Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 99 (2008) 2116–2120
BIORESOURCE TECHNOLOGY

Transesterification of primary and secondary alcohols using *Pseudomonas aeruginosa* lipase

Manpreet Singh [a], Sawraj Singh [a], Ram Sarup Singh [b], Yusuf Chisti [c], Uttam Chand Banerjee [a,*]

[a] *Biocatalysis Laboratory, Department of Pharmaceutical Technology (Biotechnology), National Institute of Pharmaceutical Education and Research, Sector 67, S.A.S. Nagar 160 062, India*
[b] *Carbohydrate and Protein Laboratory, Department of Biotechnology, Punjabi University, Patiala, Punjab, India*
[c] *Institute of Technology and Engineering, Massey University, Private Bag 11 222, Palmerston North, New Zealand*

Received 21 February 2007; received in revised form 23 May 2007; accepted 24 May 2007
Available online 9 July 2007

Abstract

Lipases of a newly isolated *Pseduomonas aeruginosa* MTCC 5113 were assessed for transesterification of benzyl alcohol and vinyl acetate to produce the flavoring agent benzyl acetate. Crude lipase preparations that minimized the cost of the biocatalyst, achieved benzyl alcohol conversion of 89% within 3 h at 30 °C. In contrast, purified and expensive commercially available lipases of *Candida antarctica* and porcine pancreas achieved much lower conversions at 80% and 15%, respectively. A well-mixed (~800 rev · min^{-1}) batch reactor having the aqueous phase finely dispersed in heptane was used in these studies. Benzyl alcohol conversion was maximal when the enzyme-containing aqueous phase constituted about 50% of the total reactor volume. Use of solvents such as hexane, benzene, toluene and dimethyl sulfoxide reduced conversion compared with the use of heptane.

Keywords: Transesterification; *Pseudomonas aeruginosa*; Lipase; Benzyl acetate

1. Introduction

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) catalyze the hydrolysis of triacylglycerols to glycerol and fatty acids (Jaeger et al., 1999; Sharma et al., 2001). They can be used to also catalyze the reverse reaction to form ester bonds. Lipases are serine hydrolases that do not require any cofactors. Lipases are widely used for enantioselective and regioselective syntheses, resolution of chiral drugs, modification of fats and oils, and synthesis of fine chemicals such as fragrances and flavors (Jesus et al., 1995; Faber, 1997; Sharma et al., 2001; Yadav and Trivedi, 2003).

Various lipase-catalyzed transesterification reactions have been reported in which vinyl acetate as an acyl doner reacts with primary or secondary alcohols (Alder et al., 1989; Brzozowski et al., 1991; Rizzi et al., 1992; Martinelle and Hult, 1995). Here we report on lipase-catalyzed production of the flavoring agent benzyl acetate by direct transesterification in organic solvents. The lipase used was produced from an isolate of *Pseudomonas aeruginosa* that had been obtained after extensive screening and selection for enantiospecific resolution of the (±)methyl *trans*-3(4-methoxyphenyl) glycidic acid methyl ester (MPGM), an intermediate in the synthesis of cardiovascular drug diltiazem (Singh and Banerjee, 2005). The *P. aeruginosa* isolate produced enantioselective, thermostable and organic solvent stable lipase in high amounts (Sharma et al., 2003; Singh et al., 2006). The transesterification capability of the lipase was assessed using benzyl alcohol and vinyl acetate to produce benzyl acetate. Most of the studies of

* Corresponding author. Tel.: +91 172 2214682/87; fax: +91 172 2214692.
E-mail address: ucbanerjee@niper.ac.in (U.C. Banerjee).

0960-8524/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2007.05.041

this kind in the literature have relied on highly purified and expensive commercially available lipases. This work used a crude and inexpensive preparation of the lipase to successfully obtain a high conversion of the substrates at 30 °C.

2. Methods

2.1. Microorganism and chemicals

A newly isolated soil bacterium *P. aeruginosa* MTCC 5113 was used to produce the extracellular lipases (Sharma et al., 2003). The organism had been selectively isolated for its ability to enantioselectively resolve a racemic mixture of (±)methyl *trans*-3(4-methoxyphenyl) glycidic acid methyl ester (MPGM) to (−)-MPGM, as described previously (Singh and Banerjee, 2005). Minimal salt medium (MSM) consisting of disodium dihydrogen phosphate (0.2% w/v), potassium dihydrogen orthophosphate (0.1% w/v), ammonium chloride (0.04% w/v) and magnesium chloride (0.04% w/v) was used with (±)-MPGM as the sole carbon and energy source (Singh and Banerjee, 2005). Agar plates were prepared by supplementing MSM with 2 mM (±)-MPGM. The identity of the isolate was confirmed by partial sequencing of the 16s rRNA gene (Singh et al., 2006). A voucher specimen was deposited with the Microbial Type Culture Collection (MTCC), Chandigarh, India, and given the accession number MTCC 5113. The identifying gene sequence was submitted to GenBank (NCBI) under the accession number DG 104332 (Singh et al., 2006).

