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Enzymatic esterification of an acid with an epoxide using an immobilized lipase from *Mucor miehei* as catalyst: Optimization of the yield and isomeric excess of ester by statistical analysis

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We have developed the methodology for the esterification of an acid with an epoxide using 2-chlorobutyric acid and 1,2-epoxy-5-hexene catalysed by a *Mucor miehei*-immobilized lipase. Thus, this methodology could be applied to obtain 2-chloroesters. A factorial design of experiments and a central composite design have been used to optimise the synthesis of these esters. The variables chosen were temperature and initial catalyst concentration, while the responses were yield and isomeric excess of the ester. According to this study, temperature was the most important factor, having a positive influence on the yield and a small negative influence on the isomeric excess of the ester. The yield and isomeric excess of the ester show a greater dependence on temperature compared to the catalyst concentration. Although the effect of the catalyst concentration on both responses is smaller than the temperature effect, the higher selectivity presented by the biocatalyst towards the studied ester considerably decreased the final product distribution.
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Introduction

The increasing demand for specifically formulated esters with applications in the food, cosmetic, lubricant [5] and pharmaceutical [10] industries creates the need for developing specific catalysts. For this reason, the application of enzymes as catalysts is undergoing rapid development [3]. Enzymes provide many economic benefits by making processes much more efficient. Lipases (triaclyglycerol acylhydrolase, EC 3.1.1.3) are used widely in a variety of reactions that include esterification of acids with alcohols [6], oil and fat interesterification [11], production of biosurfactants [4] and resolution of racemic mixtures to produce optically active compounds [16]. Lipases have distinct advantages compared to classic chemical catalysts.

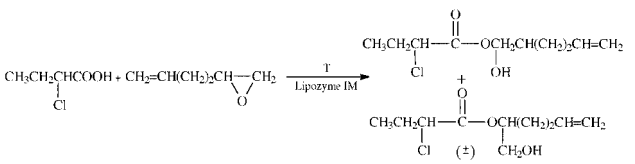
(a) Enzymes function under mild reaction conditions, avoiding the formation of side products [15].

(b) Enzyme-catalysed reactions are more efficient and easier to control.

(c) The unique specificities of lipases allow the design of synthesis routes that predetermine product structure and distribution, whereas chemical catalysts generally lead to random reaction product mixtures, and, even when the lipases are nonspecific, offer better kinetic control and milder reaction conditions [7].

Immobilized lipases are particularly interesting for industrial purposes since they can be easily handled. Unlike soluble lipases, a lipase in an immobilised form is strongly bound to a solid phase, making it easy to separate the catalysts from the reaction mixture and its reuse [14].

Enzymatic resolution of 2-substituted acids by lipases has been the subject of intense investigations. Much of the effort has centered on the production of *S*-2-hydroxyalkanoic acids [13], *R*-2-chloro and *R*-2-bromoalkanoic acids [9,8] and *S*-2-arylpropionic acids [1,12] due to their high value as products or intermediates in the synthesis of drugs or herbicides. In the present study, immobilized Lipozyme IM has been used as catalyst for esterification of 2-chlorobutyric acid with the epoxide 1,2-epoxy-5-hexene, *via* opening of the oxirane ring, to produce 2-hydroxy-5-hexenyl 2-chlorobutyrate ester as the main product and a racemic mixture of their position isomer, 1-methyldroxy-5-pentenyl 2-chlorobutyrate ester, as secondary product. This intermediate product can easily be substituted in the α-carbon by an amino group to obtain 2-aminoesters, the latter being useful as intermediate reagent in the synthesis of many drugs such as Taxol, a powerful anticancer drug. The methodology followed in the present paper could be applied to obtain this kind of regioselective β-aminoesters, from epoxide opening, to be applied as intermediates in the pharmacological industry. Below, a reaction scheme is shown:



In the present work, the use of factorial design and surface response methodology is emphasized in order to prove that the design and statistical analysis of experiments allow us to obtain an efficient model for industrial control, and in this way reduce the number and cost of experiments. As an illustration of the methodology mentioned above, the experimental study of the esterification of an acid with an epoxide was undertaken in a

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batch reactor in order to give the phenomenological relation for yield of ester and isomeric excess obtained as a function of initial catalyst concentration and temperature. The acid/epoxide molar ratio was constant in order to minimize purification steps. These variables are the most commonly used for modelling esterification reactions.

