Journal of Molecular Catalysis B: Enzymatic 75 (2012) 50–59

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Covalently immobilized lipase catalyzing high-yielding optimized geranyl butyrate synthesis in a batch and fluidized bed reactor

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ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 4 November 2011
Accepted 8 November 2011
Available online 17 November 2011

Keywords:
Geranyl butyrate
Covalent immobilization
Enzymatic esterification
Fluidized bed reactor
Optimization study

ABSTRACT

Three commercially available polymers (Sepabeads® EC-EP, Sepabeads® EC-HA and Purolite® A-109) were tested for potential application as supports for covalent immobilization of lipase from *Candida rugosa* by analyzing some critical properties of immobilized enzymes such as enzyme loading, activity and activity immobilization yield. Among them, lipase covalently immobilized on Sepabeads® EC-EP via epoxy groups appeared to show the best performance in a standard hydrolytic reaction. Therefore, it was selected and assayed in the esterification of butyric acid and geraniol to produce geranyl butyrate, first in a batch system followed by continuous geranyl butyrate synthesis in a fluidized bed reactor, as one being potentially applicable for large-scale production.

Based on statistical analysis, optimal conditions for the production of geranyl butyrate by selected, immobilized lipase in the batch system are recommended as: temperature at 25–30 °C, water concentration at 3.6% (v/v) and acid/alcohol molar ratio at 2.5. A set of optimal conditions for the ester synthesis in a fluidized bed reactor system has also been determined, specifically, flow rate at 10 mL min^{-1}, temperature at 35 °C, water concentration at 2% (v/v), substrate concentration at 0.1 M and acid/alcohol ratio at 2.0. Implementation of the optimized parameters in a batch system and in a fluidized bed reactor enabled production of target ester with high molar conversion, at > 99.9% for 48 h in the batch process, and 78.9% for 10 h in fluidized bed reactor. Although when assayed at their optimal conditions, lower molar conversion was achieved in the fluidized bed reactor system compared to the batch system, the volumetric productivity in fluidized bed reactor was more than five fold higher than that obtained in the batch system.

1. Introduction

Candida rugosa lipase (CRL) is a versatile, robust, frequently used lipase since it can be easily produced in lyophilized form and efficiently prepared in large amounts. Reports have been made describing successful CRL catalyzed synthesis of terpene esters [1–3]. In industrial scale, aromatic terpene esters are currently synthesized in a nonspecific chemical process obtaining low yields and poor quality of the product [4,5]. Also, chemical processes demand high costs for additional separation and purification steps [6]. Besides higher product yields, mild operating conditions, synthesis of products that do not need further purification, enzymatic reaction delivers "natural product", in terms of its origin, which is of a high importance, especially in the food industry [7].

However, industrial-scale synthesis using soluble enzymes is economically unacceptable, since these enzymes lack reusability as well as possibility of continuous type synthesis due to their low stability and complex separation techniques [8]. One way to address these issues is enzyme immobilization.

Covalent immobilization provides formation of a very stable catalyst via multipoint covalent attachment by maintaining enzyme's active conformation and reducing denaturing effects of environmental factors [9]. Among materials used for covalent immobilization, Sepabeds® EC series show physical and chemical stability, high protein binding capacity, low swelling tendency in

Abbreviations: CRL, *Candida rugosa* lipase; FBR, fluidized bed reactor; CCRD, central composite rotatable design.

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doi:10.1016/j.molcatb.2011.11.009

high molar solutions and resistance to the microbial degradation, making them suitable for application in industrial bioprocesses [10–13]. Specifically, Sepabeads® EC-EP particles are shown to efficiently stabilize penicillin G acylase providing hundreds-fold more stable catalyst compared to the one attached to Eupergit® C [14]. Hilterhaus et al. successfully immobilized three industrially important enzymes, endoglucanase, benzoylformate decarboxylase from *Pseudomonas putida* and lipase from *Candida antarctica* on Sepabeads® EC-EP, EC-EA and EC-BU, producing stable and active enzymes suitable for industrial application [15].

The selection of a proper reactor configuration is another important aspect in designing industrial enzymatic synthesis. Fluidized bed reactors (FBRs) are widely and successfully used in many industrial processes, like aerobic fermentation processes, catalytic reactions and biological waste-water treatment, due to continuous operational mode as well as for improved heat and mass transfer [16]. However, information on their application for ester synthesis is currently rather limited in the literature. The majority of studies concerning ester synthesis focused on batch systems and packed bed reactors [17], while ester synthesis in FBRs remains yet unexplored.

In studies conducted so far, geranyl butyrate was produced in yields between 85 and 99.9% using free or immobilized lipases [5,18–20]. Recently, it was shown that CRL immobilized on Sepabeads® EC-EP can be employed as a robust biocatalyst in esterification of geraniol with butyric acid in a low aqueous system [21]. However, these studies have been generally performed in the flasks with magnetic stirring or in the vials immersed in an orbital shaker. Analysis of the effect of reactor configuration and hydrodynamic conditions on the reaction rate and enzyme stability was not included in these contributions. To the best of our knowledge, this report presents the first study of the parameters affecting the synthesis of geranyl butyrate with the immobilized lipase in FBR system.

