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Enantioselective oxidation of (*RS*)-2-phenyl-1-propanol to (*S*)-2-phenylpropanoic acid with *Gluconobacter oxydans*: simplex optimization of the biotransformation

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Abstract

The microbial oxidation of racemic 2-phenyl-1-propanol by *Gluconobacter oxydans* DSM 50049 was investigated. Whole bacterial cells were used to produce (*S*)-(+)-2-phenylpropanoic acid with high enantiomeric excess (E>200). A simplex sequential method was employed as an experimental design to guide the optimization process. Temperature of 26–28°C, pH 6.0–6.2, substrate concentration of 20–25 mM and agitation of 150 rpm have been found the best conditions to achieve the highest reaction rates and enantioselectivities.

1. Introduction

The resolution of racemic mixtures of chiral primary alcohols is an attractive method to produce enantiomerically pure carboxylic acids, such as (*S*)-2-aryl propionic acids which are of interest as chiral building blocks and biologically active compounds.[1] One way to achieve a highly enantioselective oxidation involves the use of whole microbial cells which can exploit the action of stereospecific dehydrogenases while providing cofactors and systems for their regeneration. Acetic acid bacteria possess a number of membrane-bound dehydrogenases able to oxidize different alcohols with very high efficiency.[2] The use of acetic acid bacteria to perform enantioselective oxidations of racemic mixtures of primary alcohols has been already reported.[3–5] This approach seemed to be very promising and therefore, we have now focused our attention on obtaining the enantioselective oxidation of racemic 2-phenyl-1-propanol with acetic acid bacteria and to optimize the biotransformation. Traditional methods

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of optimization are often carried out by varying the experimental conditions independently; this process is often time-consuming and interactions between variables cannot be assessed. Simplex optimization techniques[6] are useful in improving research efficiency when more than two factors are simultaneously changing during experiments, as in microbial transformations and have been successfully employed for optimizing different processes where the trials are subsequently performed in the direction of improvement until the optimum is reached.[7] A simplex optimization was, therefore, used in this study to find the levels of variables required for the efficient and enantioselective oxidation of the racemic mixture of 2-phenyl-1-propanol.

2. Results

2.1. Selection of the strain and culture conditions

A screening to find strains able to oxidize (*RS*)-2-phenyl-1-propanol was performed with 20 acetic acid bacteria from commercial sources and our own collection. The alcohol (10 mM) was added directly onto the submerged cultures (25°C) after 24 h of growth. Most of the tested strains showed sluggish reaction rates, the only exception was *Gluconobacter oxydans* DSM 50049 which furnished the (*S*)-(+)-acid with 40% molar conversion and 97% e.e. after 24 h. This strain was used to check the influence of the growth time on the cell activity (Fig. 1).

