Modelling and optimization of ethyl butyrate production catalysed by *Rhizopus oryzae* lipase

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**Abstract**

Response surface methodology was used to model and optimise the production of ethyl butyrate, catalysed by *Rhizopus oryzae* lipase immobilised in a hydrophilic polyurethane foam. Experiments were carried out following a central composite rotatable design, as a function of reaction temperature (T; 22–38 °C), initial butyric acid concentration (A; 0.031–0.619 M) and initial molar ratio ethanol:acid (MR; 0.257–2.443). After 48 h reaction time, the production of ethyl butyrate could be fitted to a surface described by a second-order polynomial model. A maximum ethyl butyrate concentration of 0.106 M, corresponding to 47% conversion into ester and a productivity of 2.21 μmol/mL h, is expected at initial reaction conditions of T, A and MR of 33 °C, 0.225 M and 1.637, respectively. This maximum was experimentally confirmed.

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1. Introduction

Low molecular mass esters of carboxylic acids and alcohols play an important role in the food industry as flavour and aroma constituents (Liaquat and Apenten, 2000). An example is ethyl butyrate, an important component of many fruit flavours such as pineapple, passion fruit and strawberry (Rodriguez-Nogales et al., 2005).

The use of lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) as an alternative to chemical catalysts, to catalyse esterification reactions aimed at the production of flavouring esters for food purposes, has been developed. Lipases are well known enzymes widely used in biocatalysis. Their ability to catalyse synthesis in non-aqueous media has made them extensively used to produce useful esters (Aragão et al., 2011; Dias et al., 1991; Karra-Châbouni et al., 2006; Krishna and Karanth, 2002; Liaquat and Apenten, 2000; Macedo et al., 2004; Mahapatra et al., 2009; Melo et al., 2005; Pires-Cabral et al., 2005a, 2005b, 2005b, 2007, 2009, 2010; Rodriguez-Nogales et al., 2005; Tan et al., 2011). However, lipases must be used in immobilised forms, presenting both high catalytic activity and operational stability, in order to lower the costs of the biocatalyst in the process by reusing it in batch cycles or using it in continuous reactors. Lipase immobilisation in polyurethane foams has been reported by several authors (Awang et al., 2007; Dias et al., 1991; Kawamoto et al., 1987; LeJeune and Russell, 1996; Pires-Cabral et al., 2005a, 2005b, 2007, 2009, 2010). This technique consists of coupling entrapment with chemical binding during polymer synthesis. In previous studies, *Candida rugosa* lipase immobilised in hydrophilic polyurethane foams was successfully used as a catalyst for the following reactions: esterification of ethanol with butyric acid (Dias et al., 1991; Pires-Cabral et al., 2005a, 2005b, 2007, 2009, 2010), glycerolysis (Ferreira-Dias and Fonseca, 1993, 1995a, 1995b; Ferreira-Dias et al., 2003) and hydrolysis of olive oil and olive residue oils (Ferreira-Dias and Fonseca, 1995c; Ferreira-Dias et al., 1999).

In this study, a commercial *Rhizopus oryzae* lipase was immobilised in hydrophilic polyurethane foam and tested as a biocatalyst for the production of ethyl butyrate by esterification in n-hexane. Response surface methodology (RSM) was used to model and optimise the production of ethyl butyrate, as a function of temperature (T), initial butyric acid concentration (A) and initial molar ratio ethanol/butyric acid (MR).

2. Materials

2.1. Enzyme

The commercial powdered lipase from *R. oryzae* was kindly donated by Amano, U.K. This enzyme presents a minimum activity of...
150 U/mg (one lipase unit, U, releases one μmol of fatty acid from a triglyceride in one minute at 37 °C). In hydrolysis, it is highly active from pH 6.5 to 7.5 (with an optimum pH of 7) and its optimum temperature is 40°C.

