Lipase-Catalyzed Synthesis of Isoamyl Butyrate: Optimization by Response Surface Methodology

S. Hari Krishna[a], B. Manohar[b], S. Divakar[a], and N.G. Karanth[a,]*

[a]Fermentation Technology and Bioengineering Department and [b]Food Engineering Department, Central Food Technological Research Institute, Mysore 570 013, India

ABSTRACT: Immobilized lipase from *Mucor miehei* (Lipozyme IM-20) was employed in the esterification of butyric acid and isoamyl alcohol to synthesize isoamyl butyrate in *n*-hexane. Response surface methodology based on five-level, five-variable central composite rotatable design was used to evaluate the effects of important variables—enzyme/substrate (E/S) ratio (5–25 g/mol), acid concentration (0.2–1.0 M), alcohol concentration (0.25–1.25 M), incubation period (12–60 h), and temperature (30–50°C)—on esterification yield of isoamyl butyrate. In the range of parameters studied, the extent of esterification decreased with temperature, lower E/S ratios, and incubation periods. Excess acid and alcohol concentrations (i.e., acid/alcohol > 1.4 or alcohol/acid > 1.4) were found to decrease yield probably owing to inhibition of the enzyme by acid or alcohol, the former being more severe. The optimal conditions achieved are as follows: E/S ratio, 17 g/mol; acid concentration, 1.0 M; incubation period, 60 h; alcohol concentration, 1.25 M; and temperature, 30°C. With these conditions, the predicted value was 1.0 M ester, and the actual experimental value was 0.98 M.

Paper no. J9224 in *JAOCS 76,* 1483–1488 (December 1999).

KEY WORDS: Central composite rotatable design, enzymatic synthesis, esterification, isoamyl butyrate, lipase, Lipozyme IM-20, *Mucor miehei,* response surface methodology.

Esters of short-chain fatty acids and alcohols are extremely important aroma compounds. For instance, ethyl butyrate and isoamyl acetate/butyrate are found, respectively, in the aroma of strawberry and banana. Esters of isoamyl alcohol, especially acetate and butyrate, are valuable, high-demand flavor and fragrance compounds widely used in the food, beverage, cosmetic, and pharmaceutical industries. Currently, most of the flavor components are synthesized by chemical methods or extracted from plant materials. Recent trends in consumer preference toward natural products indicate that biocatalysts may have an advantage over their chemical counterparts, as products of biocatalysis may obtain a "natural" label (1).

Some attempts have been made to evaluate the feasibility of producing isoamyl butyrate using lipases from various sources (2–4). However, the need for the use of high lipase and low substrate concentrations has been a significant drawback. Considering the high demand and benefits, an optimized process with high yields for the economic enzymatic synthesis of isoamyl butyrate is important.

The goal of the present investigation was to optimize the process for enzymatic synthesis of isoamyl butyrate using response surface methodology employing a five-level, five-variable central composite rotatable design (CCRD). The variables affecting the esterification that were considered were enzyme/substrate (E/S) ratio, acid concentration, incubation period, alcohol concentration, and reaction temperature.

MATERIALS AND METHODS

Lipase. Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Lipozyme IM-20, 25 BIU/g) from *Mucor miehei* (presently named *Rhizomucor miehei*) supported on macroporous weak anionic resin beads was kindly provided by Novo Nordisk (Bagsvaerd, Denmark).

Solvent and substrates. n-Hexane obtained from S.D. Fine Chemicals (Mumbai, India) was used as the organic solvent. Isoamyl alcohol was purchased from Fluka Chemie AG (Buchs, Switzerland). Butyric acid, methanol, and sodium hydroxide were procured from S.D. Fine Chemicals. All the chemicals were analytical reagent grade. Soluble impurities, boiling fractions, and excess water were removed by distillation.

Esterification. Lipozyme IM-20, which is insoluble in organic solvents, was employed as a biocatalyst to perform the esterification of isoamyl alcohol by butyric acid. Ester synthesis was performed in stoppered flasks with a working volume of 10 mL. An appropriate amount of enzyme was added into the flask containing freshly prepared solution of isoamyl alcohol and butyric acid dissolved in *n*-hexane. The reaction mixtures were incubated in an orbital shaker (Lab-Line Instruments Inc., Melrose Park, IL) at 150 rpm and at specified temperature.

