Biochemical Engineering Journal 48 (2010) 246–252

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

# Esterification activity and operational stability of *Candida rugosa* lipase immobilized in polyurethane foams in the production of ethyl butyrate

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ARTICLE INFO

*Article history:*
Received 7 April 2009
Received in revised form
20 September 2009
Accepted 27 October 2009

*Keywords:*
Operational stability
Immobilized lipase
Polyurethane foams
Batch reactor
Continuous packed-bed reactor

ABSTRACT

Ethyl butyrate is a fruity flavor ester widely used in food and pharmaceutical products. The synthesis of ethyl butyrate in *n*-hexane, catalyzed by *Candida rugosa* lipase immobilized in two hydrophilic polyurethane foams ("HYPOL FHP 2002" and "HYPOL FHP 5000") was performed. In this study, the effects of (i) the immobilization supports, (ii) the initial substrate concentrations and (iii) the water content of the system, on the activity and operational stability of *C. rugosa* lipase in both foams, during the esterification in continuous packed-bed reactor (PBR) and in repeated batches, were investigated. When low substrate concentrations were used, no deactivation was observed for both biocatalysts, along the continuous 30-d PBR operation. Conversely, under high substrate concentrations, a fast deactivation of the biocatalysts was observed. In consecutive batches, the deactivation was faster for the lipase in the less hydrophilic foam ("FHP 5000") with a half-life of 53 h against 170.3 h for the other counterpart. Water molecules in the microenvironment did not present a deactivation effect on the biocatalysts. The low operational stability can be ascribed to the inhibitory effect of ethanol, which tends to accumulate inside the foams.



## 1. Introduction

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3) constitute one of the most important groups of biocatalysts. In spite of their high catalytic activity, lipases are inherently labile. Many attempts have been made to improve the catalytic activity and operational stability of the industrial enzymes in order to make the enzymatic processes competitive with the chemical processes. Lipase immobilization is known to allow better operation control, easier product recovery, flexibility of reactor design and, in some cases, enhanced storage and operational, thermal and conformational stability. In fact, the activity and operational stability of a biocatalyst depend on several parameters such as the enzyme itself, the type of support and immobilization method, as well as medium reaction conditions.

Polyurethanes are well known for their versatility and ability to immobilize enzymes. Encouraging results, in terms of catalytic activity and operational stability, have been achieved on the hydrolysis, esterification and interesterification reactions catalyzed by lipases immobilized in these supports [1–11].

Also, the bioreactor configuration and operation mode will affect the operational stability of the biocatalysts. In batch stirred tank reactors (BSTRs), the change in substrate and product concentrations with reaction time is similar to the change occurred in packed-bed reactors (PBRs) with reactor length. In addition, PBRs are the preferred reactors in the presence of product inhibition, substrate activation and reaction reversibility, because the enzyme contacts with high substrate and low product concentrations [12].

In this study, the synthesis of ethyl butyrate was carried out by esterification in *n*-hexane, at 30 °C, catalyzed by the lipase from *Candida rugosa* immobilized in two polyurethane foams with different hydrophilicities ("FHP 2002" and "FHP 5000" foams). In these systems, the free water molecules, remaining from polyurethane polymerization and produced in the reaction, as well as the substrates, tend to migrate towards the microenvironment and accumulate inside the foams, while the produced ester migrates to the organic medium [6,7].

The focus of the present article is to investigate the effects of (i) the immobilization supports, (ii) the initial substrate concentrations and (iii) the water content of the system, on the activity and operational stability of *C. rugosa* lipase in "FHP 2002" and "FHP 5000" foams, during the esterification reaction in continuous PBR and in repeated batches.

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doi:10.1016/j.bej.2009.10.021

**Nomenclature**

| | |
|---|---|
| $a_w$ | thermodynamic water activity |
| $A$ | kinetic deactivation constant (%) |
| $A_n$ | residual activity (%) of the biocatalyst at batch $n$ |
| $BA$ | butyric acid concentration (M) |
| $BA_{micro}$ | microenvironmental butyric acid concentration (M) |
| BSTR | batch stirred tank reactor |
| CSTR | continuous well stirred tank reactor |
| $ESTER$ | ethyl butyrate concentration (M) |
| $EtOH$ | ethanol concentration (M) |
| $EtOH_{micro}$ | microenvironmental ethanol concentration (M) |
| "FHP 2002" | foamable hydrophilic polyurethane pre-polymer "Hypol FHP 2002™" from Dow Chemicals, UK |
| "FHP 5000" | foamable hydrophilic polyurethane pre-polymer "Hypol FHP 5000™" from Dow Chemicals, UK |
| $k_d$ | deactivation rate constant ((batch number)$^{-1}$) |
| $K_{EtOH}$ | ethanol constant (M) |
| $K_{EtOH_{micro}}$ | ethanol constant in the microenvironment (M) |
| $K_{I,EtOH}$ | ethanol inhibition constant (M) |
| $K_{I,EtOH_{micro}}$ | ethanol inhibition constant in the microenvironment (M) |
| PBR | packed-bed reactor |
| $t_h$ | half-life time of biocatalyst |

## 2. Materials

### 2.1. Enzyme

The lyophilised *Candida rugosa* lipase (lipase AY 30) was a generous gift from Amano Enzyme Europe Ltd., UK.