2.2. Polyurethane pre-polymer

The polyurethane foam for lipase immobilisation was prepared from a toluene diisocynate (TDI) pre-polymer (“Hypol FHP 2002™”) kindly donated by Dow Chemical Company Limited, UK. This immobilisation support presents a porosity of about 0.64 (Dias et al., 1991) and a density of 884 ± 25 kg/m³, estimated from the weight and the respective final true volume of the foam (Pires-Cabral et al., 2007).

2.3. Reagents

Butyric acid, ethanol, ethyl butyrate, n-hexane and 4-methyl 2-pentanol (used as internal standard) were analytical grade and obtained from various commercial sources.

3. Methods

3.1. Preparation of immobilised lipase

Hydrophilic polyurethane foams were prepared by mixing the polyurethane pre-polymer (0.60 g of “Hypol FHP 2002™”) with the aqueous phosphate buffer solution (0.020 M KH₂PO₄ + 0.027 M Na₂HPO₄; i.e., 0.023 M, pH 7.0, ionic strength 0.1), containing 0.35 g of lipase powder, in a ratio of 1:1 (w/w) (Ferreira-Dias et al., 1999). The amount of lipase used corresponded to the maximum load (60%, w/w) above which severe internal mass transfer limitations are encountered (Ferreira-Dias et al., 1999; Pires-Cabral et al., 2005a). After preparation, the “Hypol FHP 2002™” (or FHP 2002) foams containing immobilised lipase molecules were cut in cuboids (~0.07 cm³) and used immediately. The hydrophilic FHP 2002 foam has an aquaphilicity value of 3.2 (Pires-Cabral et al., 2005a), which is an indicator of the affinity of the immobilisation support for water (Reslow et al., 1988).

3.2. Time-course esterification experiments

The immobilised lipase was immersed in 14 cm³ n-hexane solution with an initial butyric acid concentration of 0.325 M and a molar ratio ethanol/butyric acid of 1.350. These conditions correspond to the central point of the experimental design followed for reaction modelling and optimisation (c.f. 3.3.). A load of 12% (w/v) of immobilised biocatalyst was used in the reaction medium. The esterification reaction was carried out at 30°C in a thermostated-capped cylindrical glass vessel under magnetic stirring at 1400 rev/min. In a time-course experiment, samples of 500 μL of organic medium were withdrawn along 4 h reaction time and assayed for ethanol, butyric acid and ethyl butyrate content. These samples were added to equal volumes of 0.4 M 4-methyl-2-pentanol (internal standard) in n-hexane, prior to the analysis by gas chromatography, as previously described (Pires-Cabral et al., 2005a). The initial esterification rate was calculated by linear regression on five data-points (time, ester concentrations). Volumetric productivity was calculated by the ratio between ethyl butyrate concentration and reaction time. The conversion into ethyl butyrate was defined as the ratio between ethyl butyrate concentration and the initial concentration of the limiting substrate in the organic medium.

3.3. Experimental design experiments

Response Surface Methodology (Gacula and Singh, 1984; Haaland, 1989) was used to model and optimise the esterification of ethanol with butyric acid, catalysed by the lipase from R. oryzae immobilised in polyurethane foam. The effect of the temperature (T), initial butyric acid concentration (A) and initial molar ratio eth-anol/butyric acid (MR) on ester production (ESTER) was investigated. A total of 17 esterification experiments (3 central points, 4 factorial points and 4 stars points) were carried out following a central composite rotatable design (CCRD), where a five-level space filling design was used (Gacula and Singh, 1984; Montgomery, 2000). The standard deviation of the central point provides an independent estimate of the experimental error. The coded and decoded levels considered in the CCRD are presented in Table 1 and each independent variable was tested within the following ranges: T varied from 21.6 to 38.4 °C, A from 0.031 to 0.619 M and MR from 0.257 to 2.443. The experiments were performed in a thermostated-capped cylindrical glass vessel under magnetic stirring at 1400 rev/min, for 48 h, as previously described (c.f. 3.2.).

