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Optimization of lipase-catalysed synthesis of butyl butyrate using a factorial design

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Summary

A lipase from *Candida rugosa* immobilized on styrene-divinylbenzene copolymer was used to catalyse the direct esterification of butanol and butyric acid. A factorial design was employed to evaluate the effects of temperature (37–50 °C), substrate molar ratio of butyric acid to butanol (0.6 to 2.0) and enzyme amount (0.2–0.4 g) on the ester yield. The main effects were fitted by multiple regression analysis to a linear model and maximum ester yield could be obtained working at 41 °C with 0.4 g of lipase. The mathematical model obtained, representing the ester yield has been found to describe adequately the experimental results. Under optimal conditions, concentration of 32.4 g butyl butyrate/l that corresponds to a yield of 75% was obtained.

Introduction

Esters of short chain fatty acids and alcohols are important components of natural aromas used in the food industry. Currently, most of the flavour and fragrance components are obtained by traditional methods, which include chemical synthesis or extraction from natural sources. The concept of a natural ester made by enzymatic synthesis with lipase and natural substrate components is an attractive alternative to those routes (Yahya *et al.* 1999; Sharma *et al.* 2001).

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are an important group of enzymes that catalyse the breakdown of oils and fats, with subsequent release of free fatty acids, acylglycerols and glycerol (Vulfson 1994; Castro *et al.* 2004). In addition, lipases can also catalyse the hydrolysis and synthesis of a broad range of natural and unnatural esters, while retaining high enantio or regioselectivity (Pandey *et al.* 1999). This combination of broad substrate range and high selectivity makes lipases ideal catalysts for organic synthesis (Lai & O'Connor 1999; Villeneuve *et al.* 2000).

Ester synthesis by lipases can be performed at room temperature and pressure, providing an energy-saving procedure under neutral pH in reaction vessels operated either batchwise or continuously (Lai & O'Connor 1999). Therefore, the products obtained are qualitatively purer than those obtained by chemical catalysis, which tends to be unspecific, and consequently generates several byproducts (Roy & Bhattacharyya 1993). The use of lipases to carry out esterification alleviates the necessity of a wide variety of complex post-reaction separation processes and thus leads to lower overall operation costs (Yahya *et al.* 1998).

However, lipase-catalysed reactions, have, herewith a major inconvenience mainly associated with low conversions when compared to traditional chemical processes if crude commercial enzyme preparations are employed. These intrinsically low volumetric productivities may lead to products quantitatively less pure than those obtained via chemical synthesis. Such a drawback can be coupled with biocatalyst inhibition by products and/or substrates and biocatalyst inactivation by heat (thermal inactivation) or by several compounds (chemical inactivation) (Halling 1994; Yahya *et al.* 1998).

To overcome such limitations, immobilization of lipases is often recommended (Balcão *et al.* 1996; Tischer & Kasche 1999; Villeneuve *et al.* 2000). A promising approach to lipase immobilization makes use of hydrophobic matrices such as styrene-divinylbenzene copolymer (STY-DVB). This type of support can be easily recognized by the lipases, at molecular level, as solid surfaces. Thus, lipases can be adsorbed on such supports, leading to open immobilized structures (Villeneuve *et al.* 2000).

In accordance with this methodology, microbial lipase from *Candida rugosa* was successfully immobilized on STY-DVB by physical adsorption rendering active and stable immobilized samples (Oliveira *et al.* 2000a). In the present work, the ability of this immobilized preparation to catalyse the direct esterification of butanol and

butyric acid has been investigated. The product obtained, butyl butyrate, is in high demand as a component of pineapple flavour in the food, beverage and pharmaceutical industries. Considering the high demand and benefits, an attempt has been made to optimize the reaction parameters for maximization of butyl butyrate yields. The conventional method of optimization involves the variation of only one parameter while keeping the others constant. This is a time-consuming procedure and often does not bring about the interaction effect of various parameters when compared to statistical methods (Box *et al*. 1978). Response surface methodology (RSM) is a useful technique for studying the effect of several factors that influence the responses by varying them simultaneously and carrying out a limited number of experiments. The present investigation aimed to optimize the process for enzymatic synthesis of butyl butyrate using lipase from *Candida rugosa* immobilized on STY-DVB by applying RSM.

