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Response Surface Methodology Approach for the Synthesis of Ethyl Butyrate

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Summary

Response surface methodology was used to determine optimum conditions for the esterification of ethanol and butyric acid to produce a flavour ester using immobilized lipase. Various reaction parameters including butyric acid concentration, enzyme concentration, temperature and ethanol/butyric acid molar ratio affecting ethyl butyrate production were investigated using a fractional factorial design 2^{4-1}. Based on the results from the first factorial design, all of the variables which were significant in the process were selected to be used in a 2^4 central composite rotatable design (CCRD). The optimum conditions for the enzymatic reaction were obtained at a 90 mM butyric acid concentration using a 7.7 g/L enzyme concentration at 45 °C and the ethanol/butyric acid molar ratio of 1:1 for 3 h. The esterification percentage, under these conditions, was 87 %.

Key words: ethyl butyrate, immobilized lipase, esterification, optimization, enzymatic synthesis

Introduction

Flavour is usually the result of the presence, within complex matrices, of many volatile and nonvolatile components with diverse chemical and physicochemical properties. Whereas the nonvolatile compounds contribute mainly to taste, the volatile ones influence both taste and aroma. A vast array of compounds may be responsible for the aroma of food products, such as alcohols, aldehydes, esters, dicarbonyls, short to medium chain-free fatty acids, methyl ketones, lactones, phenolic compounds and sulphur compounds (*1*).

Low molecular mass esters are responsible for the aroma of many types of fruit and mainly make up short-chain fatty acid derivatives such as acetate, propionate, butyrate and isobutyrate. For example, ethyl butyrate and isoamyl isobutyrate are present in strawberry and banana aromas, respectively (*2*).

Most of the commercial esters can be obtained directly by extraction from plant materials, but the high cost and low quantity of the obtained product make this technique inadequate for industrial applications. Thus, the industrial production of these kinds of compounds has been traditionally carried out by chemical synthesis. In the last decade, biotechnology has been considered for the production of esters used in the food industry, due to the fact that the obtained flavour can be labelled as 'natural'. Thus, the enzymatic synthesis using lipases seems to be a competitive alternative to traditional chemical synthesis. Although biotechnological processes are more expensive than the chemical ones, they have clear environmental advantages since inorganic acids (used as catalysts in chemical synthesis) are avoided, and the enzymes can be reused, minimizing the reaction residue (*3*).

The use of enzymes to improve the traditional chemical processes of food manufacture has been developed in the past few years. Lipases (triacylglycerol lipases, EC

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3.1.1.3) are the most versatile biocatalysts and bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis and aminolysis (*4*).

The most commonly used reaction media in enzymatic synthesis are still organic solvents such as hexane or heptanes (*5,6*), even though supercritical medium (*7*) and solvent-free systems (*8,9*) have also been explored.

Since lipases are widely used in food industries, it is necessary to study their performance during the esterification reaction. Accurate control of lipase concentration, alcohol and/or acid concentration, temperature and reaction time is required to maximize the production of flavour ester (*9–14*).

The classical method of reaction optimization involves changing one variable at a time, keeping the others at fixed levels. Being single-dimensional, this laborious and time consuming method often does not guarantee the determination of optimal conditions (*15,16*). On the other hand, carrying out experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required (*17*).

The use of factorial design and response surface analysis is important to determine the optimal conditions (*18*). Factorial design of a limited set of variables is advantageous in relation to the conventional method with manipulation of a single parameter per trial, as such an approach frequently fails to determine optimal conditions due to its failure to consider the effect of possible interactions between factors (*19,20*). Response surface methodology (RSM) is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments (*21,22*). The factorial design makes it possible to study many factors simultaneously as well as to quantify the effect of each of them and to investigate their possible interaction (*15*).