3.4. Microenvironmental substrate concentrations

For each experiment of CCRD, the substrate concentrations in the microenvironment of the biocatalyst were estimated (Table 1) using the models previously established for a similar system, where the lipase from Candida rugosa immobilised in the same foam was used as a biocatalyst (Pires-Cabral et al., 2005a).

3.5. Statistical Analysis

For every experiment of CCRD, the ethyl butyrate concentration (ESTER) was analysed using the software “Statistica™”, version 5, from Statsoft, Tulsa, USA. Linear and quadratic effects of the independent variables and their linear interactions on ESTER were calculated and their significance was evaluated by analysis of variance. A four-dimensional surface, described by a second-order polynomial equation as a function of the three independent variables, was fitted to ESTER values. First- and second-order coefficients of this equation are usually unknown and, therefore, were estimated from the experimental data by using the statistical principle of least squares. The fit of the models was evaluated by the determination coefficients (R²) and adjusted R² (R²adj) (Gacula and Singh, 1984; Weisberg, 1985). The R² value provides a measure of how much of the variability in the observed response values can be explained by the experimental factors and their interactions. The R²adj takes into account that the number of residual degrees of freedom in the polynomial regression changes as the order of the polynomial changes. R²adj is an unbiased estimate of the coefficient of determination and is always smaller than R². In practice, R² should be at least 0.75 or greater; values above 0.90 are considered to be very good (Haaland, 1989).

3.6. Validation of the esterification model

To study the applicability of the model established by RSM to describe ester production, an esterification experiment was carried out in triplicate under the predicted optimised initial conditions. Along 48 h reaction time, samples of 500 μL of organic medium were taken and assayed for substrates and product as previously described (c.f. 3.2.) and the obtained results were compared to the theoretical values predicted by the model.
### 4. Results and discussion

The time-course of the esterification reaction catalysed by commercial *R. oryzae* lipase immobilised in polyurethane foam, tested under the conditions of the central point of the experimental design performed for reaction modelling, is shown in Fig. 1. After 48 h reaction time, quasi-equilibrium was attained and the conversion into ethyl butyrate reached 32%. The initial esterification rate and the volumetric productivity obtained were 148 μmol/h/g lipase and 2.13 μmol/mL/h, respectively.

#### 4.1. Modelling ethyl butyrate production

The ester production obtained in the 17 esterification experiments of the CCRD, catalysed by the commercial lipase from *R. oryzae* immobilised in polyurethane foam, is shown in Table 1. To model the esterification reaction and optimise the temperature and initial medium composition, linear and quadratic effects of *T*, *A* and MR and the respective linear interactions (*T* × *A*), (*T* × MR) and (*A* × MR), on ethyl butyrate concentration were calculated (Table 2). For a given factor (*T*, *A* or MR), a positive or negative linear effect on a response (ESTER) indicates that an increase in the value of that factor is accompanied by a corresponding increase or reduction in the response, respectively. ANOVA results are presented in Table 3.

The observed experimental data (ESTER) could be fitted to a four-dimensional response surface described by a second-order polynomial equation, as a function of the factors with important effects on them. The significant effects (*p < 0.05*), and those having a confidence range smaller than the standard deviation, were included in the model equation (Haaland, 1989). Therefore, the quadratic effect of *T* on ESTER was ignored.

This 4-dimensional response surface is presented in three three-dimensional response surfaces, as a function of two factors (temperature and butyric acid concentration or molar ratio and butyric acid concentration), keeping the third factor constant at its central value (i.e., MR = 1.35 or A = 0.325 M or *T* = 30 °C, respectively) (Fig. 2). This convex response surface fitted to the production of ethyl butyrate (ESTER) showed a maximum in the experimental domain and is described by the following second-order polynomial equation:

\[
\text{ESTER} = 0.0786 - 0.0064T + 1.681A - 0.8096A^2 - 0.0745MR
\]

\[
-0.03447MR^2 - 0.02847A + 0.00787MR - 0.2752A^2
\]

The high values of both *R*² (0.802) and *R*ₐδ² (0.604) show a good fit of the model to the experimental points.

