Journal of Molecular Catalysis B: Enzymatic 100 (2014) 78–83

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Lipase activity and enantioselectivity of whole cells from a wild-type *Aspergillus flavus* strain

Carmen Solarte[a,1], Edinson Yara-Varón[a,1], Jordi Eras[a], Mercè Torres[b], Mercè Balcells[a], Ramon Canela-Garayoa[a,*]

[a] Department of Chemistry, University of Lleida, Rovira Roure 191, Lleida 25198, Spain
[b] Department of Food Science and Technology, University of Lleida, Rovira Roure 191, Lleida 25198, Spain

ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form
18 November 2013
Accepted 9 December 2013
Available online 17 December 2013

Keywords:
Aspergillus flavus
(*R*)-1-Phenylethyl acetate
Ethyl laurate
2-Chloro-1-(chloromethyl)ethyl acrylate
2,3-Dichloro-1-propyl acrylate

ABSTRACT

This study reports the high enantiomeric preference of whole cell lipase from *Aspergillus flavus* wild-type that allows the preparation of a chiral secondary alcohol. Whole cells prepared from a wild-type *Aspergillus flavus* strain were used as biocatalysts to prepare (*R*)-1-phenylethyl acetate. (*R*)-1-Phenylethanol was esterified into (*R*)-1-phenylethyl acetate with a 94.6% enantiomeric excess (*ee*) within 24 h at 40 °C and (*S*)-1-phenylethanol remained in the reaction medium with a >99%*ee*. Besides, this biocatalyst allows the preparation of ethyl laurate and a mixture of 2-chloro-1-(chloromethyl)ethyl acrylate and 2,3-dichloro-1-propyl acrylate. The ethyl laurate yield was 96%, whereas the synthesis of a mixture of the acrylate regioisomers, 2-chloro-1-(chloromethyl)ethyl acrylate and 2,3-dichloro-1-propyl acrylate gave similar yields to those obtained using commercial lipases.

1. Introduction

Recent years have witnessed the rapid development of lipase applications for the preparation of esters and chiral alcohols. This growth, in part, is driven by EU and US legislation stating that natural flavouring and fragrance products can be prepared from natural sources only by physical processes (extraction from natural sources) or by biotechnological transformations that involve precursors isolated from nature [1]. Indeed, several biotransformation processes using either enzymes or whole cells have been implemented to conduct the kinetic resolution of 1-phenylethanol a compound used in various pharmaceutical and personal care products [2–5].

Biocatalysts have also been reported for the preparation of ethyl laurate, which has a coconut aroma [6]. A regioisomeric mixture of 2-chloro-1-(chloromethyl)ethyl acrylate and 2,3-dichloro-1-propyl acrylate (DCPA), have been used as monomers to prepare poly(dichloropropyl acrylates), which have applications in many fields [7].

Lipase-catalyzed biotransformation is one of the most popular and practical enzymatic technologies. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze a broad spectrum of reactions, such as the hydrolysis of ester bonds and transesterification and ester synthesis, at the interface between substrate and water or in non-aqueous organic solvents [8]. Consequently, lipases are used in a wide range of industrial processes, including food, chemical, pharmaceutical and detergent production. In many cases, however, these enzymes have been used in an immobilized form, which is costly and time-consuming, thus hindering the widespread use of enzymatic processes. Lipases from various microorganisms have been reported. There are two types of lipase preparation: (i) extracellular lipase, which is secreted into culture broth and (ii) intracellular or whole cell lipase, which remains either inside the cell or in the cell-wall [9]. Many of these have been purified and their properties are described. To date, several lipases are commercially available, and their applications have been extensively described [10,11]. However, while most of these are extracellular enzymes, little research has been devoted to intracellular or cell-bound lipases.

