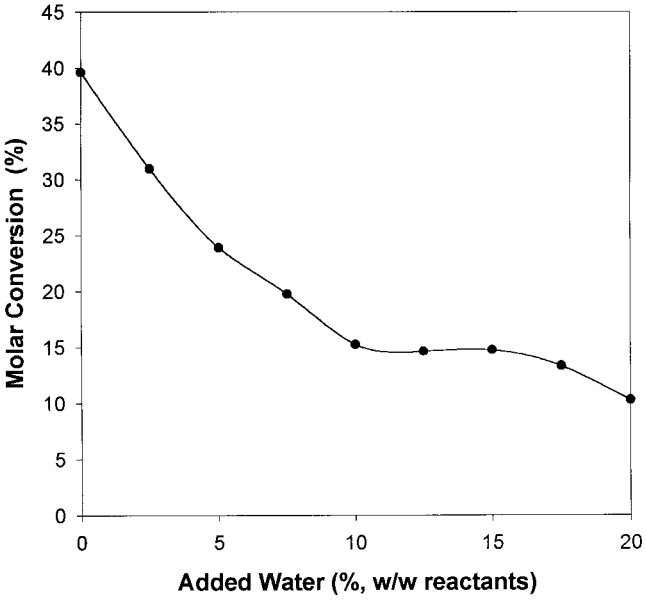

FIG. 2. Effect of added water on lipase IM77-catalyzed synthesis of citronellyl butyrate. The reaction was carried out in 3 mL hexane containing 100 mg citronellol, 112.7 mg butyric acid (molar ratio of citronellol to butyric acid of 1:2), 30 mg lipase IM77, and reaction time of 7 h at 45°C.

TABLE 2
Analysis of Variances for Full Model and Reduced Model[a]

	Full model		Reduced model	
	df	*P*-value	df	*P*-value[a]
Time (x_1)	6	0.0004	5	0.0002
Temperature (x_2)	6	0.0037	5	0.0158
Enzyme % (x_3)	6	0.0035	5	0.0001
Substrate molar ratio (x_4)	6	0.1096*	5	—
H_2O % (x_5)	6	0.0051	5	0.0001

[a]*P*-value = level of significance; df = degrees of freedom. *Not significant at *P* = 0.05.

TABLE 3
Analysis of Variance for Synthetic Variables Pertaining to Response Percent Molar Conversion

Source	df	Sum of squares	Prob > *F*
Model	14	18314	0.0001
Linear	4	16236	0.0000
Quadratic	4	760.48	0.1890
Cross product	6	1317.36	0.1217
Lack of fit	11	1540.26	0.1268
Pure error	6	324.18	
Total error	17	1864.44	
R^{2a}	0.9067		

[a]R_2 = coefficient of determination; for other abbreviation see Table 2.

small *P*-values (data not shown) in the final reduced model, respectively, and were considered as indicators of effectiveness and economical performance. The molar ratio of substrates (x_4) was kept constant without significant effect on the response in the optimization studies. The result supported our previous findings that substrate molar ratio was a less important factor than the others (reaction time, temperature, enzyme amount, and added water amount) in the synthesis of geranyl butyrate by lipase AY from *C. rugosa* (11).

Furthermore, enzyme percentage (20, 30, 40%) and added water percentage (0, 5, 10%) were kept inside experimental five-dimensional space. Such an application could be adopted to study the variables of synthesis simultaneously. Contour plots (Fig. 3) were obtained from the predicted model (Eq. 2).

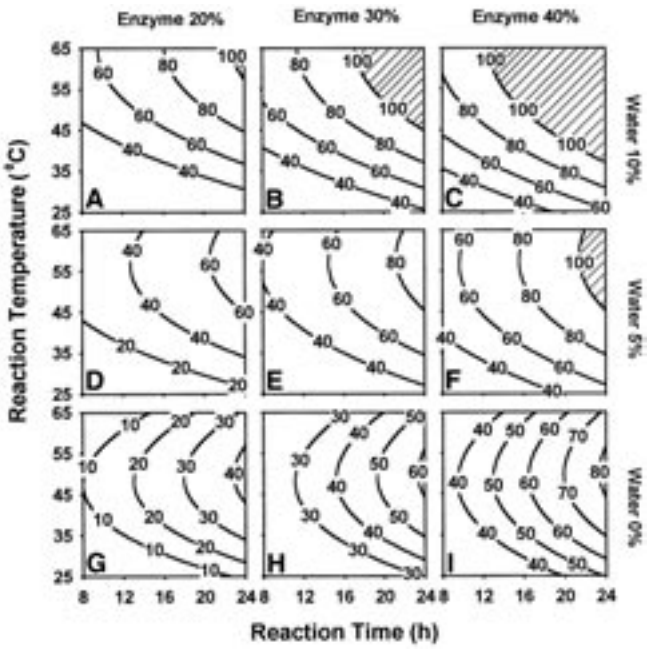

FIG. 3. Contour plots of percent molar conversion of citronellyl butyrate at substrate molar ratio, citronellol/butyric acid = 1:1.5. Enzyme amount and added water amount were by weight of citronellol. The numbers inside the contour plots indicate molar conversions at given reaction conditions.

Figure 3A, B, and C represent the same added water content (0%); and A, D, and G represent the same enzyme amount (20%). In general, all nine contour plots in Figure 3 exhibited similar behaviors in that a greater reaction time gave an increased predicted percent molar conversion. However, when the amount of added water was more, higher reaction temperature did not increase percent molar conversion. When the enzyme amount was 20% by weight of citronellol, the optimal reaction temperature for reactions conducted without added water was ~60°C in hexane. The optimal temperature, and the yield, fell at higher water levels (Fig. 3A, D, and G). As expected, reaction with high enzyme amount gave higher percent molar conversion compared to reaction with less enzyme. Thus, water inhibits the enzymatic activity of lipase IM77, or stimulates the hydrolysis of citronellyl butyrate reducing ester yields. This is consistent with the result of Figure 2. The data of Figure 3 indicate that reaction conditions of 24 h, 60°C, 20% enzyme, 0% added water, and a substrate molar ratio of 1:1.5 [citronellol/butyric acid] should result in optimal quantitative esterification using the minimum amount of enzyme examined here. In our previous report, optimal conditions for geranyl butyrate synthesis by transesterification with lipase AY were reaction time 9 h, temperature 35°C, enzyme amount 50%, and added water 10% (11). In the present study, the optimal reaction time (24 h) is longer than in the previous work (9 h), however, less enzyme was employed here (20%). Stamatis *et al*. (3) reported a high yield (80–90%) of geranyl ester by esterase isolated from *Fusarium oxysporum* using reaction times over 60 h. The ability of lipase IM77 from *M. miehei* to synthesize geranyl esters appears greater than that of esterase isolated from *F. oxysporum.*

The adequacy of the model derived here was examined by additional independent experiments at suggested optimal synthetic conditions. The predicted value was 100% molar conversion and the actual experimental value was 98.95 ± 0.14%. A chi-square test (*P*-value = 0.999, degrees of freedom = 6) indicated that the generated model adequately predicted the percent molar conversion (16). Thus, the optimal conditions for citronellyl butyrate production by lipase IM77 were successfully developed by CCRD and RSM.

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