In the present work, the esterification percentage of the enzymatic synthesis of ethyl butyrate production has been evaluated by using factorial design and response surface methodology. In order to establish the optimal conditions of esterification, temperature, ethanol/butyric acid molar ratio, enzyme concentration and butyric acid concentration were first evaluated in a fractional factorial design (2^{4-1}) followed by a 2^4 central composite rotatable design (CCRD) and response surface methodology.

Materials and Methods

Materials

The lipase used in the experiment (Lipozyme RM IM, 4.6 U/g, supplied by Novo Nordisk, Bagsvaerd, Denmark) was extracted from *Mucor miehei* and immobilized on macroporous weak base anionic resin beads. An activity unit (U) is defined as the amount of enzyme that 1 μmol of fatty acid releases per minute per gram (g) of immobilized enzyme used, and is determined by the method based on the titration of fatty acids released by the enzyme action on olive oil triglycerides, emulsified in gum arabic (*18*).

Butyric acid, ethanol and *n*-heptane were supplied by Vetec (Rio de Janeiro, Brazil) and Merck Co. (Darmstadt, Germany). All chemicals used were of analytical reagent grade. The solvent and substrates were used without any pretreatment (without dehydration).

Esterification reaction

Ester synthesis was carried out in a 100-mL stoppered flask with a working volume of 40 mL of *n*-heptane containing ethyl alcohol and butyric acid. The enzyme was added to the freshly prepared reaction mixture, which was incubated in an orbital shaking incubator (Tecnal TE-420, Piracicaba, Brazil) at 180 rpm. The temperatures and amounts of ethyl alcohol, butyric acid and enzyme were established according to the experimental designs (Tables 1 and 2).

Determination of esterification percentage

An aliquot of 1 mL of the reaction mixture was withdrawn after 3, 6 and 24 h, and after each withdrawal 10 mL of ethanol were added. Ethanol is used as a quenching agent. Along with it, 2–3 drops of phenolphthalein indicator were added and titrated against standard po-

Table 1. Values of coded levels and real values (in parenthesis) used in fractional factorial design 2^{4-1}

Trial	Variable levels				Esterification/%		
	X_1/°C	X_2/(g/L)	X_3/mM	X_4	3 h	6 h	24 h
1	–1 (30)	–1 (3)	–1 (60)	–1 (1:1)	57.82	70.31	77.51
2	+1 (50)	–1 (3)	–1 (60)	+1 (3:1)	71.31	74.53	86.73
3	–1 (30)	+1 (20)	–1 (60)	+1 (3:1)	81.76	80.13	88.94
4	+1 (50)	+1 (20)	–1 (60)	–1 (1:1)	87.55	88.69	89.62
5	–1 (30)	–1 (3)	+1 (180)	+1 (3:1)	28.97	33.01	46.31
6	+1 (50)	–1 (3)	+1 (180)	–1 (1:1)	55.21	56.89	66.55
7	–1 (30)	+1 (20)	+1 (180)	–1 (1:1)	90.03	90.65	91.38
8	+1 (50)	+1 (20)	+1 (180)	+1 (3:1)	80.88	83.76	91.42
9	0 (40)	0 (11.5)	0 (120)	0 (2:1)	92.60	93.45	94.16
10	0 (40)	0 (11.5)	0 (120)	0 (2:1)	93.02	92.56	93.55
11	0 (40)	0 (11.5)	0 (120)	0 (2:1)	92.44	91.80	93.32

X_1=temperature, X_2=enzyme concentration, X_3=butyric acid concentration, X_4=ethanol/butyric acid molar ratio

Table 2. Values of coded levels and real values (in parentheses), experimental and predicted values of percentage of esterification in the CCRD