Methods

Equipment

Experiments were carried out in a 250-cm^3 batch stirred reactor (BSTR). This reactor was provided with temperature and speed controllers. The impeller speed was set at 600 rpm to avoid mass transfer limitations [7].

Materials

2-Cholorobutyric acid was supplied by Fluka Química, Madrid, Spain, while 1,2-epoxy-5-hexene was supplied by Aldrich Química, Madrid, Spain. The catalyst used was a nonspecific triacylglycerol lipase (EC 3.1.1.3) from *Mucor miehei* (Lipozyme IM). This enzyme was supplied by Novo Nordisk Bioindustrial, Madrid, Spain, with water content of 1–2% wt/wt. The activity of the enzyme preparation was 60 batch interesterification units (BIU). One batch interesterification unit is defined as 1 μmol of palmitic acid incorporated into triolein per minute at standard conditions, after 15 min at 60°C.

Analytical method

Reaction products were monitored by gas chromatography/mass spectrometry (GC/MS) and quantitatively determined by capillary column GC. GC/MS data were recorded on a 6890 Series Hewlett-Packard instrument. GC was performed using a fused silica chiral capillary column β-cyclodextrin-permetilated Supelco β-Dex (60 m length, 0.25 mm i.d., 0.25 μm film). A Hewlett-Packard gas chromatograph was equipped for split injections (100:1). The GC/MS operating conditions were: ionization energy 200 eV, mass range 50–700 amu. The GC column oven temperature was held at 120°C for 7 min, then raised at 10°C/min to 200°C and maintained at that temperature until all components had eluted. Quantitative gas chromatographic analyses were performed on a Hewlett-Packard Series 6890 instrument, using the column and conditions described above in the CG/MS analysis. A flame ionization detector (FID) was used at 270°C. The injection system was split. The carrier gas was helium at a flow rate of 1 ml/min. The external patron technique has been used in order to quantify the amount of the chemical species. Figure 1 shows a typical chromatographic analysis.

Procedure

Acid and the catalyst were added to the reactor, fitted with a reflux condenser. When the set temperature was reached, the epoxide was introduced in the reactor. Samples were taken at regular intervals and analyzed by GC. The total reaction time was 2 h. During the experiments, temperature, pressure impeller speed and acid/epoxide molar ratio remained constant.

Results

Experiments were carried out at two different stirring speeds (600 and 900 rpm) and no significant changes in the conversion values were observed. In consequence, mass transfer limitations were considered negligible and the stirring speed was fixed at 600 rpm. The initial acid/epoxide molar ratio was fixed at 1.

This study was carried out in order to determine the effects of temperature and catalyst concentration on the yield of 2-hydroxy-5-hexenyl 2-chlorobutyrate ester and the isomeric excess of the products formed and their optimum levels for obtaining the maximum yield of ester and the isomeric excess. We have used a response surface methodology [2] for this purpose.

Response surface methodology consists of several steps. The first step is to design a set of experiments in order to obtain reliable values of the responses. Factorial design was used for this purpose. The second step is to establish a suitable second-order model to