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**Table 1**
Coded and decoded central composite rotatable design (CCRD) matrix as a function of reaction temperature (*T*), the initial butyric acid concentration (*A*) and the molar ratio ethanol/butyric acid in the organic medium (MR), the respective ethanol (EtOH) and butyric acid (*A*) estimated concentrations in the microenvironment of the lipase (Cmicr), and the experimental results of ethyl butyrate production (ESTER) after 48 h reaction time.

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Coded matrix</th>
<th>Decoded matrix</th>
<th>Cmicr (M)</th>
<th>EtOH</th>
<th>A</th>
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<tbody>
<tr>
<td><em>T</em></td>
<td><em>A</em></td>
<td>MR</td>
<td><em>T</em> (°C)</td>
<td><em>A</em> (M)</td>
<td>MR</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>35</td>
</tr>
</tbody>
</table>

n.a – not applicable.

**Table 2**
Linear and quadratic effects and respective *p*-levels (values between brackets) of the reaction temperature (*T*), the initial butyric acid concentration (*A*), the molar ratio ethanol/butyric acid (MR) and the respective interactions (*T* × *A*), (*T* × MR) and (*A* × MR) on the production of ethyl butyrate (ESTER) catalysed by *Rhizopus oryzae* lipase immobilised in FHP 2002.

<table>
<thead>
<tr>
<th>Factor</th>
<th>ESTER</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T</em> (linear term)</td>
<td>–0.0521 (0.071)</td>
</tr>
<tr>
<td><em>T</em> (quadratic term)</td>
<td>0.0031 (0.896)</td>
</tr>
<tr>
<td>A (linear term)</td>
<td>–0.024 (0.311)</td>
</tr>
<tr>
<td>A (quadratic term)</td>
<td>–0.048 (0.075)</td>
</tr>
<tr>
<td>MR (linear term)</td>
<td>–0.031 (0.177)</td>
</tr>
<tr>
<td>MR (quadratic term)</td>
<td>–0.028 (0.263)</td>
</tr>
<tr>
<td><em>T</em> × A</td>
<td>–0.04 (0.209)</td>
</tr>
<tr>
<td>T × MR</td>
<td>0.050 (0.173)</td>
</tr>
<tr>
<td>A × MR</td>
<td>–0.062 (0.061)</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Time-course of the production of ethyl butyrate (ESTER) by esterification of ethanol (EtOH) with butyric acid (A), catalysed by *Rhizopus oryzae* lipase immobilised in FHP 2002 foam, under the conditions of the central point of the experimental design.
Substrate concentrations in the microenvironment of R. oryzae lipase immobilised in FHP 2002 were also estimated (Table 1) using the models previously established for a similar system with a lipase from C. rugosa immobilised in the same polyurethane foam (Pires-Cabral et al., 2005a), assuming that similar interactions between the lipase and the immobilisation support occurred. However, the model equation proposed to estimate ethanol concentration (EtOH) in the microenvironment could not be used for the experiment 11 of CCDR (A = 0.031 M and EtOH = 0.042 M), because those models were established for a different range of initial concentrations of A (0.078 to 0.572 M) and EtOH (0.105 to 1.0 M).

For all the tested experiments, the substrate concentrations in the microenvironment were higher than those observed in the bulk, with the exception for experiment 13 of the CCDR (A = 0.325 M and EtOH = 0.084 M). Therefore, as previously observed (Pires-Cabral et al., 2005a), in the presence of high initial butyric acid concentration and an excess of ethanol, the migration of substrates for biocatalyst microenvironment is promoted due to the high hydrophilicity of the support.