Trial	Variable levels				Esterification/%		Relative deviation
	X_1/°C	X_2/(g/L)	X_3/mM	X_4	Experimental	Predicted	%
1	–1 (35)	–1 (7.7)	–1 (50)	–1 (1:1)	78.21	83.59	–6.88
2	+1 (45)	–1 (7.7)	–1 (50)	–1 (1:1)	76.89	76.83	0.08
3	–1 (35)	+1 (17.3)	–1 (50)	–1 (1:1)	81.03	74.37	8.22
4	+1 (45)	+1 (17.3)	–1 (50)	–1 (1:1)	78.91	80.97	–2.61
5	–1 (35)	–1 (7.7)	+1 (90)	–1 (1:1)	89.36	83.15	6.95
6	+1 (45)	–1 (7.7)	+1 (90)	–1 (1:1)	89.69	92.47	–3.10
7	–1 (35)	+1 (17.3)	+1 (90)	–1 (1:1)	56.24	65.73	–16.87
8	+1 (45)	+1 (17.3)	+1 (90)	–1 (1:1)	91.46	88.41	3.33
9	–1 (35)	–1 (7.7)	–1 (50)	+1 (2:1)	84.44	83.41	1.22
10	+1 (45)	–1 (7.7)	–1 (50)	+1 (2:1)	83.34	76.65	8.03
11	–1 (35)	+1 (17.3)	–1 (50)	+1 (2:1)	81.63	81.55	0.10
12	+1 (45)	+1 (17.3)	–1 (50)	+1 (2:1)	85.89	88.15	–2.63
13	–1 (35)	–1 (7.7)	+1 (90)	+1 (2:1)	75.37	75.97	–0.80
14	+1 (45)	–1 (7.7)	+1 (90)	+1 (2:1)	82.61	85.29	–3.24
15	–1 (35)	+1 (17.3)	+1 (90)	+1 (2:1)	69.94	65.91	5.76
16	+1 (45)	+1 (17.3)	+1 (90)	+1 (2:1)	91.15	88.59	2.81
17	0 (40)	0 (12.5)	0 (70)	0 (3:2)	80.98	80.69	0.36
18	0 (40)	0 (12.5)	0 (70)	0 (3:2)	79.72	80.69	–1.22
19	0 (40)	0 (12.5)	0 (70)	0 (3:2)	78.94	80.69	–2.22
20	0 (40)	0 (12.5)	0 (70)	0 (3:2)	78.02	80.69	–3.42
21	–2 (30)	0 (12.5)	0 (70)	0 (3:2)	68.65	–	–
22	+2 (50)	0 (12.5)	0 (70)	0 (3:2)	90.19	–	–
23	0 (40)	–2 (3)	0 (70)	0 (3:2)	52.07	–	–
24	0 (40)	+2 (22)	0 (70)	0 (3:2)	76.09	–	–
25	0 (40)	0 (12.5)	–2 (30)	0 (3:2)	55.56	–	–
26	0 (40)	0 (12.5)	+2 (110)	0 (3:2)	90.05	–	–
27	0 (40)	0 (12.5)	0 (70)	–2 (1:2)	70.74	–	–
28	0 (40)	0 (12.5)	0 (70)	+2 (5:2)	90.80	–	–

X_1=temperature, X_2=enzyme concentration, X_3=butyric acid concentration, X_4=ethanol/butyric acid molar ratio

tassium hydroxide to determine the residual acid content (*3*).

The percentage of esterification was calculated from the concentration of acid consumed in the reaction mixture determined from the titration values obtained for the blank as well as the test samples (*23*) using the following equation:

$$\text{Esterification (in \%)} = \frac{c_0 - c}{c_0} \times 100 \qquad /1/$$

where c_0 is the concentration of free fatty acid residues in 0 h, and c is the concentration of free fatty acid residues in time t.

Experimental design

The effects of temperature (30–50 °C), ethanol/butyric acid molar ratio (1:1 to 3:1), enzyme concentration (3–20 g/L) and butyric acid concentration (60–180 mM) on the synthesis of ethyl butyrate were studied by using a fractional design of 2^{4-1} trials plus 3 central points, which means a total of 11 trials. The reaction time was not considered a significant variable in this experimental design, since the experiments were performed in three different time frames in order to study the reaction kinetics and calculate the esterification percentage.