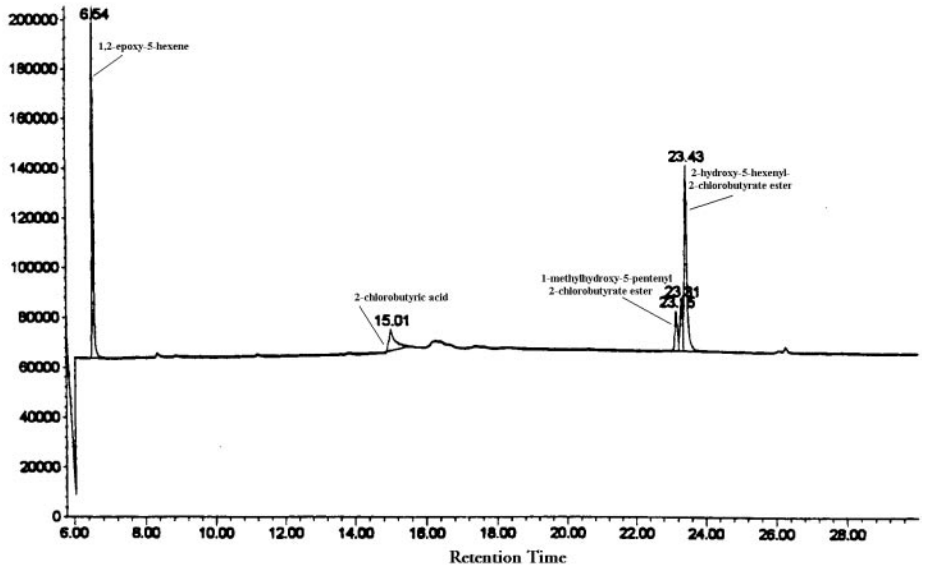

Figure 1 Chromatographic analysis of the reaction.

find out the optimum conditions of the independent variables, which produced the maximum value of the responses.

Responses

The responses chosen were the yield of 2-hydroxy-5-hexenyl 2-chlorobutyrate ester, *Y*, and the isomeric excess previously defined.

Factors

Selection of the factors was based on considerations related to the chemical aspect of the system. The factors chosen were temperature, X_T, and initial catalyst concentration, X_C. Initial acid/epoxide molar ratio and stirring were fixed.

Levels

Selection of the levels was carried out on the basis of results obtained in a preliminary study, considering the working limit conditions for each chemical species. These considerations made it possible to fix the upper temperature level at 80°C in order to avoid the loss of enzymatic activity caused by temperature effect. Temperatures under 60°C do not permit an effective enzymatic activity; therefore, this was chosen as the minimum level for temperature. Because of commercial specifications on this catalyst, initial catalyst concentrations were 5% and 10% in each case.

Linear stage

The experimental design applied was a 2^2 factorial design. Four central points were added to evaluate the experimental error. The standard experimental matrix for the factorial design is shown in Table 1. Columns 2 and 3 represent the ±1 coded factor levels in a dimensionless scale, and columns 4 and 5 represent the factor levels on a natural scale. Experiments were run at random to minimize errors due to possible systematic trends in the variables. In addition, Table 1 also shows the results of the yield of ester and the isomeric excess after 2 h. A statistical analysis was carried out with these experimental values, and the main effects and interaction effects of the variables temperature and initial catalyst concentration were calculated.

Table 2 shows the analysis of the main effects and interactions for the chosen responses yield of ester and isomeric excess together with the test of statistical significance, a two-sided test with a 95% confidence level. The best-fitting response functions for the main effects and interactions are given in Table 2.

Table 1 Experimental matrix for the CCD

Experiment	T (°C)	C (%)	X_T	X_C	Y[a] (%)	i.e.[b] (%)
1	60	5	−	−	34.8	35.8
2	60	10	−	+	37.8	36.3
3	80	5	+	−	47.5	33.7
4	80	10	+	+	48.2	33.7
5	70	7.5	0	0	36.8	37.7
6	70	7.5	0	0	36.9	38.9
7	70	7.5	0	0	37.1	37.3
8	70	7.5	0	0	36.5	38.3
9	70	4	0	$-\alpha$	38.1	39.2
10	70	11	0	$+\alpha$	30.4	39.8
11	56	7.5	$-\alpha$	0	28.2	40.0
12	84	7.5	$+\alpha$	0	53.1	37.4

[a]Yield of the ester.
[b]Isomeric excess of the ester.

Table 2 2^2 factorial design: statistical analysis

Response: yield of ester and isomeric excess after 2 h	
Main effects of interactions	
X_T=11.6, X_C=1.9, X_{TC}=−1.2	X_T=−2.3, X_C=0.2, X_{TC}=−0.3
Significance test; confidence level: 95%	
Yield of ester	Isomeric excess
Y=42.1	i.e.=34.9
S^a=1.50	S^a=1.61
t^b=3.181	t^b=3.181
Confidence interval	
±0.40	±1.11
Significant main effects and interactions	
X_T, X_C, X_{TC}	X_T
Significance of curvature	
Curvature: $C = Y_c - Y_m$	
C=5.25	C=3.17
Confidence curvature interval	
±0.56	±2.5
Significance	
Yes	Yes

[a]Standard deviation.
[b]Student's t value.