Cell-bound lipases are economically attractive because they can be produced at low cost. The biomass can be used directly, thus avoiding isolation, purification and immobilization procedures. Moreover, the biocatalyst can be easily recovered by filtration. Also,

Abbreviations: a_w, water activity; PDA, potato/dextrose/agar; *r*, specific activity; *R*-1-PEA, (*R*)-1-phenylethyl acetate; ee_s, enantiomeric excess substrate; ee_p, enantiomeric excess product; DCPA, mixture of regioisomers (2-chloro-1-(chloromethyl)ethyl acrylate and 2,3-dichloro-1-propyl acrylate); CALB, lipase B from *Candida antarctica*.

* Corresponding author. Tel.: +34 973 702843; fax: +34 973238264.
E-mail address: canela@quimica.udl.cat (R. Canela-Garayoa).
[1] These authors contributed equally to this paper.

1381-1177/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2013.12.005

naturally immobilized lipases show potential for applications. Nagy et al. [3], studied thirty-eight filamentous fungi cultivated under solid state fermentation (SSF). The majority of these preparations proved to be effective as enantiomer selective biocatalyst and some were successfully applied in preparative scale kinetic resolution of secondary alcohols.

The use of naturally immobilized lipases has been proposed in the oil and fat industry [12,13], in the preparation of flavours [1,14] and, recently, in the synthesis of biodiesel [15,16]. Recombinant microbial whole-cell biocatalysts expressing lipases have also been proposed for enantioselective transesterification in non-aqueous medium. Nevertheless, the increasing concern regarding non-natural approaches for the preparation of additives and fragrances has prompted us to look for wild microorganisms that could be used to prepare aroma and fragrance compounds.

In our attempt to obtain a new fungal cell biocatalyst from our environment, we isolated a lipolytic *Aspergillus flavus* strain from sunflower seeds collected in a sunflower mill. Several studies under submerged fermentation and solid-state fermentation has been performed to produce whole cell lipase from *A. flavus*. Its stability and activity has been reported [17–21]. Here we describe several characteristics of the whole cell lipase prepared from the isolated wild-type *A. flavus* strain. These whole cells were used as biocatalysts to prepare (*R*)-1-phenylethyl acetate, ethyl laurate and DCPA. In this study we describe the high enantiomeric preference of a wild-type whole cell lipase from *A. flavus* that allows the preparation of a chiral secondary alcohol.

2. Materials and methods

A strain of *Aspergillus flavus* was isolated from sunflower seeds and was maintained on potato/dextrose/agar (PDA) at 4 °C. The microorganism was deposited in the culture collection "Colección Española de Cultivos Tipo" (Burjassot, Valencia-Spain), reference number CECT 20475.

2.1. Reagents and solvents

Asparagine, K_2HPO_4, $MgSO_4$, glucose, thiamine hydrochloride, $Fe(NO_3)_3.9H_2O$, $ZnSO_4{\cdot}7H_2O$, isopropenyl acetate, *rac*-1-phenylethanol, (*S*)-1-phenylethanol, lauric acid, and toluene were purchased from Sigma–Aldrich (Sigma–Aldrich Quimica, S.A., Madrid, Spain). $MnSO_4{\cdot}H_2O$ was supplied by Fisher Scientific (Madrid, Spain). Oleic acid was acquired from Merck (Barcelona, Spain). Ethyl laurate, ethyl acrylate, methyl palmitate and methyl oleate were from Fluka (Sigma–Aldrich, Madrid, Spain). Ethanol and hexane were supplied by J.T. Baker (Quimega, Lleida, Spain). Isooctane and sodium carbonate were purchased from Panreac (Barcelona, Spain). 1-Propanol was from Acros Organics (Barcelona, Spain).

