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Enantioselective oxidation of (±)-2-phenyl-1-propanol to (*S*)-2-phenyl-1-propionic acid with *Acetobacter aceti*: influence of medium engineering and immobilization

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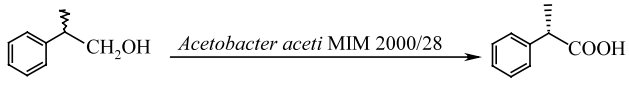
Abstract—The enantioselective oxidation of (*RS*)-2-phenyl-1-propanol to (*S*)-2-phenylpropionic acid catalysed by *Acetobacter aceti* occurred under very mild conditions. The rate, substrate tolerance and enantioselectivity (enantiomeric ratio, E >200) can be increased dramatically simply by adding cyclodextrins to the reaction medium. Cells immobilized in calcium alginate showed good operational stability and good enantioselectivity (e.e. 94%); the catalyst can be re-used at least five times without e.e. diminution. © 2002 Published by Elsevier Science Ltd.

1. Introduction

Acetic acid bacteria have been employed traditionally for the selective oxidation of primary and secondary alcohols.[1] These bacteria have an incomplete oxidative metabolism and, therefore, can efficiently perform chemo-, regio- and stereoselective biotransformations of primary alcohols, affording good yields of the corresponding carboxylic acids, while aldehydes are not normally isolated. As a consequence, acetic acid bacteria can also be conveniently employed for the kinetic resolution of racemic alcohols affording the corresponding carboxylic acid in enantiomerically pure form.[2–5]

Hydrophobic substrates are often converted only slowly in aqueous biotransformations, since their dissolution in the aqueous medium may be the rate controlling step.[6] Several procedures have been described to increase the reaction rate of these substrates such as using organic solvents or additives which help the availability of the organic substrate in aqueous medium.[6–9] This last solution is interesting because the deactivation of the biocatalyst is often reduced. Among the supramolecular additives, cyclodextrins (CD) are particularly attractive since they are commercially available and their internal hydrophobic cavities can be suited for achieving a controlled release of organic compounds in aqueous media. The use of cyclodextrin to ameliorate the rate and selectivity of aqueous bioconversions has been successfully applied for the modification of steroids and vitamins and can be a useful tool for bioconversions in plant cell biotechnology.[7,8,10]

We have recently reported the use of different acetic acid bacteria for enantioselective oxidation of primary alcohols and diols.[5] A strain of *Acetobacter aceti* 2000/28 MIM turned out to be quite versatile and efficient as a biocatalyst and was employed in this work for studying the enantioselective oxidation of (*RS*)-2-phenyl-1-propanol to (*S*)-2-phenylpropionic acid (Scheme 1).



Scheme 1.

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2. Results and discussion

Acetobacter aceti 2000/28 MIM was selected among recently isolated acetic acid bacteria for its oxidative activity towards (*RS*)-2-phenyl-1-propanol. Fig. 1 shows the relationship between growth and molar conversion obtained after 24 h; (*S*)-2-phenylpropionic acid was always produced with enantiomeric excess of 92%. Incomplete enantioselectivity can be due to enzymatic racemization, which was ruled out by the observation that enantiomerically pure (*S*)-2-phenylpropionic acid added to *A. aceti* MIM 2000/28 was not racemized even after 24 h.

Cells of *A. aceti* MIM 2000/28 showed the highest molar conversions after 24 h of growth. Experiments aimed at the improvement of the reaction rates and enantioselectivity were carried out with cells grown for 24 h and re-suspended in different buffers and under different reaction conditions (pH, temperature and substrate concentration). While the pH and temperature (the best results in terms of conversion were obtained at 28°C and initial pH 7.0) did not significantly influence the stereobias, the initial substrate concentration strongly affected the enantioselectivity since high enantiomeric excesses (>97%) could be obtained with low substrate concentration (Table 1).