Previous research of Saponjic et al. regarded application of CRL immobilized on Sepabeads® EC-EP for amyl caprylate synthesis in FBR [22]. The study revealed that both batch and continuous synthesis of amyl caprylate can be accomplished in a high yield. Continuous esterification had slightly improved kinetics since 90.2% yield was achieved within 14 h compared to the batch synthesis where almost complete conversion was observed within 24 h [22]. As a further research, we aimed to prove robustness and versatility of immobilized CRL and efficiency of the reaction optimization pathway by employing them for geranyl butyrate synthesis. Using a different reaction model which includes acid substrate of three fold higher polarity and synthesis of a higher polarity ester would pose additional limitations to the ongoing esterification, since lipase activity is known to be affected by changes in substrate partitioning between organic phase and water layer surrounding the enzyme [23,24].

Focus was set on designing an economical fluidized-bed-immobilized-enzyme system for geranyl butyrate synthesis employing an efficient and inexpensive commercial support and immobilization method. In the first part of the study, characteristics of CRL covalently immobilized on Sepabeads® EC-EP have been compared to those obtained with other two commercial supports. Some critical properties of immobilized enzymes such as protein loading, activity, specific activity and immobilization yield were considered. Selected highly active immobilized lipase was used for statistical assessment of relevant process conditions in the batch system and in further study as a biocatalyst and constituent of the FBR. The bioreactor hydrodynamic characteristics and the reaction conditions have been investigated in order to improve the process performance.

2. Experimental

2.1. Materials

Nonspecific lipase AY, Type VII, L 1754, in powdered form from *C. rugosa*, lipase substrate (stabilized olive oil emulsion), Triton X-100, bovine serum albumin (BSA) and terpene alcohol, geraniol (98.0%) were purchased from Sigma–Aldrich Inc. St. Louis, MO, USA. Butyric acid from Merck, Darmstadt, Germany was used as acid substrate while isooctane (HPLC grade) from Arcos Organics, New Jersey, USA, was used as organic solvent. Sepabeads® EC-EP and Sepabeads® EC-HA were donated by Resindion S.R.L., Mitsubishi Chemical Corporation, Milan, Italy. Purolite® A-109 was purchased from Purolite International Ltd. (Llantrisant, United Kingdom). All other chemicals were purchased from Merck, Darmstadt, Germany.

2.2. Immobilization method

Immobilization of CRL on epoxy-Sepabeads® (EC-EP): Immobilization of CRL on epoxy-activated support, Sepabeads® EC-EP, was achieved by direct lipase coupling to the polymer via epoxy groups in the presence of very high salt concentration (standard protocol recommended by Resindion S.R.L., Mitsubishi Chemical Corporation). Immobilization was performed in 70 mL of 1.25 M potassium phosphate buffer, pH = 8.0 at 22.5 °C with orbital mixing for 48 h. Immobilized enzyme was then washed with water and buffer and kept at 4 °C followed by drying *in vacuo* for 48 h, prior to the reaction.

Immobilization of CRL on Sepabeads® EC-HA and Purolite® A-109: The immobilization procedure on amino-supports consisted of two steps: (1) oxidation of the lipase by sodium periodate; and (2) coupling of the oxidized enzyme to the amino-supports.

Therefore, first step was the lipase oxidation by sodium periodate following the methodology previously described [25]. According to this protocol, 1 mg mL^{-1} of crude enzyme solutions, corresponding to 0.25 mg mL^{-1} of pure protein determined by the Bradford method [26] were incubated in 5 mM solution of sodium periodate in sodium acetate buffer, pH = 5.0, for 6 h in the dark at 4 °C. The reaction mixture was stirred occasionally and the reaction was quenched with 10 mM ethylene glycol for 30 min. To remove by-products, the oxidized lipase solution was then dialyzed against 50 mM sodium acetate buffer, pH = 5.0, for 18 h.

Polymers with amino groups (1.0 g) were incubated with 35 mL of oxidized lipase solution in sodium acetate buffer at pH = 5.0 and 4 °C for 48 h. Afterwards, obtained biocatalysts were washed with water and sodium phosphate buffer, pH = 7.0 and stored in the same buffer at 4 °C until use. In previous research, it was reported that Schiff's bases formed between oxidized enzyme and aminated supports proved as very stable, therefore, additional reduction step was not applied to the immobilized preparations [25,27]. The lipase oxidation was checked using FT-IR spectrometry as described bellow.

2.3. Immobilization parameters

Protein loading, P_g defined as the amount of the pure protein coupled to the supports (mg of protein/g of the supports) is calculated as a difference between protein amount (mg) added in the immobilization process and the protein amount (mg) found in the filtrate and wash-through after immobilization. The protein loading efficiency, Y_P (%) was calculated according to Eq. (1):

$$Y_P\,(\%) = \frac{C_0V_0 - (C_fV_f + C_wV_w)}{C_0V_0} \quad (1)$$

where C_0 is the protein concentration of the initial immobilization solution (mg mL^{-1}); V_0 its volume (mL); C_f the protein concentration in the filtrate (mg mL^{-1}); V_f the filtrate volume (mL); C_w the