

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

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Abstract

Lipases of a newly isolated *Pseudomonas 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 ($\sim 800 \text{ rev} \cdot \text{min}^{-1}$) 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.

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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 donor 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 (\pm)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

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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 (\pm)-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 (\pm)-MPGM as the sole carbon and energy source (Singh and Banerjee, 2005). Agar plates were prepared by supplementing MSM with 2 mM (\pm)-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 CaCl₂ (75 mM). The mixture was pre-incubated at 30 °C for 5 min and 100 μ l 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 μ mol/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 \times 250 mm, 5 μ m, 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.

3. Results and discussion

The specific activity of the crude preparation of *P. aeruginosa* lipase was $893 \text{ U} \cdot \text{mg}^{-1}$ of total protein. This was substantially greater than the specific activities of purified commercial lipases of *C. antarctica* (specific activity of $859 \text{ U} \cdot \text{mg}^{-1}$) and porcine pancreas (specific activity of $300 \text{ U} \cdot \text{mg}^{-1}$). Benzyl alcohol conversion versus reaction time data during production of benzyl acetate with these lipases are shown in Fig. 1. The reactions were carried out at identical values of the enzyme activity concentration in the reactors. By the end of the reaction (3-h), *P. aeruginosa* lipase-catalyzed reactor had attained a benzyl alcohol conversion of nearly 90% compared with a conversion of 80% obtained with the lipase of *C. antarctica*. The porcine lipase produced a conversion of only 15%. This reaction liberates acetaldehyde that is known to deactivate certain enzymes (Castaing et al., 1987; Wang et al., 1988). Pancreatic lipase was probably susceptible to acetaldehyde and this might be an explanation for its relatively quite poor performance. Production of acetaldehyde can be prevented by using esters such as ethyl acetate instead of vinyl acetate in this reaction, but the important advantage of vinyl acetate is that the reverse equilibrium reaction is suppressed because the enol co-product of the reaction involving vinyl acetate is immediately and irreversibly transformed to acetaldehyde or acetone (Ishii et al., 1996; Kita et al., 1996).

Conversion of benzyl alcohol with *P. aeruginosa* lipase was not affected by increasing the incubation temperature from 30 °C to 60 °C. Four different constant temperatures were tested in increments of 10 °C. Over this entire range the conversion was $88.8 \pm 0.2\%$. Because conversion was not particularly sensitive to temperature over the range tested, all subsequent work was carried out at 30 °C.

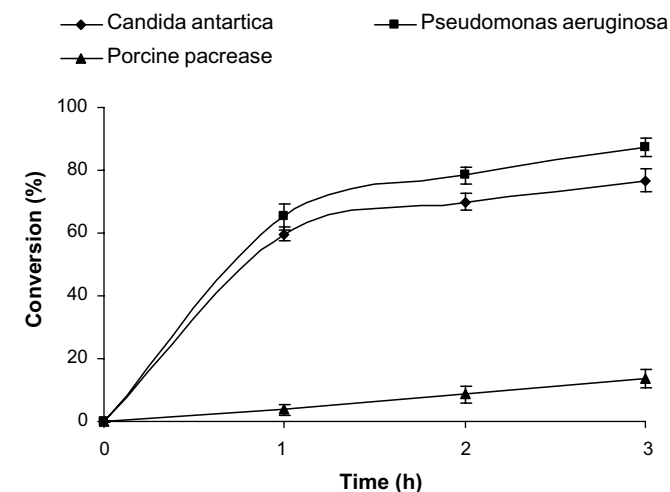


Fig. 1. Benzyl alcohol conversion versus reaction time for lipases of *P. aeruginosa*, *C. antarctica* and porcine pancreas at 30 °C. All reaction mixtures had 50% v/v heptane as the organic solvent. The enzyme activity used was identical at 700 U/ml. Benzyl alcohol (0.02 M) and vinyl acetate (0.05 M) were the substrates.

Organic solvents are known to affect the activity and specificity of lipases. Organic solvents with low $\log P$ (P is octanol–water partition coefficient for the solvent) values negatively influence hydrolytic activity of lipases because activity depends on the amount of water that can be absorbed to the enzyme in the organic phase. Solvents with low $\log P$ values are more hydrophilic and they tend to strip away the water molecules present on the surface of the enzyme (Zaks and Klibanov, 1986). This reduces the catalytic activity. Solvents with high $\log P$ values also stabilize lipases. In view of these effects of solvents, five solvents (i.e., hexane, heptane, toluene and benzene) were assessed for the lipase-catalyzed production of benzyl acetate. In all cases the solvent volume fraction used was 50% v/v. As shown in Fig. 2, the transesterification reaction did occur in all solvents, but heptane and hexane were distinctly better solvents. Solvents affected the rate of reaction and therefore the final conversion attained in a given time. The reaction was most rapid in heptane (Fig. 2), affording a conversion of more than 80% within 3-h. In view of its superior performance, heptane was used in all subsequent work.