Determination of esterification yield. Aliquots of the reaction mixture were withdrawn periodically. Samples were assayed both by titrimetry and by gas chromatography (GC). Samples were titrated against sodium hydroxide to determine the residual acid content using phenolphthalein as indicator and methanol as quenching agent. The percentage esterification and the moles of acid reacted were calculated from the values obtained for the blank and test samples. GC analyses

*To whom correspondence should be addressed.
E-mail: ferm@cscftri.ren.nic.in

Copyright © 1999 by AOCS Press

TABLE 1
Coded and Actual Levels of Variables Taken for Design of Experiment

Variable	Unit	Coded level of variable				
		−2	−1	0	1	2
X_1: enzyme/substrate ratio[a]	g/mol	5	10	15	20	25
X_2: acid concentration	M	0.2	0.4	0.6	0.8	1.0
X_3: incubation period	h	12	24	36	48	60
X_4: alcohol concentration	M	0.25	0.50	0.75	1.00	1.25
X_5: temperature	°C	30	35	40	45	50

[a]Enzyme (g/L)/substrate (mol/L).

TABLE 2
Coded Level Combinations for a Five-Level Five-Variable Central Composite Rotatable Design (CCRD)

Test run no.	X_1	X_2	X_3	X_4	X_5	Ester formed (M)	Predicted ester (M)
1	−1	−1	−1	−1	1	0.161	0.145
2	1	−1	−1	−1	−1	0.387	0.397
3	−1	1	−1	−1	−1	0.302	0.315
4	1	1	−1	−1	1	0.275	0.211
5	−1	−1	1	−1	−1	0.322	0.287
6	1	−1	1	−1	1	0.314	0.358
7	−1	1	1	−1	1	0.124	0.102
8	1	1	1	−1	−1	0.518	0.527
9	−1	−1	−1	1	−1	0.285	0.263
10	1	−1	−1	1	1	0.319	0.333
11	−1	1	−1	1	1	0.266	0.275
12	1	1	−1	1	−1	0.740	0.701
13	−1	−1	1	1	1	0.213	0.224
14	1	−1	1	1	−1	0.383	0.475
15	−1	1	1	1	−1	0.596	0.591
16	1	1	1	1	1	0.426	0.487
17	−2	0	0	0	0	0.162	0.204
18	2	0	0	0	0	0.581	0.526
19	0	−2	0	0	0	0.185	0.144
20	0	2	0	0	0	0.299	0.326
21	0	0	−2	0	0	0.323	0.400
22	0	0	2	0	0	0.553	0.503
23	0	0	0	−2	0	0.206	0.245
24	0	0	0	2	0	0.550	0.497
25	0	0	0	0	−2	0.557	0.630
26	0	0	0	0	2	0.208	0.274
27	0	0	0	0	0	0.489	0.452
28	0	0	0	0	0	0.476	0.452
29	0	0	0	0	0	0.482	0.452
30	0	0	0	0	0	0.487	0.452
31	0	0	0	0	0	0.479	0.452
32	0	0	0	0	0	0.478	0.452

of the samples were performed on a Shimadzu instrument (model GC 15-A; Shimadzu Corp., Tokyo, Japan) equipped with a Carbowax 20M column (3 m length, 3.175 mm i.d.) and a flame-ionization detector. Nitrogen was used as a carrier gas with a flow rate of 30 mL/min. Column oven, injection port, and detector temperatures were maintained at 100, 200, and 250°C, respectively. The percentage esterifications calculated by GC analysis (which showed product formation) and by titrimetry (which showed acid consumption) were found to be in good agreement.

Experimental design. A five-level, five-variable CCRD was adopted in this study (5). 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 6 center points. The variables and their levels selected for the study are represented in Table 1. Table 2 shows the actual experiments that were carried out for developing the model. For creating response surfaces, the experimental data obtained based on the above design were fitted to a second-order polynomial equation of the form

$$Y = b_0 + \sum_{i=1}^{5} b_i X_i + \sum_{i=1}^{5} b_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} b_{ij} X_i X_j \qquad [1]$$

where Y = ester formed, M; X_1 = E/S ratio, g/mol; X_2 = acid concentration, M; X_3 = incubation period, h; X_4 = alcohol concentration, M; X_5 = temperature, °C; b_0 = constant; b_{ii} = quadratic term coefficients; b_{ij} = cross term coefficients. The regression analyses, statistical significances, and response surfaces were done using Microsoft Excel software (version 5.0; Redmond, WA). Optimization of the reaction parameters for maximum ester yield were obtained using Microsoft Excel's Solver program (version 5.0), which used Newton's search method.