The observation that the enantioselectivity of the reaction drops with higher substrate concentrations is often encountered when using whole microbial cells and it is often due to the simultaneous action of different dehydrogenases, which display different enantioselectivities.[11,12]

Different strategies can be used to reduce the concentration of an organic substrate available for a biocatalyst working in an aqueous medium. These include the addition of a second phase (for example an adsorbing resin[13] or an organic solvent)[14] to partition the substrate and lower its effective concentration with respect to the enzyme; the use of additives able to complex the substrate reversibly (e.g. cyclodextrins); and the immobilization of the whole cells on a solid support. Experiments were performed in the presence of hydrophobic organic solvents (1/1 v/v), or cyclodextrins (equimolar ratio with respect to the substrate). Different cyclodextrins were employed: underivatized β-cyclodextrin (β-CD), acetyl cyclodextrin (Ac-CD), methyl-β-cyclodextrin (Me-CD), trimethylammoniumcyclodextrin (TM-CD) and hydroxypropylcyclodextrin (HP-CD). various amounts of cyclodextrins were tested and the highest rates were observed with molar ratio substrate/CD of 0.5 in all the cases. The results are reported in Table 2.

Table 1. Production of (*S*)-2-phenylpropionic acid by oxidation of (*RS*)-2-phenyl-1-propanol with *Acetobacter aceti* MIM 2000/28: enantioselectivity and molar conversion after 24 h at different substrate concentrations

Substrate concentration (g/L)	Molar conversion (%)	e.e. of product (%)	*E*
0.5	45	>97	>200
1.0	45	>97	>200
2.5	41	92	46
5.0	28	67	6.5
7.5	12	46	2.9
10.0	>5	–	–

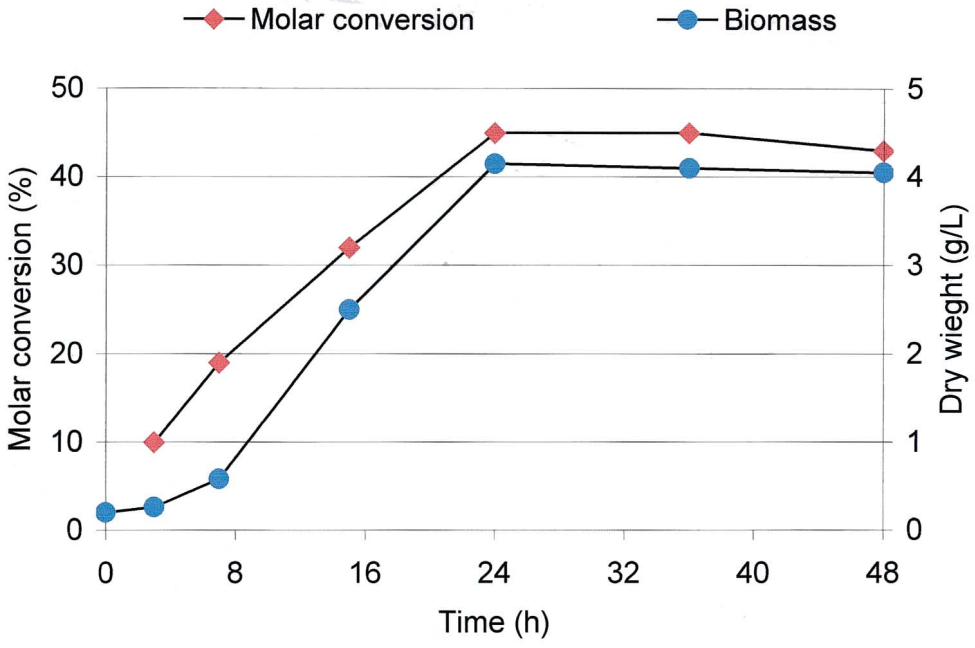

Figure 1. Growth of *Acetobacter aceti* MIM 2000/28 and molar conversion of (*RS*)-2-phenyl-1-propanol into phenylpropionic acid. Molar conversion after 24 h.