Also, high concentration of substrates in microenvironment, particularly ethanol, in the experiments 4, 8, 12 and 14 of CCDR (Table 1), conducted to low values of ethyl butyrate concentration, suggesting that ethanol has an inhibitory effect on the activity of R. oryzae immobilised lipase. Similar results were reported for C. rugosa lipase immobilised in the same foams, under high ethanol concentrations in the microenvironment [Pires-Cabral et al., 2005a, 2007, 2010]. Also, as suggested by Manjón et al. (1991), a decrease of biocatalyst activity may occur due to dehydration of the enzyme protein induced by the ethanol in the microenvironment of the enzyme.

At high microenvironment substrate concentrations, the increase of reaction temperature leads to lower values of ester production. Conversely, at low microenvironment substrate concentrations, a temperature increase conducts to high values of ethyl butyrate concentrations.

By partial differentiation of the polynomial equation for ESTER, a maximum ester production of 0.106 M is expected, after 48 h esterification reaction, at 32°C, when using 0.225 M of A and 1.637 of MR. Also, under these predicted optimised conditions, substrate concentrations in the microenvironment of the biocatalyst were estimated, using the model equations previously established (Pires-Cabral et al., 2005a): C_{micro}^{A} of 1.920 M and C_{micro}^{EtOH} equal to 0.644 M. The volumetric productivity and the conversion into ester, after 48 h esterification reaction, calculated with these predicted values are 2.21 μmol/mL.h and 47%, respectively.

In order to validate the model, an esterification experiment was carried out under the predicted initial optimised conditions for ester production. The time-course of the reaction is presented in Fig. 3. The obtained values were compared with the theoretical values predicted by the models. After 48 h reaction time, ethyl butyrate concentration was 0.112 ± 0.002 M, corresponding to a
conversion into ester of 50% and a volumetric productivity of 2.33 μmol/mL h. These values are similar to those predicted by the models, confirming the goodness of fit of the model to the experimental data, validating it.

4.2. Comparison with other studies

The esterification of ethanol with butyric acid catalyzed by immobilised lipases was previously described (Aragão et al., 2011; Dave and Madamwar, 2006; Dias et al., 1991; Manjón et al., 1991; Pires-Cabral et al., 2005a, 2005b, 2007, 2009, 2010; Rodriguez-Nogales et al., 2005). The experimental conditions followed and the most important results obtained in these studies are presented in Table 4. When C. rugosa lipase was immobilized in FHP 2002 foam and used as a catalyst for the esterification of equimolar amounts of butyric acid and ethanol (0.3 M), in n-hexane or n-heptane, final ester concentrations of 0.21 M and 0.23 M, corresponding to volumetric productivities of 8.58 μmol/mL h and 9.63 μmol/mL h, were obtained, respectively (Dias et al., 1991). When the same biocatalyst was used at 30 °C for ethyl butyrate production, using 0.35 M of butyric acid and 0.52 M of ethanol, 0.243 M of ester was obtained after 24 h reaction time, corresponding to 69.5% of conversion into ester and a volumetric productivity of 10.1 μmol/mL h (Pires-Cabral et al., 2007). Dave and Madamwar (2006) used the same lipase immobilized on silica-gel as a catalyst for the esterification of initial equimolar substrate concentrations (0.2 M), in n-hexane at 37 °C. Under these conditions, 0.19 M of ethyl butyrate was obtained after 96 h reaction time (volumetric productivity of 1.98 μmol/mL h). Using RSM, Rodriguez-Nogales et al. (2005) predicted a maximum conversion to ethyl butyrate of 72.9%, corresponding to 0.03 M ester production, upon 96 h esterification reaction, at 34 °C in n-heptane (0.312 μmol/mL h), when a commercial immobilized preparation of Candida antarctica (Novozym 435, Novozymes, Denmark) was used with initial equimolar substrate concentrations of 0.04 M. When Rhizomucor miehei lipase immobilized in Celite was used to catalyze the esterification of 0.2 M of ethanol with 0.1 M of butyric acid in n-hexane, 98 % conversion into ester was achieved, corresponding to a volumetric productivity of 4.1 μmol/mL h (Manjón et al., 1991). Aragão et al. (2011) obtained around 88% conversion into ethyl butyrate after 3 h reaction time (26.4 μmol/mL h), with the commercial immobilized lipase from R. miehei (Lipozyme RM IM, Novozymes, Denmark). Esterification reaction was carried out at 45 °C with an enzyme concentration of 7.7 g/L in n-heptane and initial equimolar substrate concentrations of 0.09 M.