### 2.2. Immobilization matrix

The hydrophilic polyurethane pre-polymers ("Hypol FHP 2002™" and "Hypol FHP 5000™"), for lipase immobilization, were kindly donated by Dow Chemical Company Limited, UK. "Hypol FHP 2002™" is a toluene diisocyanate (TDI) pre-polymer and "Hypol FHP 5000™" contains diphenylmethane-4,4′-diisocyanate (4,4′-MDI) groups. "Hypol FHP 2002™" foams are more hydrophilic than "Hypol FHP 5000™" [6–8].

### 2.3. Reagents

Butyric acid (*BA*), ethanol (*EtOH*), ethyl butyrate (*ESTER*), *n*-hexane and 4-methyl 2-penthanol (used as internal standard) were analytical grade and obtained from various commercial sources; HYDRANAL® - Coulomat AG-H was from Riedel-de-Häen, Germany.

## 3. Methods

### 3.1. Preparation of immobilized lipase

Hydrophilic polyurethane foams were prepared by mixing the polyurethane pre-polymer (0.60 g of "Hypol FHP 2002™" or 0.35 g of "Hypol FHP 5000™") with the aqueous phosphate buffer solution (0.020 M $KH_2PO_4$ + 0.027 M $Na_2HPO_4$; pH 7.0), containing lipase powder (0.35 g or 0.30 g for "Hypol FHP 2002™" and "Hypol FHP 5000™" foams, respectively), in a ratio of 1:1 (w:w), as previously described [4,6–8].

### 3.2. Operational stability tests

#### 3.2.1. Continuous packed-bed reactor

A continuous packed-bed reactor, consisting of a thermostated glass column (1.6 cm in internal diameter and 20 cm in height) was tested for the esterification, at 30 °C. An amount of 1560 mg lipase in "FHP 2002" and "FHP 5000" foams was used. The "FHP 2002" or "FHP 5000" foams containing immobilized lipase molecules were cut in cuboids (~0.07 $cm^3$) and PBR was filled with the biocatalyst (bed volume of 14.2 $cm^3$). Under these conditions, the biocatalyst load was about 60 mg of lipase/mL of reaction medium circulating throughout the immobilized bed. The biocatalysts were used with their original water activity ($a_w$ = 0.98), which showed to favor the esterification reaction [6]. Reaction media were continuously pumped upwards at a flow rate of 0.1 mL/min, which leads to a residence time of about 260 min. When a quasi steady-state was reached, after the start-up of the reactor (a time equal to at least 1.5 residence time) and along the continuous PBR operation, 500 μL effluent samples were taken and assayed for ethanol (*EtOH*), butyric (*BA*) 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 [7,8]. The conversion into ester was calculated as the ratio of ethyl butyrate concentration and the initial concentration of the limiting substrate (in percentage). The ester conversion, obtained when a quasi steady-state was reached, was considered as the initial activity of the biocatalyst. The observed activities were compared to the initial activity for operational stability assessment. From the knowledge of the amount of ester produced, productivities were calculated in μmol/mL h of ester produced and specific productivities in μmol/mL h mg of lipase.

Reaction Medium I (0.35 M *BA* and 0.53 M *EtOH*, in *n*-hexane) was used in the reactor filled with the immobilized lipase in "FHP 2002" foams. This medium presents the composition previously optimized by Response Surface Methodology to maximize ester production, when the lipase is used in FHP 2002 foams [8]. Also, a reaction medium containing low substrate concentrations (Reaction Medium II: 0.078 M of *BA* and 0.105 M of *EtOH*) was tested for both biocatalyst preparations. In previous studies carried out batchwise [13], the highest conversion into ester was observed, when this medium was used.

#### 3.2.2. Repeated batch reactors

The operational stability of the immobilized lipases in "FHP 2002" and "FHP 5000" foams, was also assayed in 12 successive batches carried out at 30 °C in a batch reactor consisting of a thermostated cylindrical glass vessel (25 mL) closed with rubber stopper under magnetic stirring at 1400 rev/min.

The "FHP 2002" or "FHP 5000" foams containing 0.35 g or 0.30 g of immobilized lipase molecules, respectively (c.f. Section 3.1), were immersed in 12 $cm^3$ of *n*-hexane solution of 0.35 M of *BA* and 0.53 M of *EtOH* (Reaction Medium I) or 0.53 M *BA* and 0.40 M of *EtOH* (Reaction Medium III), respectively. As for reaction medium I, the composition of reaction medium III corresponds to that previously optimized by Response Surface Methodology to maximize ester production, when the lipase in FHP 5000 foams is used [8].

Along the 47-h reaction time, samples of 500 μL were taken to assay for substrates and ester concentrations in bulk solution. At the end of each batch, the immobilized lipases were removed by paper-filtration from the reaction mixture and rinsed twice with *n*-hexane to remove substrate molecules retained in the matrix. One hour later (length of time required for evaporation of the solvent), foams were immersed in fresh medium and reused. The biocatalyst activities were estimated at the end of each batch as the conversion into ester. The first batch was used as the reference (100% activity). The residual activity (in percentage) of the biocatalyst at the end of