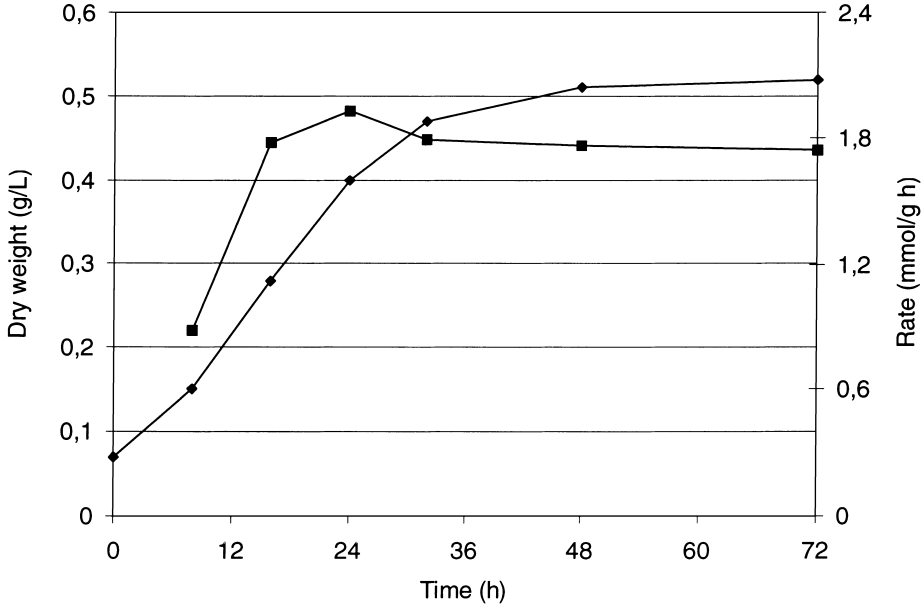

Figure 1. Growth (dry weight, ◆) and oxidative activity towards 2-phenyl-1-propanol (■) of *Gluconobacter oxydans* DSM 50049

The highest activity was observed with cells grown for 24 h. No marked differences in the stereobias were observed by using cells with different growth times, (*S*)-(+)-2-phenylpropionic acid being always obtained with an e.e.>95%.

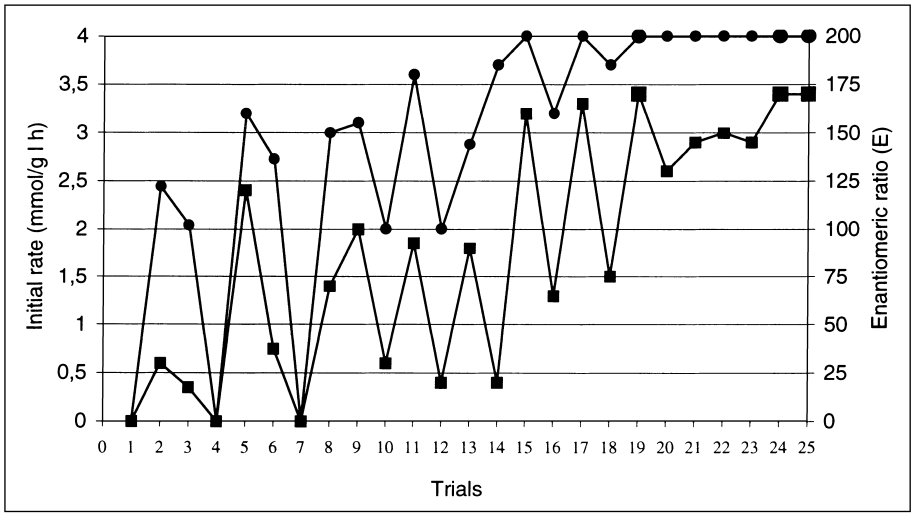

Figure 2. Oxidation of 2-phenyl-1-propanol by *Gluconobacter oxydans* DSM 50049: experimental responses (■ initial rate, ● enantiomeric ratio) as function of sequential trials

2.2. Optimizaton of the biotransformation

Cells of *Gluconobacter oxydans* DSM 50049 grown for 24 h were used in experiments aimed at the optimization of biotransformation. The conditions of the sequential experimental trials were selected employing the Multisimplex® 2.0 software. Two response variables were chosen for the optimization of 2-phenyl-1-propanol oxidation, namely the initial reaction rate and the enantiomeric ratio (E).[8,9] The control variables (temperature, pH, substrate concentration, agitation) and their initial levels were selected on the basis of previous experiences on biotransformations carried out with acetic acid bacteria.[10] After the initial trials the simplex process is sequential, with the addition and evaluation of one new trial at a time. The results of the sequential experiments aimed at the optimization of initial rate and E are reported in Fig. 2.

A progressive increase towards the optimum responses can be observed. The highest initial rate and enantioselectivity were achieved in trials **19**, **24** and **25**, with slightly lower responses in trials **15** and **17**. The response approached conditions near the optimum rapidly (trial **15**), and the program used later experiments attempting to locate other optima for activity and enantioselectivity by making major changes in the levels of control variables. It is noteworthy that after trial **19** the optimum for E was always achieved.

The variations of the values of the individual parameters as a function of the iterative sequence of experiments are reported in Figs. 3–6.