In the present study, the ethyl butyrate volumetric productivity obtained under optimized conditions is higher than the values reported by Rodriguez-Nogales et al. (2005) and by Dave and Madamwar (2006) but considerably lower than the values attained with C. rugosa lipase immobilized in polyurethane (Dias et al., 1991; Pires-Cabral et al., 2007) or with the commercial immobilized lipase from R. miehei, Lipozyme RM IM (Aragão et al., 2011). In a forthcoming work, polyurethane foams with lower hydrophobicity will be tested as supports for R. oryzae lipase in order to avoid substrate inhibitory concentrations in the microenvironment and promote ester production.

5. Conclusions

The present study shows that (i) the R. oryzae lipase immobilized in polyurethane foams can be used as a biocatalyst for the production of ethyl butyrate, (ii) the optimal reaction conditions for ethyl butyrate production may be achieved through the use of response surface models and that, (iii) in optimization, special attention must be given to the inhibitory effect of ethanol on this lipase.

References


Table 4

Volumetric productivities and ethyl butyrate concentrations, reported by several authors, obtained by esterification of ethanol (EtOH) with butyric acid (A) using other biocatalysts and under different reaction conditions and the corresponding results of the present work.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Biocatalyst</th>
<th>Solvent</th>
<th>Substrate concentrations (M)</th>
<th>Ester concentration (M)</th>
<th>Ester conversion (%)</th>
<th>Volumetric productivity (μmol/mL h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The present work</td>
<td>R. oryzae lipase</td>
<td>n-hexane</td>
<td>0.225 A; 0.368 EtOH</td>
<td>0.112</td>
<td>49.8</td>
<td>2.33</td>
</tr>
<tr>
<td>Aragão et al. (2011)</td>
<td>Lipozyme RM IM</td>
<td>n-heptane</td>
<td>0.09 A; 0.09 EtOH</td>
<td>0.079</td>
<td>87.8</td>
<td>26.4</td>
</tr>
<tr>
<td>Dave and Madamwar (2006)</td>
<td>C. rugosa lipase immobilised on silica-gel</td>
<td>n-heptane</td>
<td>0.2 A; 0.2 EtOH</td>
<td>0.190</td>
<td>95.0</td>
<td>1.98</td>
</tr>
<tr>
<td>Dias et al. (1991)</td>
<td>C. rugosa lipase immobilised in FHP 2002</td>
<td>n-heptane</td>
<td>0.3 A; 0.3 EtOH</td>
<td>0.210</td>
<td>70.0</td>
<td>8.58</td>
</tr>
<tr>
<td>Manjón et al. (1991)</td>
<td>R. miehei lipase immobilised in Celite</td>
<td>n-heptane</td>
<td>0.1 A; 0.2 EtOH</td>
<td>0.098</td>
<td>98.0</td>
<td>4.10</td>
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<tr>
<td>Pires-Cabral et al. (2005a, 2005b, 2007, 2009, 2010)</td>
<td>C. rugosa lipase immobilised in FHP 2002</td>
<td>n-heptane</td>
<td>0.35 A; 0.52 EtOH</td>
<td>0.243</td>
<td>69.5</td>
<td>10.1</td>
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<tr>
<td>Rodriguez-Nogales et al. (2005)</td>
<td>Novozym 435</td>
<td>n-heptane</td>
<td>0.04 A; 0.52 EtOH</td>
<td>0.030</td>
<td>75.0</td>
<td>0.312</td>
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