A central composite design (CCRD; 2^4 plus axial and central points) with four replicates at the central point (a total of 28 trials) was used for temperature (30–50 °C), ethanol/butyric acid molar ratio (1:2 to 5:2), enzyme concentration (3–22 g/L) and butyric acid concentration (30–110 mM), with the esterification percentage at 3 h as a response.

The variables and their levels for the fractional factorial design 2^{4-1} are presented in Table 1. Table 2 shows the CCRD that was carried out to develop the model using the STATISTICA v. 5.0 software (Statsoft Inc., Tulsa, OK, USA).

In order to generate response surfaces, the experimental data obtained based on the CCRD design were fitted to a first order model, which was adjusted to the independent variables using the following equation:

$$Y=A_0+A_1X_1-A_2X_2+A_3X_1X_2+A_4X_1X_3-A_5X_2X_3+A_6X_2X_4-A_7X_3X_4 \quad /2/$$

where Y is the dependent variable (response variable) to be modelled, X_1-X_4 are the independent variables (factors), and A_0-A_7 are the regression coefficients of the model. Where it was possible, the model was simplified by dropping terms which were not statistically significant ($p<0.1$) in an analysis of variance (ANOVA). The lack-of-fit test was used to determine whether the constructed model was adequate to describe the observed data (*24*). The R^2 statistics indicates the percentage of optimization of variability parameters that is explained by the model (*15*). Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent ones.

Results and Discussion

Fractional factorial design

Experiments using the 2^{4-1} fractional factorial design were carried out with three values of each independent variable (Table 1). The esterification was performed for 3, 6 and 24 h. An estimate of the main effect is obtained by evaluating the difference in process performance caused by a change from low (–1) to high (+1) levels of the corresponding variable (*25*).

The percentage of esterification varied according to the synthesis conditions, from around 28 to 93 % in 3 h (Table 1). As can be seen in Table 3, all variables had a statistically significant effect ($p<0.05$). Changes in the temperature and enzyme concentration from level –1 to level +1 led to an increase in the esterification percentage, while an increase in the butyric acid concentration and ethanol/butyric acid molar ratio led to a decrease of the response. The percentage of esterification was more significantly affected by enzyme concentration (21 % average positive effect after 24 h), followed by butyric acid concentration, temperature and ethanol/butyric acid molar ratio during the enzymatic synthesis.

Table 3. Estimated effects of variables on esterification percentage for the fractional factorial design 2^{4-1}

Variables	Effect	Standard error	t(2)	p-value
		3 h		
X_1	9.09	0.21	42.93	0.00054
X_2	31.73	0.21	149.79	0.00004
X_3	–10.84	0.21	–51.16	0.00038
X_4	–6.92	0.21	–32.68	0.00093
		6 h		
X_1	7.44	0.58	12.74	0.00061
X_2	27.12	0.58	46.44	0.00046
X_3	–12.34	0.58	–21.13	0.00223
X_4	–8.78	0.58	–15.03	0.00439
		24 h		
X_1	7.54	0.31	24.58	0.00165
X_2	21.06	0.31	68.63	0.00021
X_3	–11.78	0.31	–38.39	0.00068
X_4	–2.91	0.31	–9.50	0.01091

X_1=temperature, X_2=enzyme concentration, X_3=butyric acid concentration, X_4=ethanol/butyric acid molar ratio

Central composite rotatable design (CCRD)

The levels of CCRD were defined based on the results obtained in the fractional factorial design, where it was indicated that there should be an increase in the temperature and enzyme concentration ranges and a decrease in the levels of ethanol/butyric acid molar ratio and butyric acid concentration. In spite of the optimum temperature strip for Lipozyme being in the order of 70 °C, according to the data supplied by the manufacturer (*26*), the same variation was maintained due to limitations of the process where high rates of evaporation of the reagent medium can happen when the reaction is performed at higher temperatures (*27*). A small increment was considered in the enzyme concentration based on the range mentioned in literature. There was a reduction of the studied levels in the ethanol/butyric acid molar ratio as well as in the butyric acid concentration.