The addition of cyclodextrins generally reduced the reaction times, whilst affording similar yields and an improvement in the enantioselectivity. Modified cyclodextrins were more efficient, which can be attributed to the different size and hydrophobicity of the modified cyclodextrin cavity compared to that of unmodified cyclodextrins, thus improving the recognition of hydrophobic chiral alcohol molecules. The substrate molecules are released from the cyclodextrin cavity in a more controlled fashion, and as a result the enzymatic machinery is not saturated, leading to similar yields in shorter reaction times. Hydrophobic organic solvents, working as reagent reservoirs, increased the enantioselectivity, but low yields were achieved even after reaction times of 24 h. This result is likely to be due to the reduction of the activity of the cells by the hydrophobic organic solvent.[9]

Table 2. Production of (*S*)-2-phenylpropionic acid by oxidation of (*RS*)-2-phenyl-1-propanol with *Acetobacter aceti* MIM 2000/28 in the presence of solvents or cyclodextrins. [substrate] = 2.5 g/L

Solvent or additive	Molar conversion (%)	Time (h)	e.e. (%)	*E*
None	44	24	92	51
Heptane	23	24	96	103
iso-Octane	32	24	97	100
Pentadecane	31	24	97	100
β-CD	38	6	>97	>200
Ac-CD	47	6	>97	>200
Hp-CD	47	6	>97	>200
Me-CD	48	6	>97	>200
TM-CD	48	6	>97	>200

The specific activities using methyl cyclodextrin and different substrate concentrations were compared with those obtained without cyclodextrin (Fig. 2).

No formation of the *R*-enantiomer could be detected in all the experiments performed in the presence of CD, and the reaction occurred with good rates even at high substrate concentrations (10 g/l) where no reaction could be observed in experiments carried out without additives. Experiments with cells of *A. aceti* immobilized in alginate beads with Ca^{2+} or Ba^{2+} as counterions were also carried out (Table 3).

Simple immobilization of the cells did not improve the enantioselectivity or the yield compared to free whole cells. The biotransformation was also carried out with immobilized cells in the presence of methyl-β-cyclodextrin or *iso*-octane. The addition of *iso*-octane to immobilized cells resulted in lowered reaction productivity and a small improvement of the enantiomeric ratio, while no reaction occurred in the presence of cyclodextrins. This finding can be explained by the observation that alginate gels attract the cyclodextrin molecules, which then have a reduced capacity to bind organic molecules.[15]

Table 3. Production of (*S*)-2-phenylpropionic acid by oxidation of (*RS*)-2-phenyl-1-propanol with immobilized *Acetobacter aceti* MIM 2000/28

Alginate counterion	Solvent or additive	Molar conversion (%)	Time (h)	e.e. (%)	*E*
Ca^{2+}	None	35	24	94	54
Ba^{2+}	None	32	24	94	50
Ca^{2+}	*iso*-Octane	25	24	97	66