Materials and Methods

Enzyme

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3) from *Candida rugosa* (Type VII, Sigma Chemical Co., St Louis, MO, United States) supported on resin beads (styrene-divinylbenzene copolymer macro porous structure) was prepared according to methodology previously established in our laboratory (Oliveira *et al*. 2000a). The properties of the support were the following: 45% divinylbenzene; 100 mesh granulometry average pore diameter (291 Å); surface area BET (0.99 $m^2\ g^{-1}$) and porous volume (0.72 $cm^3\ g^{-1}$). Some properties of the immobilized derivative were: bound activity yield (65%); bound enzyme activity (75 U mg^{-1}); water content (10%); storage stability (little determined loss of activity at 4 °C for 6 months; biocatalyst half-life time (620 h). Further information about this immobilized preparation is given elsewhere (Oliveira *et al*. 2000a, b).

Chemicals

n-Butanol (98% pure) and butyric acid (98% pure) were obtained from Merck Chemical Co (Darmstadt, Germany). Molecular sieve (4 Å) was purchased from Sigma-Aldrich Chemical (St. Louis, USA). *n*-Heptane was obtained from Reagen Chemical Co (São Paulo, Brazil). All other chemicals were of analytical grade. All reagents and *n*-heptane were dehydrated by molecular sieve (4 Å) for 24 h.

Esterification reaction

The reaction was carried out in a 100 ml stoppered conical flask with a working volume of 20 ml *n*-heptane containing the required amount of *n*-butanol (300 mM). Different concentrations of butyric acid (200–600 mM) were added followed by different amounts of lipase. The mixtures of butanol, butyric acid and lipase immobilized on STY-DVB were stirred in an orbital shaking water bath (150 rev min^{-1}) at different reaction temperatures. Based on previous work (Oliveira *et al*. 2000b), reaction time was fixed for 24 h and samples were analysed at the initial and final time for each test. The influence of the several factors on the formation of the butyl butyrate using the lipase immobilized on STY-DVB was verified using a 2^3 full factorial design with triplicates at the central point. The range and the levels of the variables investigated in this study were: temperature from 37 to 50 °C, biocatalyst mass (0.2–0.4 g) and molar ratio of butyric acid (Abut) to butanol (ButOH) of 0.6 to 2.0. The butyl butyrate yield (Y%) was taken as the dependent variable or response of the design experiments. Where possible, the model was simplified by the elimination of statistically insignificant terms. The 'Statistica' (version 5.0) software was used for regression and graphical analyses of the obtained data. The statistical significance of the regression coefficients was determined by *Student's* test, the second order model equation was determined by *Fischer's* test and the proportion of variance explained by the obtained model was given by the multiple coefficient of determination, R^2. Based on the results, a new 2^2 factorial design with centred face was built, with the objective of determining parameters levels that will provide the highest conversion of the starting materials (butanol and butyric acid) into butyl butyrate.

Analytical methods

Butyl butyrate formation was determined by injecting a 1 μl aliquot into a gas chromatograph (Varian 3400, USA) equipped with a flame ionization detector (FID) and column (6 ft 5% DEGS on a Chromosorb WHP 80/10 mesh, Hewlett Packard, USA). Hexanol was employed as an internal standard. The oven temperature was maintained at 70 °C and nitrogen was used as carrier gas. The ester yield (molar conversion) was defined as (mol butyl butyrate formed/mol initial butanol) × 100% and was estimated by peak area integration using on-line software. An aliquot of 0.2 ml of the reaction mixture was periodically withdrawn and an equal volume of ethanol was added. Ethanol is used as a quenching agent. Along with this, 2–3 drops of phenolphthalein indicator were added and the mixture was titrated with standard potassium hydroxide to determine the residual acid content. Water concentrations in liquid and solid phases were measured by the Karl Fischer method using the Karl Fischer titrator (Mettler DL 18).

Results and discussion

The first step in the process of seeking optimum conditions was to identify the input variables that have the greatest influence on the response. As a preliminary step,

Table 1. Matrix for a 2^3 full factorial design and experimental results.

Run	x_1	x_2	x_3	T (°C)	[E] (g)	MR	Y (%)
1	−	−	−	37	0.2	0.6	47.40
2	+	−	−	50	0.2	0.6	3.74
3	−	+	−	37	0.4	0.6	49.04
4	+	+	−	50	0.4	0.6	12.21
5	−	−	+	37	0.2	2.0	48.76
6	+	−	+	50	0.2	2.0	10.36
7	−	+	+	37	0.4	2.0	53.59
8	+	+	+	50	0.4	2.0	16.45
9	0	0	0	43	0.3	1.3	57.60
10	0	0	0	43	0.3	1.3	58.70
11	0	0	0	43	0.3	1.3	49.10

MR: initial acid/alcohol molar ratio. Reaction fixed conditions: butanol concentration (300 mM), substrate volume (20 ml); incubation time (24 h).

the effects of different variables (temperature, biocatalyst initial level and molar ratio) on butyl butyrate synthesis were simultaneously investigated using a 2^3 full factorial design. The experimental matrix for the factorial design is shown in Table 1, together with the data for the response factor. The first three columns of data give the factors levels (+) or (−) in the dimensionless scale.