The results for esterification as a function of time in the CCRD were obtained after 3, 6 and 24 h. The statistical analysis was performed with data obtained after 3 h, due to the fact that there was no significant increase in the esterification percentage after this period for most of the trials.

The experimental results had not been adjusted to a second order model, therefore an evaluation of the first order model was carried out, using the central composite design (CCD) trials 1 to 20 in Table 2. Temperature, enzyme concentration, butyric acid concentration, ethanol/butyric acid molar ratio and the values predicted by the model provided by Eq. 1 for the four studied variables are presented in Table 2.

In this second design, esterification percentage varied from 52 up to 91 % in 3 h. The best percentage of esterification was obtained in trials 8 and 16. The conditions in trial 8 were: temperature at level +1 45 °C, enzyme concentration at level +1 17.3 g/L, butyric acid concentration at level +1 90 mM and ethanol/butyric acid molar ratio at level –1 1:1. In trial 16, the conditions were: temperature at level +1 45 °C, enzyme concentration at level +1 17.3 g/L, butyric acid concentration at level +1 90 mM and ethanol/butyric acid molar ratio at level +1 2:1, resulting in an esterification percentage of around 91 % for both trials.

ANOVA was applied using the values of esterification percentage presented in Table 4 for CCD. ANOVA classifies and cross-classifies statistical results and tests whether the means of a specified classification differ significantly or not. This was carried out using Fisher's statistical test for the analysis of variance. The F-value is the ratio of the mean square due to regression to the mean square due to error, and it indicates the influence (significance) of each controlled factor on the tested model (*28*).

The mathematical model was built by means of regression based on the response results and the coded experimental plan (Table 2). Although the model coefficients obtained are empirical and cannot be associated with

Table 4. ANOVA for determination of esterification percentage for CCD

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-value	p-value
X_1 (L)	253.72	1	253.72	160.98	0.0010
X_2 (Q)	35.00	1	35.00	22.21	0.0181
$X_1 \times X_2$	178.42	1	178.42	113.21	0.0018
$X_1 \times X_3$	258.24	1	258.24	163.85	0.0010
$X_2 \times X_3$	67.35	1	67.35	42.73	0.0073
$X_2 \times X_4$	53.93	1	53.93	34.22	0.0099
$X_3 \times X_4$	48.90	1	48.90	21.92	0.0138
lack of fit	310.92	9	34.55	21.92	0.0138
pure error	4.73	3	1.58		
total SS	1211.22	19	63.75		

Regression coefficient: R=0.8598; $F_{0.90;\ 7;\ 12}$: 2.28, F-ratio (model significance)=4.86

physical or chemical significance, they are very useful for predicting the results of untested operation conditions (*17*). A good R value (R=0.8598) and an F-value twice higher than the listed value for a 90 % confidence (Table 4) were obtained. In conclusion, this model is workable and can give predictions for a range of conditions in the limits of the following model:

$$Y\ (\text{in } \%) = 80.69 + 3.98X_1 - 1.48X_2 + 3.34X_1X_2 + 4.02X_1X_3 - 2.05X_2X_3 + 1.84X_2X_4 - 1.75X_3X_4 \qquad /3/$$

where Y is the esterification percentage and X_1, X_2, X_3 and X_4 are the factors presented in Table 2.

The model for calculating esterification percentage was used to construct the response surfaces, which can be seen in Fig. 1, so as to understand the interaction among the reaction parameters, and the optimum range of each variable required for optimum ethyl butyrate esterification.