Experimental results were fitted to a linear model, and the following expressions were obtained:

$$Y = 42.08 + 5.78X_T + 0.93X_C - 0.58X_TX_C \qquad (1)$$

$$\text{i. e.} = 34.89 - 1.17X_T + 0.12X_C - 0.14X_TX_C \qquad (2)$$

To evaluate whether the factorial design was sufficient to describe accurately the esterification process, the statistical significant of the curvature was studied. The test of statistical significance is shown in Table 2. The curvature effects, defined as the difference between the average of the center points responses and the average of the factorial points, were 5.25% for yield of ester and 3.17% for isomeric excess. At 95% confidence level, the confidence intervals on curvature were ±0.56 for yield of ester and ±2.5 for isomeric excess. Therefore, the curvature effects were found to be statistically significant and it was necessary to consider a different design. This allowed us to fit our data to a second-order model.

Nonlinear stage

According to the response surface methodology, a second-order model is required because of the significant curvature effect found in the linear model. Additional experiments (star points) were added to the factorial design in order to produce a central composite design. The experimental matrix corresponding to the central composite design is shown in Table 1. It consists of factorial points, center points, and star points coded as $+\alpha$ and $-\alpha$. The value of α, which is the distance from the center point to star point, is $2^{n/4}$, where n is the number of factors. The corresponding model is the

complete quadratic surface between responses and factors, given by equation:

$$Y = a_0 + \sum_{k=1}^{2} a_k X_k + \sum_{k=1}^{2} a_{kk} X_k^2 + \sum_{k \neq j}^{2} a_{kj} X_k X_j$$

The coefficients were obtained by multiple regression analysis. This analysis includes all the independent variables and their interactions, regardless of their significance levels. The best-fitting response surfaces are given by the following equations.

Statistical models:

$$Y = 36.83 + 7.28X_T - 0.9X_C - 0.58X_T X_C + 3.07X_T^2 - 0.13X_C^2 \therefore \text{r} = 0.85 \quad (3)$$

$$\text{i. e.} = 38.03 - 1.04X_T + 0.16X_C - 0.14X_T X_C + 0.72X_T^2 - 0.32X_C^2 \therefore \text{r} = 0.50 \quad (4)$$

Industrial models:

$$Y = 125.61 - 3.39T - 1.57C - 0.02TC + 0.03T^2 - 0.02C^2 \therefore \text{r} = 0.85 \quad (5)$$

$$\text{i. e.} = 3.43 - 0.96T - 1.21C - 0.005TC + 0.007T^2 - 0.05C^2 \therefore \text{r} = 0.50 \quad (6)$$

The statistical model is obtained from coded levels giving the real influence of each variable on the process and the technological model from the real values of the variables.

Figures 2 and 3 show the response surface plots and contour plots for the predicted and experimental values for the yield and the isomeric excess of ester over the experimental range studied *versus* the temperature and the initial catalyst concentration after 2 h of reaction.