Because lipase-catalyzed reactions occur at the interface of the two liquid phases, the volumetric rate of reaction is increased by increasing the liquid–liquid interfacial area. For given characteristics of a liquid–liquid dispersion, interfacial area can be increased by changing the relative proportions of the two liquid phases. To identify the optimal ratio of the two phases under a fixed set of agitation conditions (800 rpm) in a constant volume (60 ml) reactor, final conversion (3-h) of benzyl alcohol was measured in reactors with different volume fractions of heptane. Clearly, a dispersion having 50% v/v heptane provided the highest value of conversion. Higher volume fractions

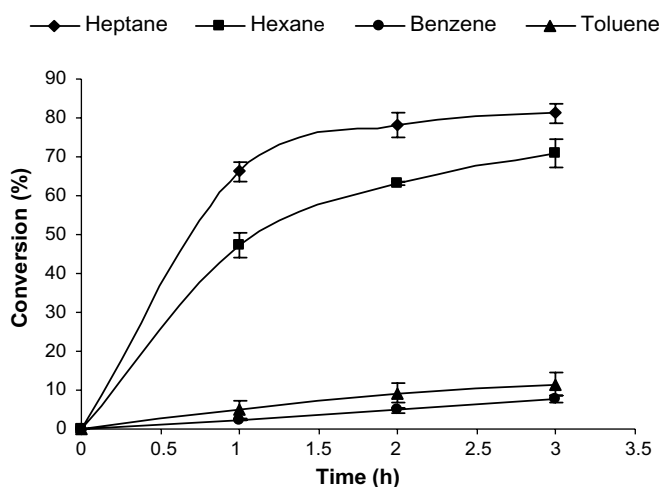


Fig. 2. Effect of solvent type on the transesterification conversion of benzyl alcohol by *P. aeruginosa* lipase at 30 °C. All reaction mixtures had 50% v/v of organic solvent, enzyme activity levels of 700 U/ml, and initial substrate concentrations of 0.02 M benzyl alcohol and 0.05 M vinyl acetate.

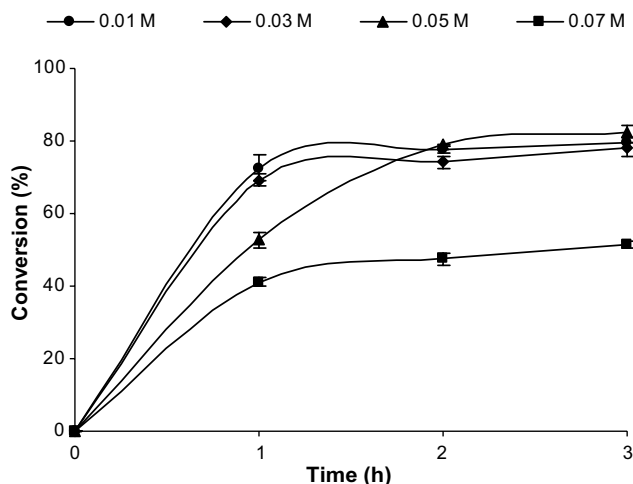


Fig. 3. Effect of benzyl alcohol initial concentration (0.01–0.07 M) on the conversion by *P. aeruginosa* lipase at 30 °C. Initial concentration of vinyl acetate was 0.05 M. Reaction mixtures had 50% v/v of heptane and enzyme activity levels of 700 U/ml.

of heptane produced dispersions that were relatively viscous and difficult to mix. Such dispersions therefore actually had lower values of interfacial areas per unit volume of the reactor when compared with the dispersion that had 50% v/v heptane present.