The formation of butyl butyrate was found to be strongly dependent on the reaction temperature. The ester yield (Y%) widely varied (from 3.74 to 58.70%) and independently of the other variables, increasing the reaction temperature level from 37 to 50 °C, substantially decreased the ester yield from 47.4 to 3.74% (runs 1 and 3). The experiment results shown in Table 1 were used to estimate the main variable effects and their interactions. According to the Student's t-test (Table 2), only the reaction temperature seems to have played a critical role in the ester formation within the studied experimental range (Table 2). Its statistical meaning was significant and negative on the response variable at 95% probability level ($P = 0.0427$).

No statistical influence was found in relation to the other variables. It is appears that the influence of reaction temperature masked the possible influence of the other variables.

The negative effect of the reaction temperature on ester synthesis is in agreement with reports of other authors dealing with production of flavour esters (ethyl hexanoate, isoamyl isovalerate, ethyl butyrate) using lipases (Lai & O'Connor 1999; Chowdary *et al.* 2000; Matsumoto & Ohashi 2003; Rodrigues-Nogalez *et al.* 2005). However, this trend is dependent on the lipase source and thermal stability data. For *Candida rugosa* lipase, which has low thermal stability (Pandey *et al.* 1999; Matsumoto & Ohashi, 2003) high temperature values usually decrease the product formation as verified in the present work, in which ester yields decreased to about 39% when the temperature level raised from 37 to 50 °C.

Table 2. Statistical analysis.

Standard design: 2^3, Number of factor: 3, Number of runs: 8, Total number of runs in experiment: 11 (3 centre points)

Main effects	t	P
$x_1 = -39.01$	−2.93	0.0427
$x_2 = 5.26$	0.40	0.7128
$x_3 = 4.19$	0.32	0.7684
$x_1x_2 = 2.02$	0.15	0.8865
$x_1x_3 = 1.24$	0.09	0.9304
$x_2x_3 = 0.20$	0.02	0.9889
Curvature = 49.88	12.43	0.0011

Similar behaviour was reported by Rodriguez-Nogales *et al.* (2005) using lipase from *Candida antarctica* immobilized on macroporous acrylic resin to catalyse ethyl butyrate at different temperatures (27–65 °C). According to their results, increasing the reaction temperature markedly reduced the esterification yield and the best results were obtained at low temperature (27 °C) and high enzyme concentration.

Analysis of variance revealed a significant value for curvature ($P < 0.05$, Table 2), indicating the non-linearity of the model and thus justifying planning a new statistical design in order to determine a mathematical model that provides the highest conversion of the starting materials (butanol and acid butyric) into butyl butyrate. For this purpose, a 2^2 factorial design with centred face was built considering the temperature (x_1) and lipase mass (x_2). In this set of runs, the molar ratio of butyric acid (Abut) to butanol (ButOH) was fixed in 2.0. Table 3 shows the standard orthogonal central composite design matrix and ester yield values.

To obtain a better data fit, natural logarithm values of the response variable were used for statistical analysis as summarized in Table 4. In the studied experimental range, statistical analysis indicated that both temperature and initial catalyst concentration played a critical role in the butyl butyrate synthesis. The results also revealed that while initial biocatalyst amount (x_2) had a significant positive effect ($P < 0.05$), the temperature exerted a negative influence on ester yield.

It can be also observed that the quadratic effect of temperature was significant, being, therefore, its corresponding coefficient included in the model. Due to their low statistical significance values, the interactions between the variables x_1 and x_2 as well as the quadratic effect of the lipase concentration were excluded from this analysis.

The mathematical model that represents the esterification yield, considering the terms that really have influenced, can be expressed by Equation 1.

$$\mathrm{LN}(Y) = 3.94 - 0.72\,x_1 - 0.95\,x_1^2 + 0.38\,x_2 \qquad (1)$$

where x_1 and x_2 represent the values coded for temperature and lipase mass, respectively.