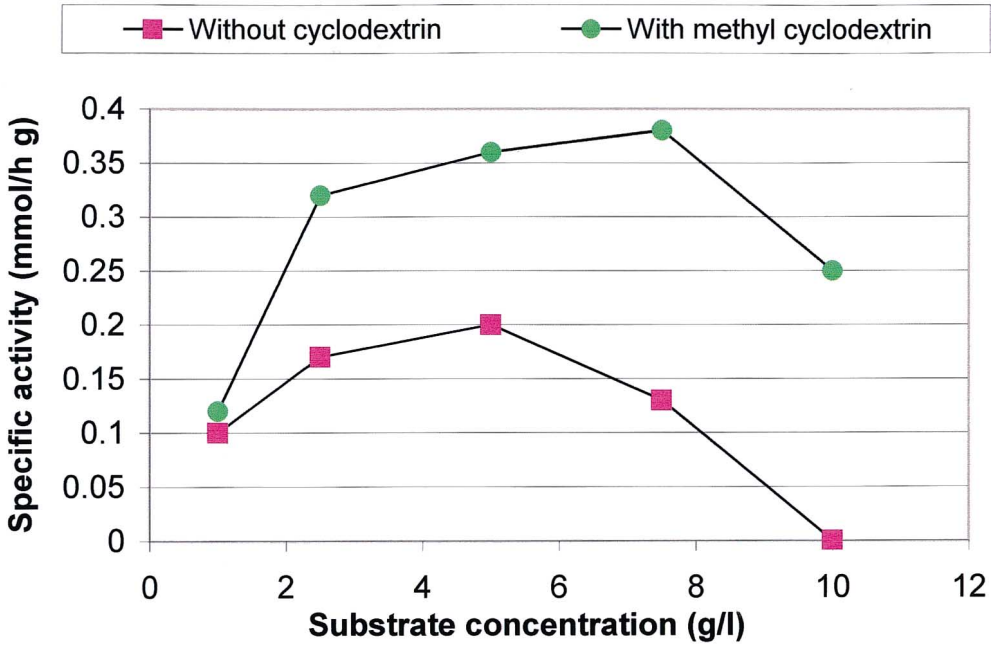

Figure 2. Activity of *Acetobacter aceti* MIM 2000/28 towards (*RS*)-2-phenyl-1-propanol at different concentrations with and without methyl cyclodextrins.

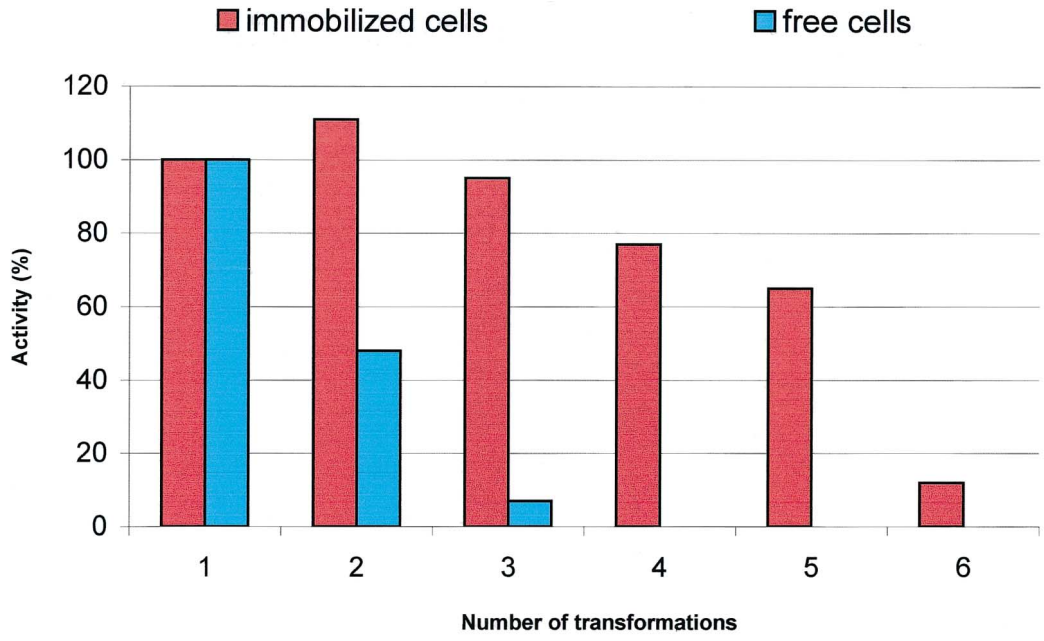

Figure 3. Re-use of free and immobilized cells for the oxidation of (*RS*)-2-phenyl-1-propanol.

Free and immobilized cells were re-used in successive experiments for gaining information about the stability of the biocatalyst (Fig. 3).

Freely suspended cells lost 50% of their activity after two batches, while cells immobilized in calcium alginate showed good operational stability, with high activity after five successive batch reactions. Additionally, the enantiomeric excess of the acid produced after repeated batches was the same (94%) showing that immobilization is a good tool for completion of this biotransformation under preparative conditions.