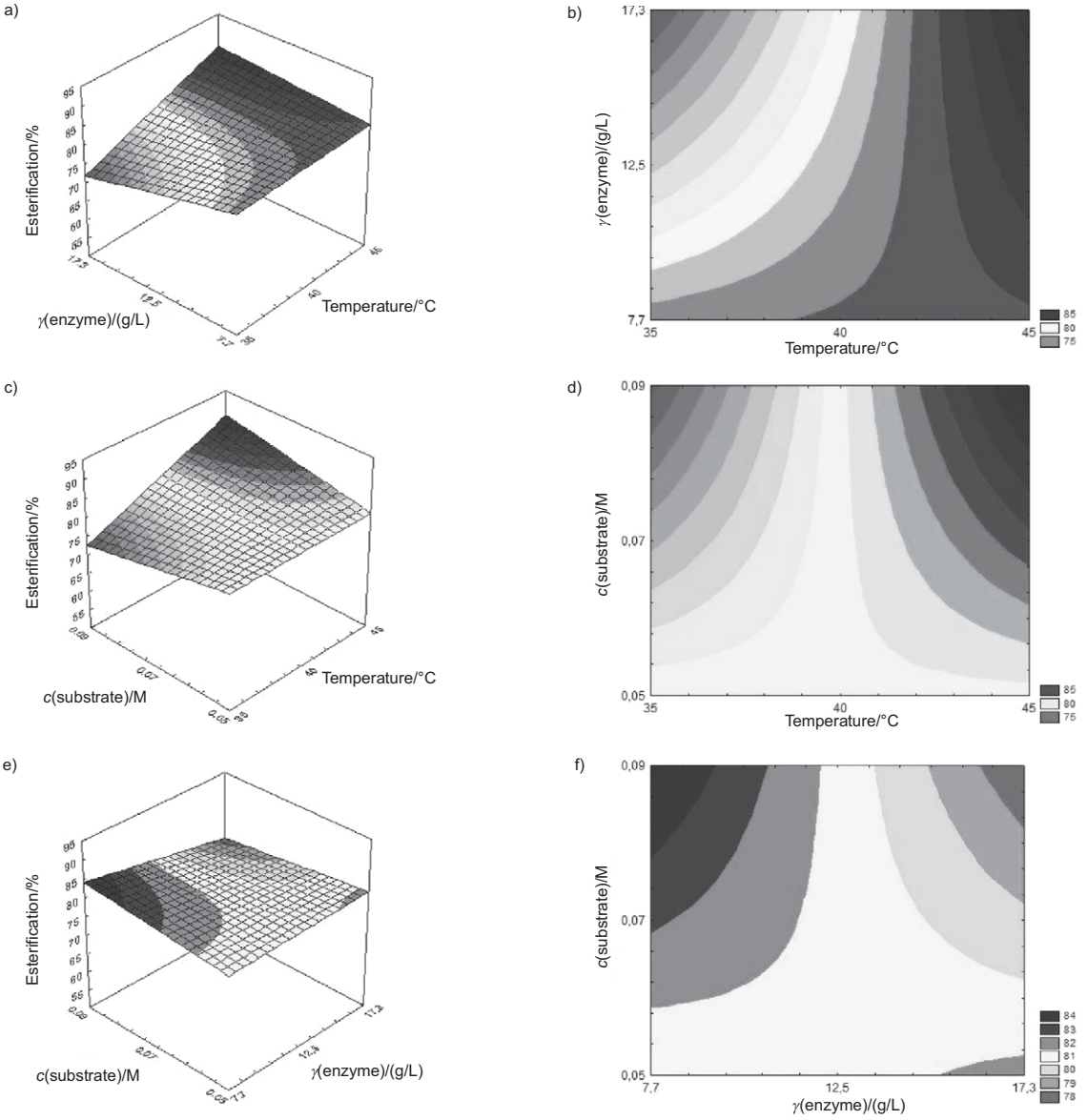

Fig. 1. Response surface and contour diagrams of esterification percentage as a function of: (a, b) temperature and enzyme concentration, (c, d) temperature and butyric acid concentration, (e, f) enzyme concentration and butyric acid concentration