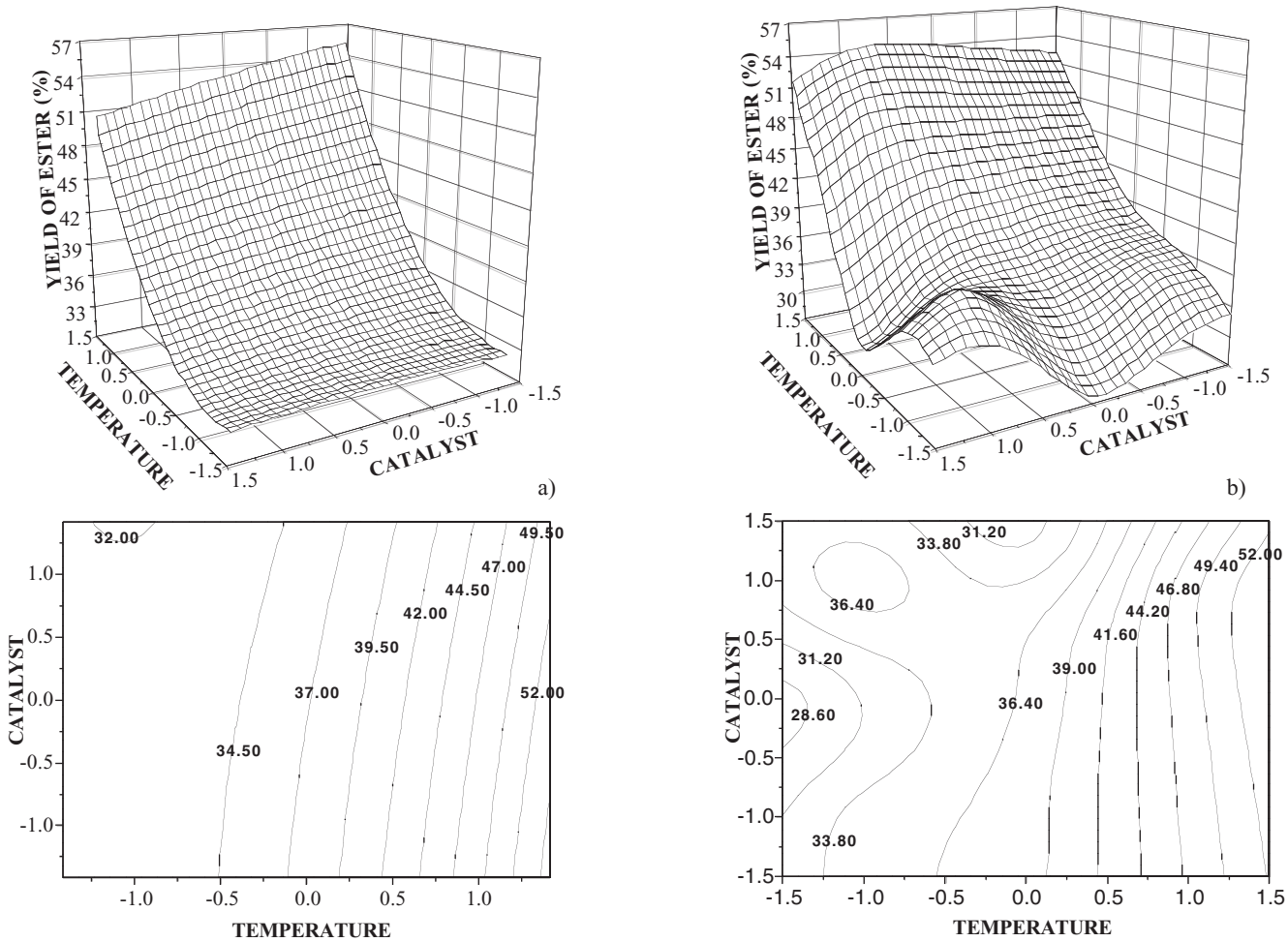

Figure 2 Response surface plot of the statistical (a) and the experimental (b) models for the ester yield *versus* catalyst concentration and reaction temperature.