The statistical significance of this model was evaluated by the F-test (Table 5), which revealed that this regression is statistically significant at 95% probability level. The model did not show lack of fit and the determination coefficient ($R^2 = 0.89$) indicates that 89% of the variability can be explained by the model.

A detailed presentation of the optimum value predicted from the results using the response surface model

Table 3. Matrix for central composite design ($2^2$with 3 centre points and centred face) and experimental results.

Run	x_1	x_2	T (°C)	[E] (g)	Y (%)
1	−1	−1	37	0.2	48.08
2	+1	−1	50	0.2	7.05
3	−1	+1	37	0.4	51.41
4	+1	+1	50	0.4	14.30
5	−1	0	37	0.3	27.70
6	+1	0	50	0.3	8.97
7	0	−1	43	0.2	21.80
8	0	+1	43	0.4	100.00
9	0	0	43	0.3	57.60
10	0	0	43	0.3	58.70
11	0	0	43	0.3	49.10

Fixed conditions: butanol concentration (300 mM), butyric acid concentration (600 mM) substrate volume (20 ml); incubation time (24 h).

Table 4. Estimated effects, standard errors and Student's t test for natural logarithm of the ester yield (%) using the factorial design.

Standard design: 2^2 Number of factor: 2, Number of runs: 8 (4 face centred points) Total number of runs in experiment: 11 (3 centre points)

Main effects	t	P
x_1 = −1.44	−18.01	0.0031
x = −1.90	−16.01	0.0039
x_2 = 0.766	9.56	0.0108

Table 5. Analysis of variance for the model that represents ester yield (%) using the 2^2 factorial design.

Source	SS	DF	MS	F	P
Model	6.4675	3	2.1559	18.39	0.001
Lack of fit	0.8012	5	0.1602	16.65	0.058
Pure error	0.0192	2	0.0096		
Total	7.2880	10			

R^2 = 0.89; SS = sum of squares; DF = degrees of freedom; MS = mean square

is given in Figure 1. The values predicted by the model and represented in the surface plot indicated that the natural logarithm of the maximum esterification yield was 4.46 ± 0.17 (where 0.17 is the 90% confidence limit), corresponding to a ester yield higher than 75%, which can be attained at 41 °C for 0.4 g lipase.

The run conducted to study particular conditions arising from the results of the experimental design, attained a concentration of 32.4 g butyl butyrate/L, which corresponds to a yield of 75%.

Conclusions

In this study, full central composite design has been applied to optimize the synthesis process of butyl butyrate. A full two-factorial design has proved to be effective in the study of the influence of the process variables.

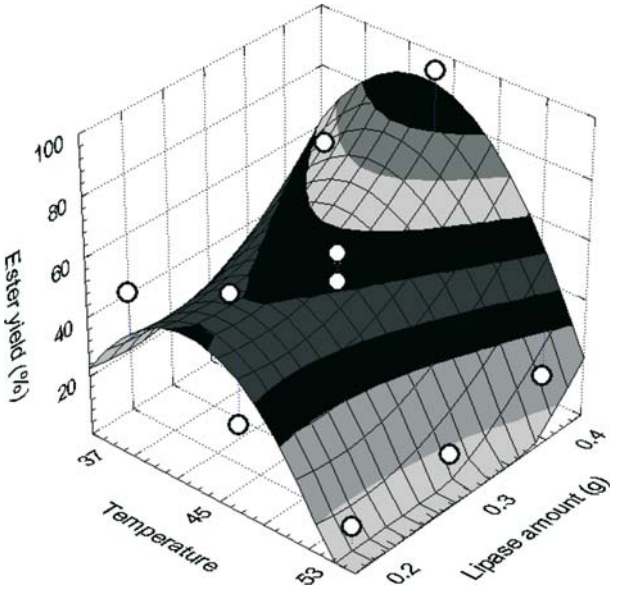

Figure 1. Response surface of the butyl butyrate yield as a function of the real values of the independent variables (temperature and lipase concentration).

Central composite design procedure has been followed to optimize the variables that determine the ester yield. A response equation has been obtained for the ester yield. From this equation, it was possible to predict adequately the operational conditions required to obtain a well-defined amount of ester. The study of the factors affecting the ester yield shows that, within the considered experimental range, the most important factors were the reaction temperature and enzyme concentration. The methodologies used (Factorial design of experiments and statistical analysis of data and the response surface methodology) well describe this process, the development and optimization of fine chemicals, leading to the development of a technological model that is simple, not limited and valid for the process scale-up.

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