Various solvents used were of HPLC grade and were obtained from Ranbaxy Fine Chemicals Limited (New Delhi, India). Media components used were obtained from Himedia (Mumbai, India). *Candida antarctica* lipase was obtained from Amano Pharmaceuticals Co. (Japan) and porcine pancreas lipase was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Benzyl alcohol, vinyl acetate and benzyl acetate were purchased from Central Drug House (P) Ltd. (New Delhi, India). All other chemicals used were of analytical grade.

2.2. Enzyme production

A single colony of the microorganism maintained on nutrient agar plates (0.5% w/v peptone, 0.15% w/v yeast extract, 0.5% w/v beef extract, 0.5% w/v sodium chloride, 1.5% w/v agar, pH 8) was transferred to 50 ml sterilized (121 °C, 20-min) nutrient broth (as above, but without the agar) to produce the seed culture (Singh and Banerjee, 2005). A 1% seed inoculum was used to inoculate 100 ml of nutrient broth in 250 ml shake flasks. The flasks were incubated at 30 °C (200 rpm, 96-h). Crude supernatant of the microbial broth was separated from the cells by centrifugation (10,000-g, 10-min). The supernatant was used directly as the crude preparation of lipases.

2.3. Transesterification reactions

Lipase-catalyzed transesterification reactions were carried out in 100 ml capacity stoppered shake flasks that were magnetically agitated (800 rpm) and held at the desired constant temperature (30–60 °C in different experiments) in an incubator. The organic solvents evaluated for the reaction were hexane, toluene, heptane and benzene in separate experiments. Unless specified, the total volume of the solvent was always 30 ml and the final reaction volume was kept constant at 60 ml. As discussed later, only for heptane was the ratio of aqueous and organic phases in the reactor varied in different experiments from 10% v/v to 60% v/v. Clear liquid was sampled from shake flasks periodically for up to 3-h to quantify the products and substrates as described in Section 2.4. Other variables studied included the specific substrates used (i.e., benzyl alcohol, 1-ethylhexanol, 2-phenylethanol) and their concentrations; the enzyme activity in the reactor; and the enzyme source. Values of these variables are specified at appropriate places in the text. The study focused mainly on benzyl alcohol as the substrate. Reactions involving alcohols were carried out at conditions that had been established as optimal for the reaction between vinyl acetate and benzyl alcohol.

2.4. Enzyme assay and analytical methods

Lipase activity was quantified according to the method of Winkler and Stuckmann (1979) modified as follows. The substrate used was *p*-nitrophenyl palmitate. The substrate was dissolved in 2-propanol (3 mg/ml). An aqueous solution (9 ml) of gum arabic (0.11% w/v) and Triton X-100 (0.44% w/v) was added. Intense agitation (approx. 800 rpm, magnetic stirred) was used to emulsify the mixture. This emulsion (0.9 ml) was mixed with 1.5 ml Tris–HCl buffer (50 mM, pH 8) and 0.5 ml $CaCl_2$ (75 mM). The mixture was pre-incubated at 30 °C for 5 min and 100 μl of appropriately diluted (in 50 mM Tris HCl buffer, pH 8.0) enzyme solution was added. Incubation was continued for a further 10 min. Samples were taken periodically and optical density was measured spectrophotometrically at 410 nm against substrate solution blanks. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol/min of *p*-nitrophenol under the above specified assay conditions.

Reaction samples were dried under reduced pressure using rotary evaporator, dissolved in acetonitrile and filtered prior to analysis. Reaction products were quantified by HPLC using a Shimadzu system that consisted of LC-10AT pump, SPD-10 A UV–VIS detector and a reverse phase column (Chiracel ODH column, 0.46 mm × 250 mm, 5 μm, Diacel; Waters, USA). The products were detected at 220 nm. The mobile phase consisted of a 60:40 (v/v) mixture of acetonitrile and water. The mobile phase flow rate was $1.0\ ml \cdot min^{-1}$. Analyses were performed at 30 °C. The identity of the product was confirmed by NMR and GC-MS.