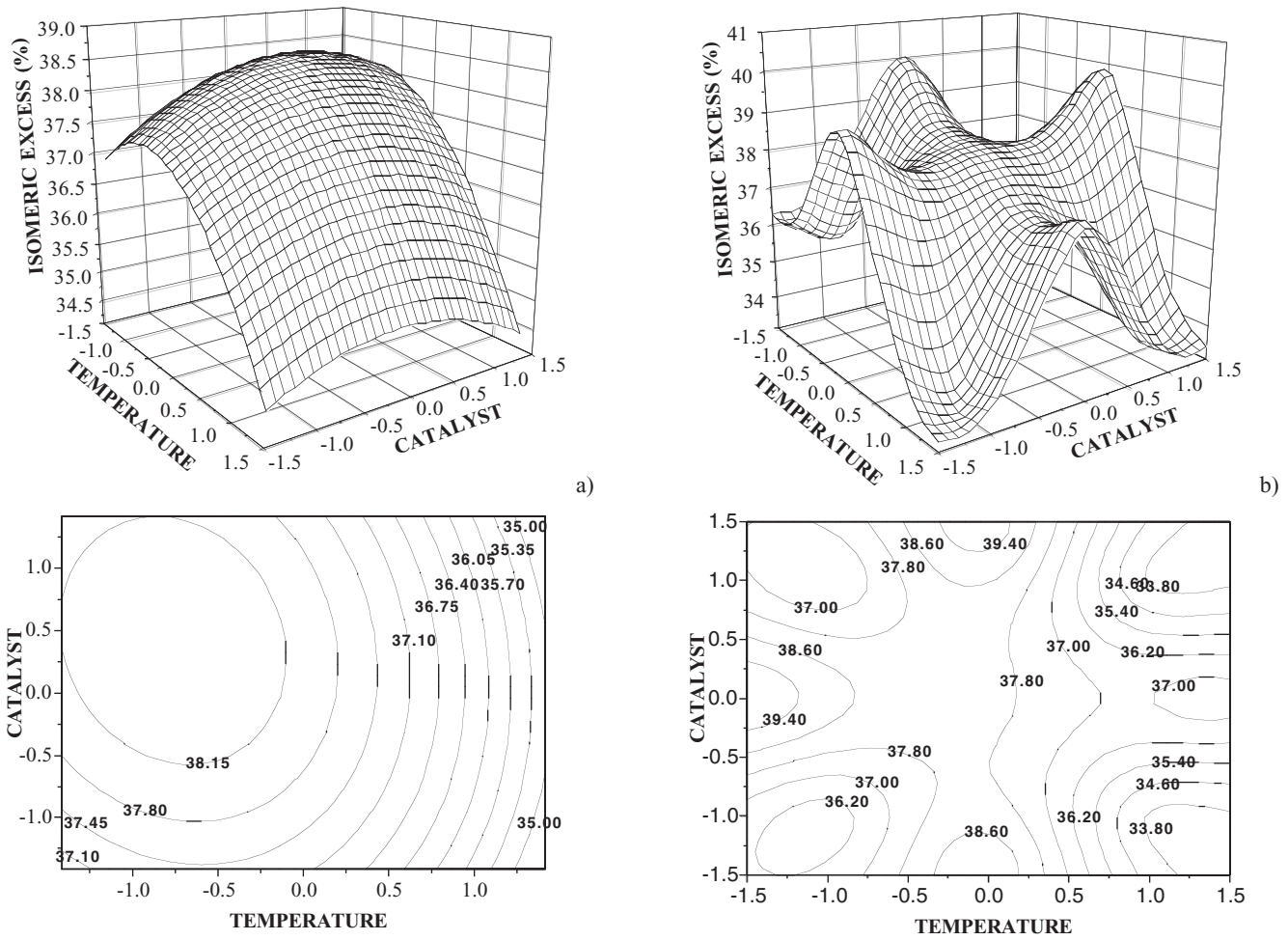

Figure 3 Response surface plot of the statistical (a) and the experimental (b) models for the isomeric excess *versus* catalyst concentration and reaction temperature.

Discussion

The influence of variables, reaction temperature and catalyst concentration on the yield and isomeric excess of ester is discussed using the statistical models shown in Eqs. (1), Eqs. (2), Eqs. (3) and Eqs. (4).

Influence of temperature

Statistical analysis of the experimental range studied identified temperature as the most important factor for both responses. It has a positive effect on the yield of ester in both statistical models, linear and nonlinear.

In both statistical models, temperature has a negative effect on the isomeric excess because with temperature increases the isomeric excess decreases. This isomeric excess loss can be explained by kinetic reasons; thus, as well, as the temperature rises, the rate of the secondary product increases.

Influence of initial catalyst concentration

In the linear model, the initial catalyst concentration has a positive effect on the yield of ester. In the nonlinear model, it has a small negative influence, probably due to the wider experimental range considered. For the isomeric excess, the initial catalyst concentration has a small positive influence in both models. However, the type of catalyst is important for the reaction. It needs to have a high selectivity towards the ester studied, avoiding the great products distribution that takes place when the reaction is carried out without any catalyst. The biocatalyst used in this study fulfilled these requirements.

Influence of the interactions

The effect of the temperature–catalyst concentration interaction, $T-C$, is slightly negative on both responses in comparison with the simple effect of the temperature.

Analysis of response: yield of ester and isomeric excess

The shapes of the three-dimensional surfaces and contour plots — representing yield of ester and isomeric excess *versus* temperature, T, and initial catalyst concentration, C, values — are shown in Figures 2 and 3.