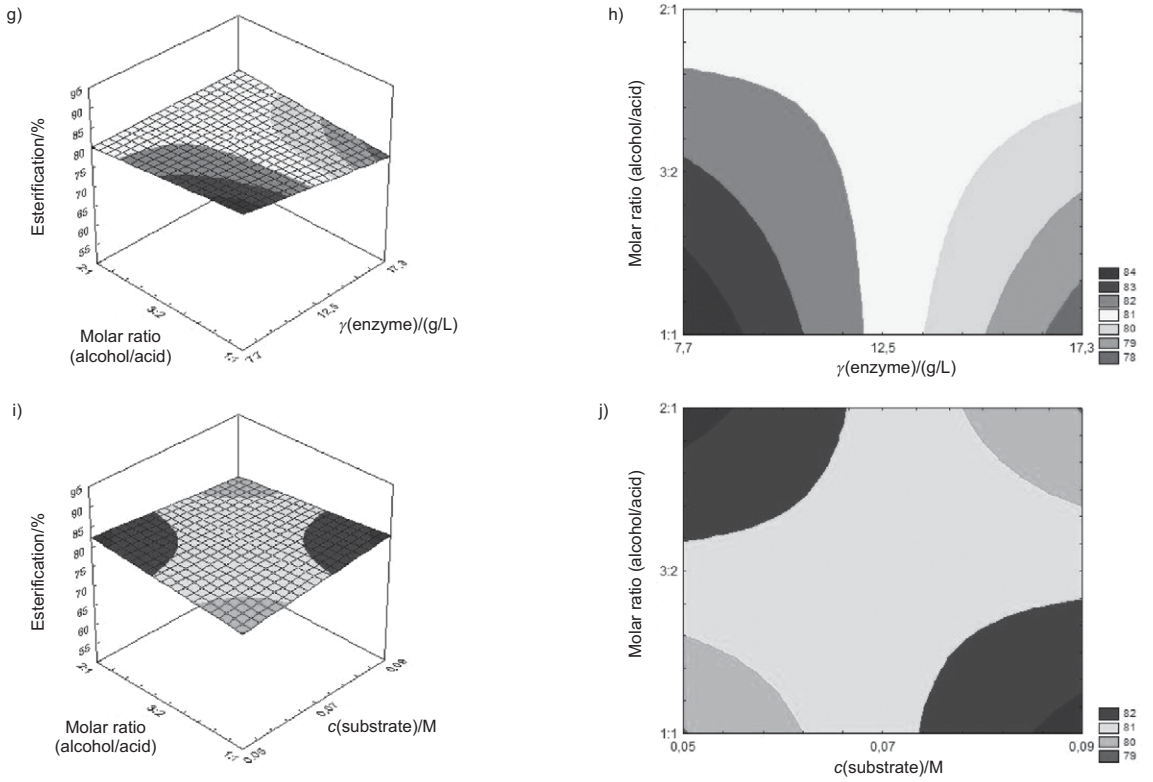

Fig. 1. – continued: (g, h) enzyme concentration and ethanol/butyric acid molar ratio, (i, j) substrate concentration and ethanol/butyric acid molar ratio

Response surfaces of the dependent variables were estimated for the response on the basis of the samples in the central composite design. It is possible to study the sensitivity of different conditions for esterification of butyric acid (temperature, enzyme concentration, butyric acid concentration and ethanol/butyric acid molar ratio) from these response surfaces. A rather flat response surface indicates that the esterification reaction can tolerate variations in the processing conditions without the responses being seriously affected, whereas a pointed surface indicates that responses are sensitive to the processing conditions used (*24*).