RESULTS AND DISCUSSION

The coefficients of the response surface model as given by Equation 1 were evaluated. Student's *t*-test indicated that all the linear coefficients, all quadratic terms except time and temperature coefficients, and only the acid–alcohol and acid–temperature interaction terms were highly significant (all $P < 0.05$). The values of the parameters and the analysis of variance (ANOVA) are presented in Table 3. The ANOVA indicates that the model is highly significant, as the F_{model} value (24.5) is very high compared to $F_{10,21}$ value (3.31). The coefficient of determination (R^2) of the model was 0.921, which indicates that the model adequately represents the real relationships among the selected reaction parameters. The lack-of-fit test (Table 3) also indicates that the model is adequate to represent the experimental data. The average absolute relative deviation is 11.22%. The normal percentage probability plot (Fig. 1) of the residuals indicates that the errors are normally distributed ($R^2 = 0.95$) and are independent of each other and that the error variances are homogeneous. Neglecting the insignificant terms, the final predictive equation obtained is given as

$$\begin{aligned} Y = {} & 0.452 + 0.080X_1 + 0.045X_2 + 0.026X_3 + 0.063X_4 \\ & - 0.089X_5 - 0.022X_1^2 - 0.054X_2^2 - 0.020X_4^2 \\ & + 0.050X_2X_4 - 0.044X_2X_5 \end{aligned} \qquad [2]$$

In the present study, the concentration of acid substrate was varied and the ester concentration formed after a specified duration was expressed with respect to conversion of the acid. While enzyme concentration is an influencing parameter, it is the E/S ratio, in g/mol, that is probably a more im-

TABLE 3
Coefficients of the Model and Analysis of Variance (ANOVA)

Regression statistics

Multiple R	0.960
R^2	0.921
Adjusted R^2	0.883
Standard error	0.053
Observations	32

ANOVA

	Degrees of freedom	Sum of squares	Mean sum of squares	F ratio	P–value
Regression	10	0.679	0.068	24.493	2.30E–09
Residual	21	0.058	0.003		
Total	31	0.737			
Lack of fit	16	0.053	0.003	2.995	N.S.
Pure error	5	0.006	0.001		

Coefficients[a]	Value	Standard error	t-Stat	P-value
b_0	0.452	0.016	27.670	5.23E–18
b_1	0.080	0.011	7.484	2.36E–07
b_2	0.045	0.011	4.232	3.73E–04
b_3	0.026	0.011	2.404	0.026
b_4	0.063	0.011	5.859	8.15E–06
b_5	–0.089	0.011	–8.270	4.82E–08
b_{11}	–0.022	0.010	–2.254	0.035
b_{22}	–0.054	0.010	–5.611	1.44E–05
b_{44}	–0.020	0.010	–2.099	0.048
b_{24}	0.050	0.013	3.762	0.001
b_{25}	–0.044	0.013	–3.307	0.003

[a]Only coefficients significant at $P < 0.05$ are presented. N.S., not significant.

portant parameter to indicate the behavior, as the requirement of enzyme is likely to be a function of the substrate (acid) concentration. Although enzyme weight percentage (with respect to alcohol and acid) has been used in the literature (6,7), E/S ratio has been adopted in the present study mainly so as to neglect the alcohol term and represent the actual quantity of enzyme present against acyl donor (acid).

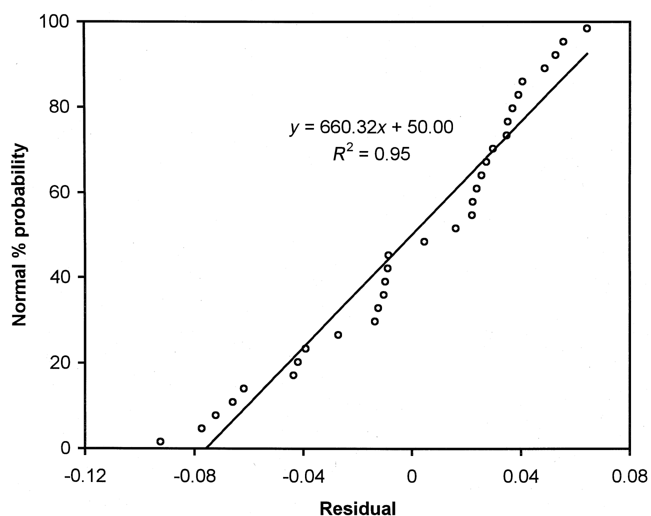

FIG. 1. Normal (percentage) probability plot of the residuals.