The maximum yield of 2-hydroxy-5-hexenyl 2-chlorobutyrate ester is obtained at high temperatures (80°C) and at low initial catalyst concentration (5%) (Figure 2a). The maximum isomeric excess is obtained at low temperatures (60°C) and high initial catalyst concentration (10%) (Figure 3a). Figures 2 and 3b show

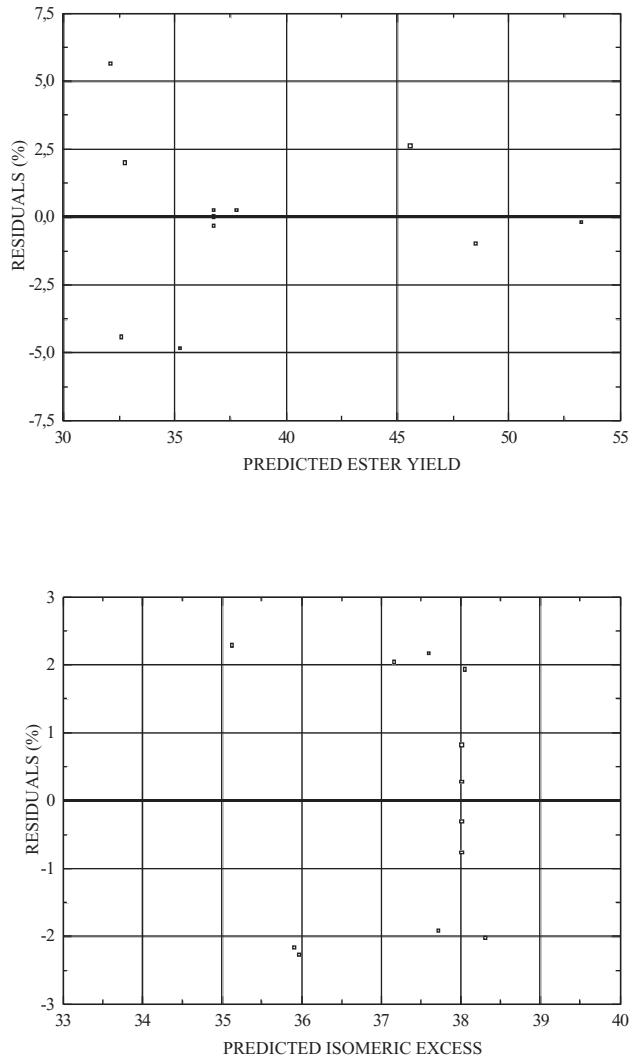

Figure 4 Plot of residuals for the second-order model.

the experimental surfaces and contour plots of the responses *versus* temperature and initial catalyst concentration. The response surface plot of the yield of ester obtained from the experiments is quite similar to values predicted with the statistical method, while for the isomeric excess, the experimental results are very different from the predicted values.

Figure 4 shows a plot of the residuals' distribution, defined as the difference between calculated and observed values over the observed values. The quality of fit is good because the residuals' distribution does not follow a trend with respect to the predicted variables. All the residuals are smaller than 5%, which indicates that the models describe adequately the conversion to 2-hydroxy-5-hexenyl 2-chlorobutyrate ester and isomeric excess over the experimental range studied.

Conclusions

In the present work, a central composite design to optimise the synthesis of 2-hydroxy-5-hexenyl 2-chlorobutyrate ester and the isomeric excess was applied using a *M. miehei*-immobilised lipase as catalyst. A two-factorial design was proved effective to study the influence of the temperature and initial catalyst concentration in the reaction process. The central composite design procedure was also studied to optimise these variables to obtain the optimum yield of both ester and isomeric excess. From these equations, it is possible to adequately predict the required reaction conditions to obtain an optimum yield for the ester and the isomeric excess.

The investigation of the variables affecting the responses indicated that within the experimental range considered, the most important factor was temperature. This temperature factor had a positive influence on the yield of the ester and negative for the isomeric excess. On the other hand, the initial catalyst concentration variable presented a small positive effect in both responses. However, the importance of the biocatalyst should be emphasised due to the great selectivity presented towards the ester. The cross-interactions had no effect on the reaction.

A first-order approach did not fit the data adequately and quadratic models were required. Second-order models were developed to predict the yield of ester and isomeric excess as a function of the variables. Analysis of residuals demonstrated the efficiency of the models investigated; using these models, the yield of ester and isomeric excess in the experimental range studied could be determined accurately. According to this study, the maximum yield of ester could be obtained when working at intermediate and low initial catalyst concentrations and high temperatures, while for the isomeric excess, the optimum conditions were obtained when working at intermediate initial catalyst concentrations and at low temperatures.

These models could be useful to determine the optimum operating conditions in an industrial process using the least number of experiments with consequent economic benefits.

Acknowledgements

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