Journal of Food Engineering 115 (2013) 475–480

Contents lists available at SciVerse ScienceDirect

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng

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

C. Grosso [a], S. Ferreira-Dias [b], P. Pires-Cabral [a,b,*]

[a] Instituto Superior de Engenharia, Universidade do Algarve, Campus da Penha, 8005-139 Faro, Portugal
[b] Instituto Superior de Agronomia, CEER-Biosystems Engineering, Technical University of Lisbon, Tapada da Ajuda, 1349-017 Lisboa, Portugal

ARTICLE INFO

Article history:
Available online 13 August 2012

Keywords:
Ethyl butyrate
Immobilised lipase
Rhizopus oryzae
Modelling
Optimisation
Polyurethane foam

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 μmole/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.

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-Châabouni 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, 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

Abbreviations: A, initial butyric acid concentration in the organic medium (M); CCRD, central composite rotatable design; *Cmicro*, initial substrate concentration in the microenvironment of the lipase (M); ESTER, ethyl butyrate concentration in the organic medium (M); EtOH, initial ethanol concentration in the organic medium (M); FHP 2002, foamable hydrophilic polyurethane pre-polymer "HYPOL FHP 2002™" from Dow Chemicals, UK; MR, initial molar ratio ethanol/butyric acid in the organic medium; R^2, coefficient of determination; R^2_{adj}, adjusted coefficient of determination; RSM, response surface methodology; U, one lipase unit.

* Corresponding author at: Instituto Superior de Engenharia, Universidade do Algarve, Campus da Penha, 8005-139 Faro, Portugal. Tel.: +351 289 800100; fax: +351 289 888405.

E-mail addresses: ppcabral@sapo.pt, pcabral@ualg.pt (P. Pires-Cabral).

0260-8774/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jfoodeng.2012.08.001

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 diisocyanate (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/m^3, 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-penthanol (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 KH_2PO_4 + 0.027 M Na_2HPO_4; 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 corresponds 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 cm^3) 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 cm^3 *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 48 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-penthanol (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 ethanol/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 (R^2) and adjusted R^2 (R^2_{adj}) (Gacula and Singh, 1984; Weisberg, 1985). The R^2 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 R^2_{adj} takes into account that the number of residual degrees of freedom in the polynomial regression changes as the order of the polynomial changes. R^2_{adj} is an unbiased estimate of the coefficient of determination and is always smaller than R^2. In practice, R^2 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.