At the lowest concentration of acid (0.2 M) with the lowest E/S ratio (5 g/mol), esterification was zero (Fig. 2). A moderate acid concentration (0.6–0.7 M) and high enzyme concentration (20–25 g/mol) favored maximal esterification at 0.5 M alcohol concentration in 24 h at 35°C. As the E/S ratio increased, ester concentration increased at all the acid concentrations. An increase in acid concentration above 0.7 M resulted in less esterification at any given E/S ratio probably due to inhibition of the enzyme by the acid beyond 0.7 M concentration.

Increase in E/S ratio resulted in increased esterification at all alcohol concentrations (Fig. 3). As alcohol concentration increased at any given E/S ratio, ester concentration increased up to an alcohol concentration of 0.75 M and thereafter decreased, probably owing to inhibition of the enzyme by the higher alcohol concentration. A similar trend was observed at all enzyme concentrations. Alcohols have been reported to be competitive inhibitors of lipases, which follow ping-pong bibi kinetic patterns in esterification reactions (8,9). Kinetic analysis of the present system should throw more light on this matter.

The effect of varying acid and alcohol concentrations at constant E/S ratio, temperature, and incubation period (all at the −1 level, which is equal to 10 g/mol, 35°C, and 24 h, respectively) is shown in Figure 4. At all acid concentrations from 0.2 to 1.0 M, an increase in alcohol concentration led to higher yields up to an alcohol concentration of 0.875 M. Further increases in alcohol concentration beyond 0.875 M drastically affected the conversions particularly at lower acid concentrations, with a maximal drop in esterification in the range of 0.875–1.25 M alcohol. With the increase in alcohol concentration (0.875 to 1.25 M), the esterification increased at

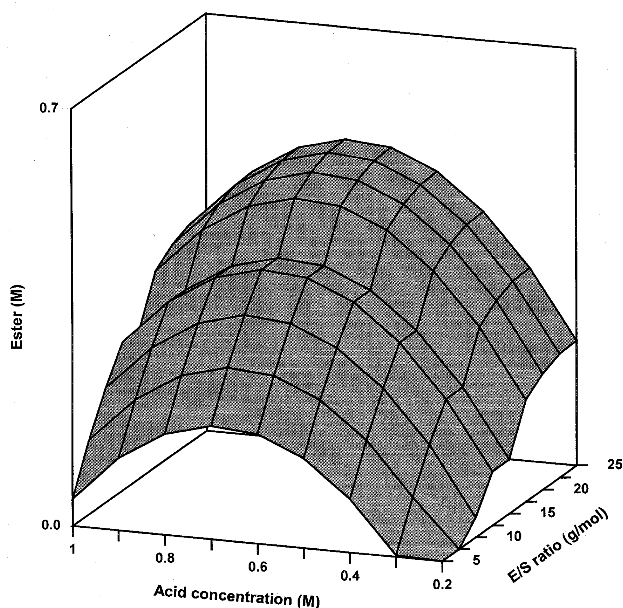

FIG. 2. Response surface plot showing the effect of acid concentration, enzyme/substrate (E/S) ratio, and their mutual interaction on isoamyl butyrate synthesis. Other variables (alcohol, incubation period, and temperature) are constant at –1 level.