For otherwise constant initial conditions, the initial concentration of benzyl alcohol was an important influence on the rate of the reaction. Benzyl alcohol conversion values at various stages of the reaction are shown in Fig. 3 for various initial concentrations of benzyl alcohol. The rate of the transesterification reaction, i.e., the time required to attain a given value of conversion, decreased as the concentration of benzyl alcohol was increased above 0.01 M; however, as expected, the final conversion was highest when

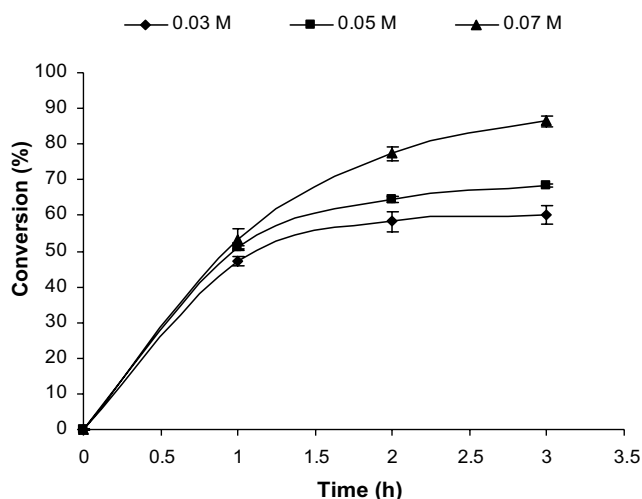


Fig. 4. Effect of vinyl acetate (0.03–0.07 M) initial concentration on conversion of benzyl alcohol by *P. aeruginosa* lipase at 30 °C. Initial concentration of benzyl alcohol was 0.05 M. Reaction mixtures had 50% v/v of heptane and enzyme activity levels of 700 U/ml.

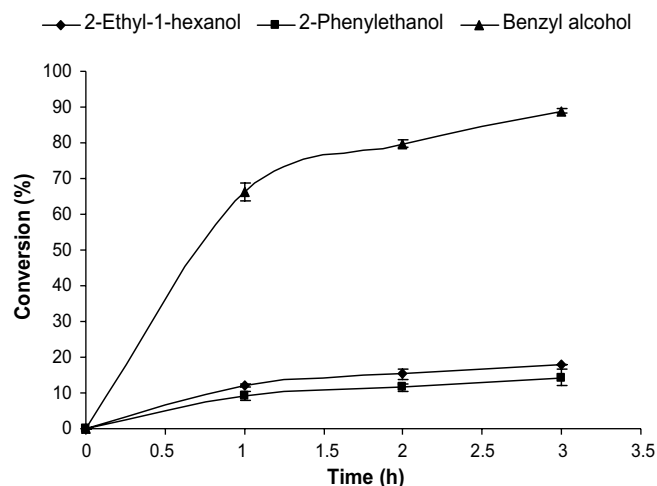


Fig. 5. Transesterification conversions for various alcohols (2-ethyl hexanol, 2-phenyl ethanol, benzyl alcohol) with vinyl acetate using *P. aeruginosa* lipase (700 U/ml) at 30 °C. Reaction mixtures contained 50% v/v heptane. Initial concentrations of all alcohols were 0.05 M. Initial concentration of vinyl acetate was 0.07 M.

equimolar initial amounts (i.e., 0.05 M) of the benzyl alcohol and vinyl acetate were provided. The data in Fig. 3 suggest that the enzyme was inhibited by benzyl alcohol, as the rate of conversion decreased with increasing initial concentration of benzyl alcohol.

Fig. 4 shows the effect on conversion of benzyl alcohol, of the initial concentration of vinyl acetate. In all cases the initial concentration of benzyl alcohol was 0.05 M. The reaction rate was not particularly sensitive to concentration of vinyl acetate. There was no clear evidence of the enzyme being inhibited by vinyl acetate.

P. aeruginosa lipase was further tested for its ability to transesterify vinyl acetate with 2-phenylethanol and 2-ethylhexanol. Compared with the conversions achieved with benzyl alcohol, the conversion values with these other alcohols were quite low at <20% by 3-h (Fig. 5). Clearly, this newly isolated enzyme is not effective in every case.

4. Concluding remarks

In comparison with the highly purified commercial lipases of *C. antarctica* and porcine pancreas, the inexpensive crude lipase preparation of *P. aeruginosa* MTCC 5113 had a distinctly higher specific activity. In production of the flavoring agent benzyl acetate from vinyl acetate and benzyl alcohol, the *P. aeruginosa* lipase afforded a substantially greater conversion of benzyl alcohol than did the other lipases. The conversion attained with *P. aeruginosa* lipase was nearly 90% within 3-h of reaction at 30 °C in a reactor that had 50% v/v heptane as the organic phase. Heptane proved a distinctly superior solvent for this reaction when compared with hexane, benzene, toluene and dimethyl sulfoxide. A 50% v/v ratio of organic to aqueous phases appeared to be optimal. Evidence suggested that *P. aeruginosa* lipase experienced possible substrate inhibition by

benzyl alcohol. No clear evidence was seen for inhibition of the enzyme by vinyl acetate.

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