Figs. 1a and b show the response surface plot for enzyme concentration and temperature. The best results were obtained when the temperature was high, the enzyme concentration was in the range up to 12.5 g/L, and esterification was approx. 85 %. Similar esterification occurred when the temperature and butyric acid concentration were high (Figs. 1c and d). Figs. 1e and f show the effects of the enzyme concentration and butyric acid concentration, which reached values of about 85 % at the low enzyme concentration and high butyric acid concentration. Figs. 1g and h show an interactive effect between the enzyme concentration and ethanol/butyric acid molar ratio. The best results (85 % esterification) were obtained when low enzyme concentration and ethanol/butyric acid molar ratio were used. When high butyric acid concentration and low ethanol/butyric acid molar ratio, or low butyric acid concentration and high ethanol/butyric acid molar ratio were used, esterification percentage was up to 80 % (Figs. 1i and j).

Enzyme concentration is known to be an important variable in esterification reactions for the synthesis of various fatty acid esters. The positive effect of the enzyme concentration on the synthesis of butyric ester in the present work is in agreement with reports of other authors dealing with the production of flavour esters using microbial lipases (*5*,*6*,*10*,*21*,*27*).

In general, the increment of butyric acid concentration lowered the esterification capacity of the lipases. This effect had been reported in the biosynthesis of isoamyl acetate (*2*), isoamyl isovalerate (*11*) and ethyl esters of short-chain fatty acids (*29*). The lowest conversions at higher butyric acid concentrations showed that there could be two reasons for acid inhibition: the accumulation of water during the progress of the reaction, which favours hydrolysis (backward reaction), or probable acid or alcohol inhibition. It was asserted that alcohols are terminal inhibitors of lipases, and acids may cause acidification of the microaqueous interface leading to enzyme inactivation (*11*,*30*).

The optimized synthesis conditions for ethyl butyrate reached better results in this work than those obtained previously by Rodriguez-Nogales *et al.* (*10*). The authors reported that the optimum percentage of esterification of ethyl butyrate with 7 % immobilized *Candida antarctica* lipase (Novozyme 435) and 0.04 M butyric acid at 34 °C in 96 h was 72.9 %.

The surfaces indicated that the high percentage of esterification can be obtained at a temperature of 45 °C, when the lowest enzyme concentration was 7.7 g/L, with a butyric acid concentration of 90 mM and ethanol/butyric acid molar ratio of 1:1, as can be seen in trial 6 in Table 2. Fig. 2 shows the results of the synthesis of ethyl butyrate carried out in triplicate, using the conditions al-

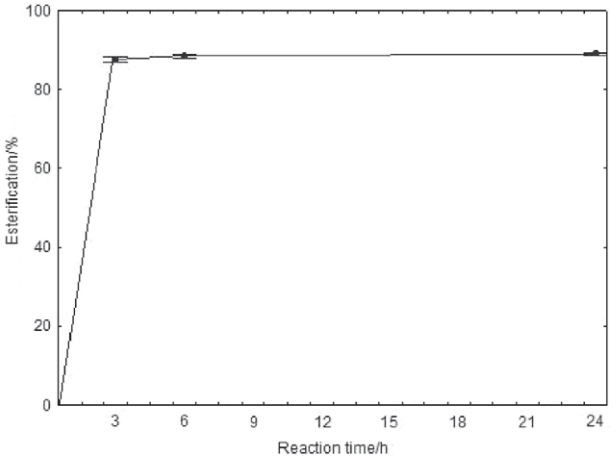

Fig. 2. Esterification percentage as a function of time for the validation of experiments under the optimized conditions

ready described for validation experiments. In this synthesis, 88 % esterification was obtained, presenting a relative deviation of around 5 %.

Conclusions

Based on the present study, it is evident that response surface methodology can be successfully used to gain knowledge to explain the relative performance of immobilized lipases during esterification reactions. The optimum conditions for maximum esterification percentage, established by using a central composite design (CCD), were at a temperature of 45 °C, enzyme concentration of 7.7 g/L, butyric acid concentration of 90 mM and ethanol/butyric acid molar ratio of 1:1. Under these conditions the esterification percentage was around 88 % in 3 h.

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