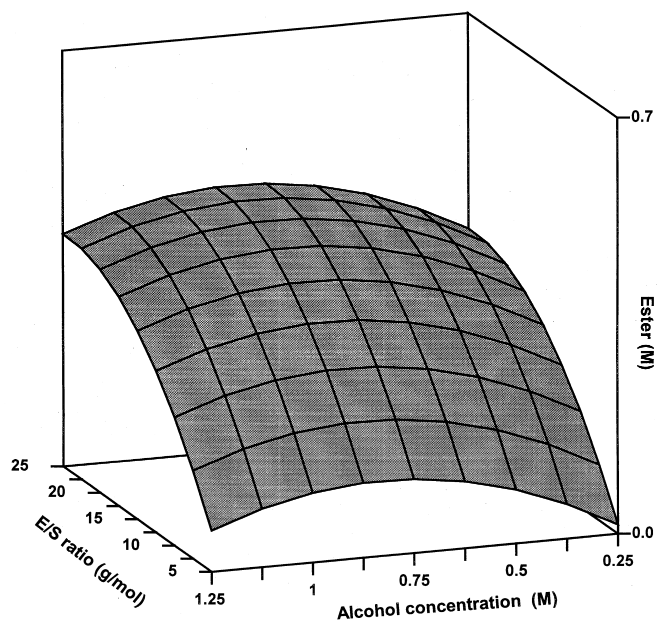

FIG. 3. Response surface plot showing the effect of alcohol concentration, E/S ratio, and their mutual interaction on isoamyl butyrate synthesis. Other variables (acid, incubation period, and temperature) are constant at −1 level. For abbreviation see Figure 2.

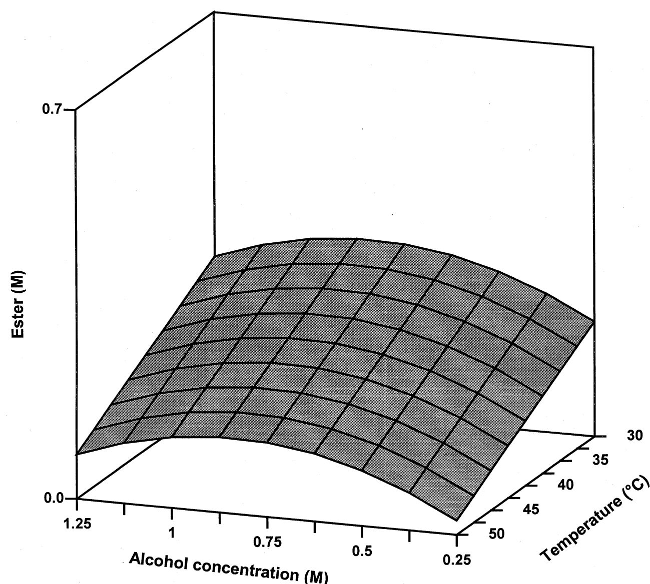

FIG. 5. Response surface plot showing the effect of alcohol concentration, temperature, and their mutual interaction on isoamyl butyrate synthesis. Other variables (E/S ratio, acid, and incubation period) are constant at −1 level. For abbreviation see Figure 2.

higher acid concentrations (>0.6 M). Below an alcohol concentration of 0.875 M, esterification showed a maximum at 0.6–0.7 M acid, which decreased slightly at higher acid concentrations.

The effects of temperature on esterification at varying concentrations of alcohol or acid were found to be slightly different (Figs. 5 and 6). Temperatures above 30°C affected esterification negatively. With an increase in temperature from 30 to 50°C, esterification decreased at all alcohol concentrations at E/S = 10 g/mol, acid = 0.4 M, and incubation period = 24 h (Fig. 5). At each temperature, esterification increased with increases in alcohol, up to 0.75–0.875 M, decreasing thereafter up to 1.25 M alcohol. The decrease in esterification beyond 0.875 M alcohol may be due to enzyme inhibition by alcohol.