3. Conclusions

The oxidation of (*RS*)-2-phenyl-1-propanol with *A. aceti* furnished (*S*)-2-phenylpropionic acid under very mild conditions. The rate, substrate tolerance and enantioselectivity are dramatically increased by adding cyclodextrins to the reaction medium. These effects might be connected with the formation of reversible complexes among the cyclodextrin and the substrate; the controlled release maintains the low concentration of the substrate in water, therefore allowing for high enantioselectivity and lowering the tendency for substrate inhibition. A four-fold increase in the reaction rate (45% molar conversion after 6 h) was achieved by using methyl cyclodextrin, with enantiomeric excess always above 97%. Cells immobilized in calcium alginate showed good operational stability, although the acid was obtained with slightly lower enantioselectivity (e.e. 94%).

The simple methodology described in the paper can be used in a traditional organic chemistry laboratory and furnishes enantiomerically pure (*S*)-2-phenylpropionic acid with higher yields than reported previously.

4. Experimental

4.1. General

All the chemicals were purchased from Fluka Chemical Co., including enantiomerically pure (*R*)- and (*S*)-2-phenylpropionic acid. Cyclodextrins were a generous gift from Wacker-Chemie GmbH.

4.2. Microorganism, growth and biotransformation conditions

A. aceti MIM 2000/28 from our collection (MIM: Microbiologia Industriale Milano) was routinely maintained on GYC slants (glucose 50 g l^{-1}, yeast extract 10 g l^{-1}, $CaCO_3$ 30 g l^{-1}, agar 15 g l^{-1}, pH 6.3) at 28°C. The strain, grown on GYC slants for 24 h at 28°C, was inoculated into 100 ml Erlenmeyer flasks containing 10 ml of the liquid medium GLY (glycerol 25 g l^{-1}, yeast extract 10 g l^{-1}, pH 5, distilled water) and incubated on a reciprocal shaker (100 spm); the flask liquid cultures of *A. aceti* MIM 2000/28 were used for inoculating a 1 L reactor with 200 mL working volume, agitation speed 250 rpm, air flow rate 1 vvm. The dry weight was determined after centrifugation of 100 ml of cultures, cells were washed with distilled water and dried at 110°C for 24 h.

Biotransformations were completed using bacteria grown directly inside the reaction vessel; in experiments with two-liquid phase systems, solvents were added to reach a 1:1 v/v ratio. Neat substrate was directly added to suspensions. Control of the pH was performed by continuous addition of aqueous NaOH via a multichannel Watson–Marlow 503 U/R peristaltic pump connected to a pH controller (pH/ORP Controller 3675, Jenco Electronics). The work-up of these biotransformation was carried according to Ref. 4.

4.3. Analytical methods

The absolute configuration of the obtained acid was determined by comparison with the specific rotation of authentic samples of the enantiomerically pure compounds. Samples (0.5 ml) were taken at intervals brought to pH 1 by addition of 5 M HCl and extracted with an equal volume of Et_2O. Phenylpropanoic acid concentrations and enantiomeric composition were routinely determined by HPLC using a Chiralcel® OD (Daicel Chemical Industries, Illkirch, France) analytical column. The stereochemical outcome of the transformations was expressed as enantiomeric excess (e.e.) of the major enantiomer or as enantiomeric ratio (E). The upper threshold for the numerical value of E is 200, since higher values of E cannot be accurately determined.

4.4. Immobilization procedures

The cell culture medium was centrifuged at 4000 g for 15 min at 4°C and the biomass was separated from the liquid. The cells were mixed with sterilized sodium alginate (20 ml, 2.0% solution) (Sigma Chemicals) and the mixture was dropped into calcium chloride solution (0.1 M, 50 ml) introduced in an ice bath using a hypodermic syringe. After hardening for 1 h, the obtained beads were rinsed with aqueous $CaCl_2$ solution (0.1 M, 50 ml), separated by filtration and the wet beads were stored at 4°C until use. Both free and immobilized cells were re-used in successive cycles. After 24 h, free cells were centrifuged and re-suspended in fresh reaction mixture and 2-phenylethanol (2.5 g/l) was added. Immobilized cells were filtered on paper and re-used after washing with a 0.1 M aqueous calcium chloride solution. The reaction was discontinued if the yield of the acid was less than 15% after 24 h.

Acknowledgements

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