2.2. Microorganism, growth media, and culture conditions

A non-aflatoxigenic strain of *A. flavus*, isolated from sunflower seeds, was cultivated in a synthetic liquid medium that contained 2.0 g asparagine, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 5.0 mg thiamine hydrochloride, 1.45 mg $Fe(NO_3)_3{\cdot}9H_2O$, 0.88 mg $ZnSO_4{\cdot}7H_2O$ and 0.31 mg $MnSO_4{\cdot}H_2O$ per litre of distilled water. The pH was adjusted to 6.0 using 1 M HCl. Two hundred and fifty millilitres of the liquid medium were sterilized in a 1 L Erlenmeyer flask at 121 °C for 15 min, and 2% of refined sunflower oil was added. The medium was inoculated with 2.5 mL of a spore suspension (5×10^6 spores/mL) of *A. flavus* grown on PDA. The medium was then incubated at 28 °C for 5 days on an orbital shaker at 200 rpm.

2.3. Preparation of whole cell lipase

Mycelium obtained from the culture medium was harvested using a Buchner funnel, washed with distilled water followed by acetone, and freeze-dried for 18 h. It was then ground to powder consistency.

2.4. Equilibration of water activity (a_w)

The a_w in the experiments was set by independently equilibrating reagents, solvent and biocatalyst with aqueous saturated solutions of LiCl ($a_w = 0.12$), $MgCl_2$ ($a_w = 0.33$), K_2CO_3 ($a_w = 0.42$), $Mg(NO_3)_2$ ($a_w = 0.54$) and NaCl ($a_w = 0.75$). Separate closed containers were used for each reactant and biocatalyst [22]. Equilibration was performed at room temperature for at least 48 h. The a_w of the biocatalyst was measured using an Aqua Lab series 3TE from Decagon Devices Inc. (Pullman, WA, USA).

2.5. Biocatalyst activity

Two mL of a 0.09 M isooctane solution of oleic acid (0.05 g) containing 0.08 g of 1-propanol was stirred for 1 h at 28 °C in the presence of various amounts of whole cell lipase ($a_w = 0.12$). Samples were collected every 15 min. These samples were diluted at *ca.* 1 mg mL^{-1} using methyl palmitate as internal standard. Resulting samples were analyzed by gas-chromatography (GC-FID) as described below. All experiments were performed in triplicate.

2.6. Evaluation of substrate adsorption by the whole cell lipase biomass

Eighty mL of a 0.022 M isooctane solution of methyl oleate (500 mg) containing 3 g of whole cell lipase ($a_w = 0.12$) were stirred for 24 h at 28 °C. Samples were collected at 0, 2, 4 and 24 h. These samples were diluted at *ca.* 1 mg mL^{-1} using methyl palmitate as internal standard and analyzed by gas chromatography (GC-FID) as described below. All experiments were performed in triplicate.

2.7. Kinetic resolution of rac-1-phenylethanol

Kinetic resolution reactions were carried out using dry conditions in flame-dried glassware following a previously described method [23]. *rac*-1-Phenylethanol was dried over a molecular sieve (4 Å) before use. Isopropenyl acetate was dried over $CaCl_2$ and distilled before use. Dry toluene was dried by refluxing under nitrogen in the presence of sodium wire and benzophenone.

Whole cell lipase (25 mg) and sodium carbonate (53 mg, 0.5 mmol) were added to a vial. The corresponding *rac*-1-phenylethanol or (*S*)-1-phenylethanol (55 mg, 0.5 mmol) dissolved in dry toluene (1 mL) was added to the vial, and the mixture was stirred for 6 min. Next, isopropenyl acetate (110 μL, 1.0 mmol) was added to the reaction. Experiments were carried out at 25, 40 and 60 °C. Samples were collected between 30 min and 44 h depending on the temperature and reagent used, and then analyzed by chiral GC as described below. A blank study was conducted without using isopropenyl acetate.

2.8. Preparation of ethyl laurate

Half a mL of a solution containing 25 mg mL^{-1} (0.125 mmol mL^{-1}) of lauric acid and ethanol 11.52 mg mL^{-1} (0.250 mmol mL^{-1}) in hexane was added to a reaction vial (1.5 mL) fitted with a PTFE-lined cap. Then 20 mg of biocatalyst ($a_w = 0.54$) was added to the vial, and the mixture was stirred and heated to 40 °C for 24 h. The resulting solution was analyzed using GC-FID.