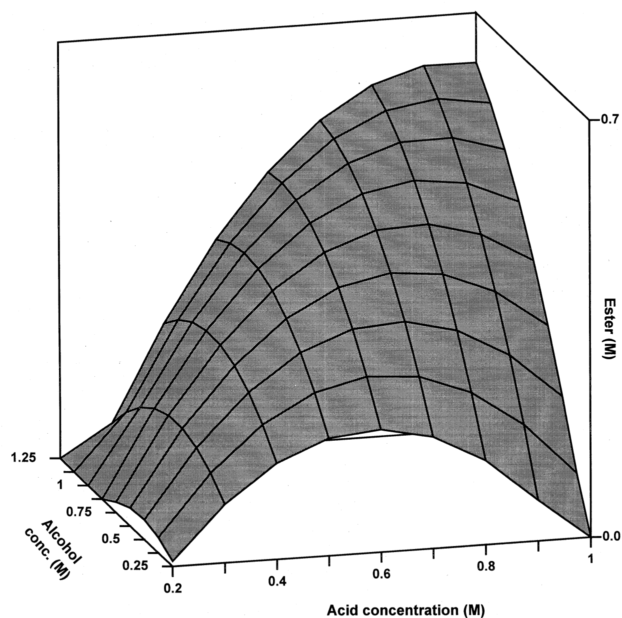

FIG. 4. Response surface plot showing the effect of acid concentration, alcohol concentration, and their mutual interaction on isoamyl butyrate synthesis. Other variables (E/S ratio, incubation period, and temperature) are constant at −1 level. For abbreviation see Figure 2.

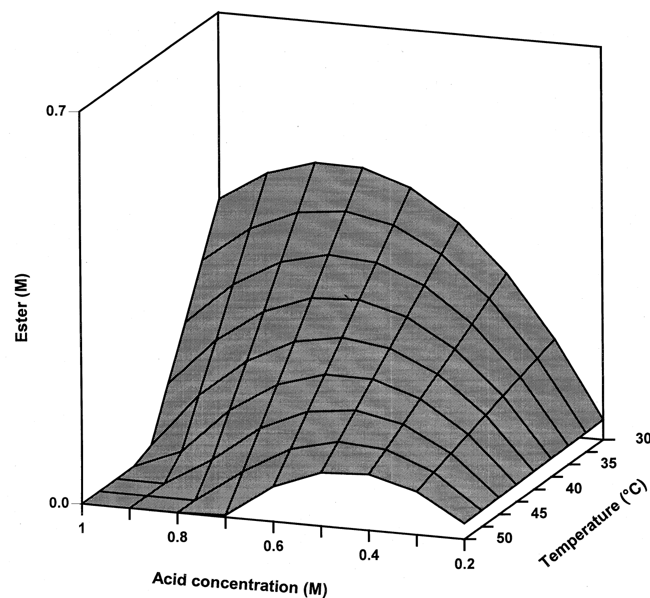

FIG. 6. Response surface plot showing the effect of acid concentration, temperature, and their mutual interaction on isoamyl butyrate synthesis. Other variables (E/S ratio, alcohol, and Incubation period) are constant at −1 level. For abbreviation see Figure 2.

Lower temperatures favor esterification at all acid concentrations between 0.2 and 1.0 M at E/S = 10 g/mol, alcohol = 0.5 M, and incubation period = 24 h (Fig. 6). However, certain other features were appreciably different from those observed in Figure 5. At all the temperatures, esterification increased with acid concentration up to a critical value, beyond which there was a drastic decrease. This critical acid concentration decreased with temperature in the range tested (0.4 M for 50°C, extent of esterification being 20%; 0.7 M for 30°C, extent of esterification being 93%). Therefore, lower temperatures not only allowed higher acid concentrations to be used but also resulted in higher conversions. Because further lowering of temperature gave very low rates of reaction, 30°C can be said to be the optimal temperature to use. Also at higher acid concentrations (>0.7 M) and higher temperatures (>45°C) zero esterification was observed.

Increases in temperature normally affect the various equilibrium processes involved in the esterification reaction—namely, alcohol, acid and ester binding and solubility and partitioning of the acid between the microaqueous enzyme–water–solvent interface and the dissociation equilibrium of the acid—which all become more pronounced at higher temperatures. While the binding equilibria decrease with the increase in temperatures, acid dissociation and solubility increase with temperature, all resulting in unfavorable esterification conditions.

Figure 7, depicting the variation of esterification with time, shows clearly that the period of incubation has only a marginal effect on the esterification behavior at E/S = 10 g/mol, acid = 0.4 M, and alcohol = 0.5 M beyond an incubation period of 12 h.

The most efficient, or optimal, condition for the present system would be to use the lowest amount of enzyme to achieve maximal conversion of the substrate in minimal time at ambient temperature. For a given temperature and acid concentration, the alcohol concentration and E/S ratio required to attain a known extent of esterification in a given time can be calculated using Equation 2. Figure 8 shows the contour plot predicting the extent of esterification for different alcohol concentrations and E/S ratios. This type of plot is quite useful experimentally to arrive at economical processing conditions to obtain the required yield. Table 4 deals with validation of experimental conditions to obtain required yields indicated by the contour plot. While several combinations of alcohol concentrations and E/S ratios can give the same conversion, from an economic viewpoint, it is desirable to choose the lowest possible E/S ratio value from Figures 8 and 9 for practical esterification. For example, 1.0 M ester (representing full esterification) could be obtained using about 17 g/mol enzyme after 60 h, but the same 1.0 M ester can be achieved using about 24 g/mol enzyme just after 38.3 h (Fig. 9). These opti-

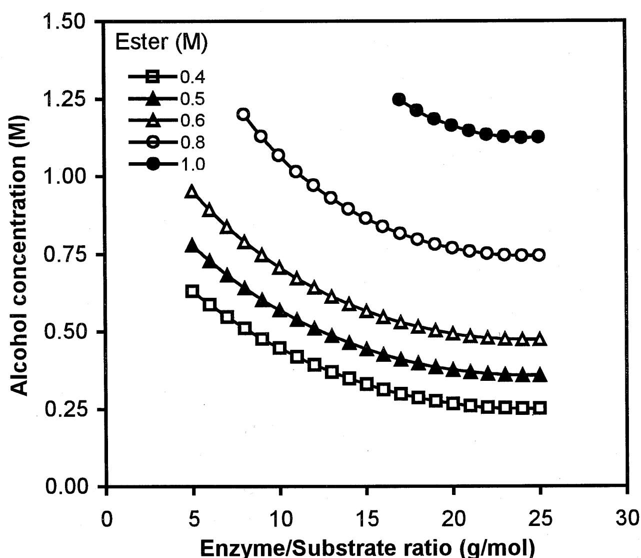

FIG. 8. Contour plot showing the ranges of E/S ratios and alcohol concentrations to obtain various ester concentrations. Other variables are constant at their respective levels as follows: acid = +2, incubation period = +2, and temperature = −2. For abbreviation see Figure 2.

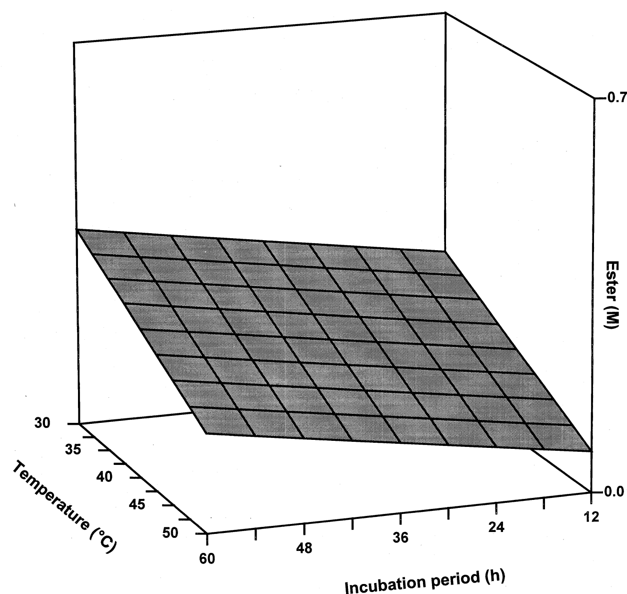

FIG. 7. Response surface plot showing the effect of temperature, incubation period, and their mutual interaction on isoamyl butyrate synthesis. Other variables (E/S ratio, acid, and alcohol) are constant at −1 level. For abbreviation see Figure 2.

TABLE 4
Model Validation Experiments[a]

X_1	X_2	X_3	X_4	X_5	Ester formed (M)	Predicted ester (M)
15	0.6	12	0.75	50	0.208	0.222
10	0.4	24	0.5	35	0.243	0.236
10	0.8	48	0.5	35	0.339	0.366
20	0.8	24	0.5	35	0.492	0.476
20	0.8	48	1.0	35	0.768	0.752
20	0.4	24	0.5	45	0.280	0.306
20	0.8	48	0.5	45	0.246	0.262
20	0.8	24	1.0	45	0.420	0.436
24	1.0	38	1.25	30	0.968	1.000
17	1.0	60	1.25	30	0.979	1.000

[a]Average absolute relative deviation = 4.697%.

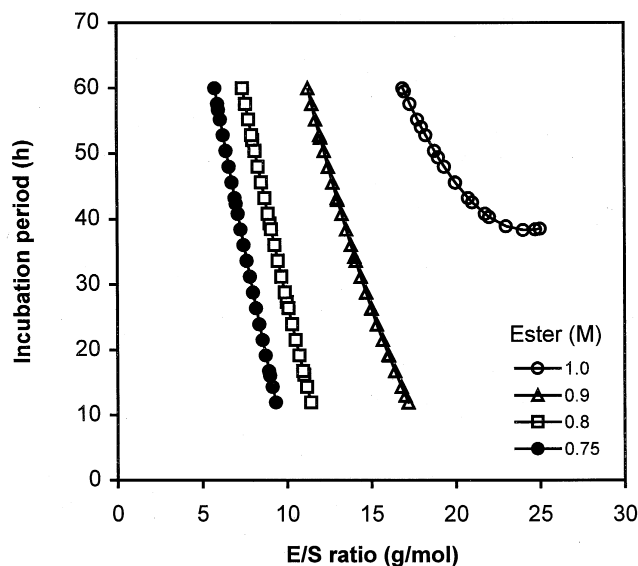

FIG. 9. Contour plot showing the ranges of E/S ratios and incubation periods to obtain various ester concentrations. Other variables are constant at their respective levels as follows: acid = +2, alcohol = +2, and temperature = −2. For abbreviation see Figure 2.

mal conditions have also been confirmed experimentally (Table 4).

Adequacy of the model was also examined at additional independent conditions that were not employed in this treatment. It was observed that the experimental and predicted values of ester concentration showed good correspondence (Table 4). The optimal conditions predicted for synthesizing 1.0 M ester were as follows: E/S ratio = 17 g/mol; acid = 1.0 M; incubation period = 60 h; alcohol = 1.25 M; and temperature = 30°C. The actual experimental value obtained was 0.98 M, which was in good agreement with the predicted value. ANOVA also indicated that the generated model adequately predicted the esterification reaction between isoamyl alcohol and butyric acid.

ACKNOWLEDGMENTS

The authors wish to thank the Director, CFTRI, Mysore, for providing facilities. The author S. Hari Krishna, is thankful to the Council of Scientific and Industrial Research, India, for the award of Senior Research Fellowship to carry out research.

REFERENCES

1. Armstrong, D.W., Selective Production of Ethyl Acetate by *Candida utilis,* in *Biogeneration of Aromas*, edited by T.H. Parliament and R. Croteau, *ACS Symp. Ser. 317*:254–265 (1986).
2. Langrand, G., C. Triantaphylides, and J. Baratti, Lipase Catalyzed Formation of Flavor Esters, *Biotechnol. Lett. 10*:549–554 (1988).
3. Langrand, G., N. Rondot, C. Triantaphylides, and J. Baratti, Short-Chain Flavor Esters Synthesis by Microbial Lipases, *Ibid. 12*:581–586 (1990).
4. Welsh, F.W., R.E. Williams, and K.H. Dawson, Lipase-Mediated Synthesis of Low Molecular Weight Flavor Esters, *J. Food Sci. 55*:1679–1682 (1990).
5. Cochran, W.G., and G.M. Cox, in *Experimental Designs*, 2nd edn., Wiley, New York, 1992, pp. 335–375.
6. Shieh, C.J., C.C. Akoh, and P.E. Koehler, Four-Factor Response Surface Optimization of the Enzymatic Modification of Triolein to Structured Lipids, *J. Am. Oil Chem. Soc. 72*:619–623 (1995).
7. Shieh, C.J., C.C. Akoh, and L.N. Yee, Optimized Enzymatic Synthesis of Geranyl Butyrate with Lipase AY from *Candida rugosa*, *Biotechnol. Bioeng. 51*:371–374 (1996).
8. Chulalaksananukul, W., J.S. Condoret, and D. Combes, Kinetics of Geranyl Acetate Synthesis by Lipase Catalyzed Transesterification in *n*-Hexane, *Enzyme Microb. Technol. 14*:293–298 (1992).
9. Chulalaksananukul, W., J.S. Condoret, and D. Combes, Geranyl Acetate Synthesis by Lipase Catalyzed Transesterification in Supercritical CO_2, *Ibid. 15*:691–698 (1993).

[Received April 29, 1999; accepted August 26, 1999]