The best results were achieved with a pH between 6.0 and 6.2, temperature 26–28°C and substrate concentration 20–25 mM. Only agitation levels notably differed in these trials ranging from 130 to 200 rpm, showing that this control variable exerted minor effects on the overall performances. Conditions from trial **24**, having the highest substrate concentration, were selected to follow the time course of the biotransformation (Fig. 7).

The difference in the reactivity of the two enantiomers was quite large since very slow reaction rates were observed after 45–50% molar conversion.

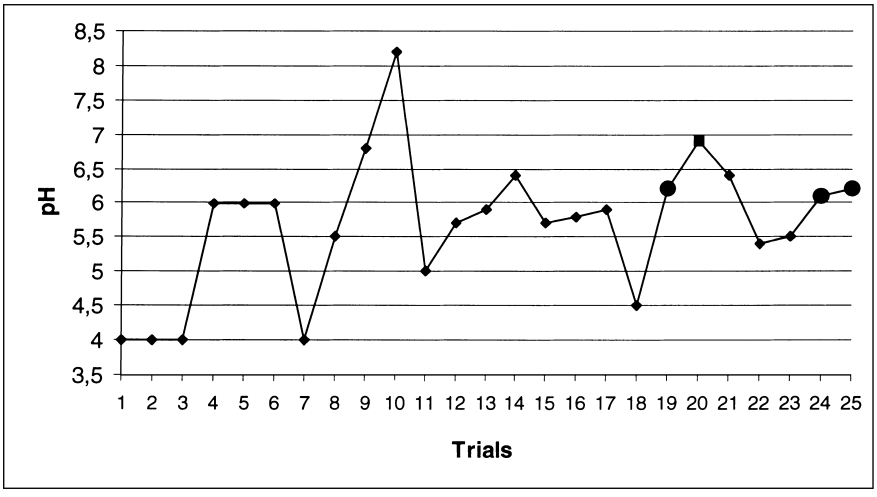

Figure 3. Oxidation of 2-phenyl-1-propanol by *Gluconobacter oxydans* DSM 50049: variation of pH as a function of sequential trials. Circles correspond to the experiments giving the best enantioselective transformation

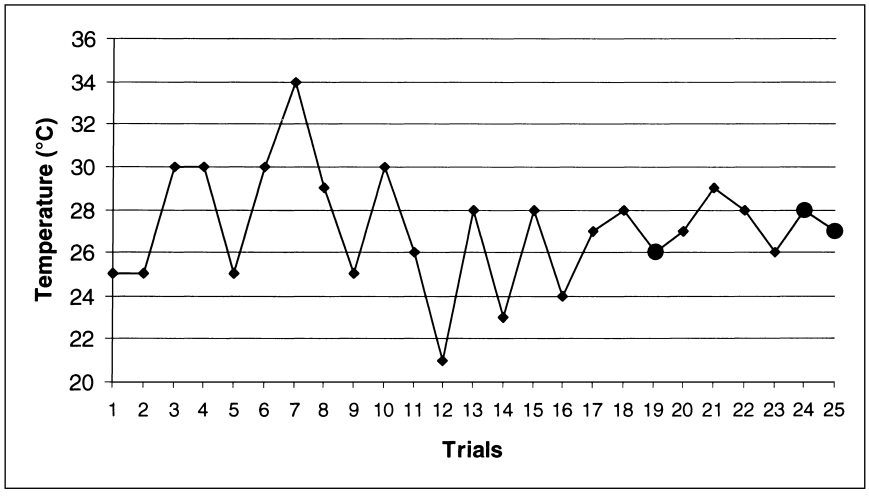

Figure 4. Oxidation of 2-phenyl-1-propanol by *Gluconobacter oxydans* DSM 50049: variation of temperature as function of sequential trials. Circles correspond to the experiments giving the best enantioselective transformation

3. Conclusion

Whole cells of *Gluconobacter oxydans* DSM 50049 promoted highly enantioselective oxidation of (*RS*)-2-phenyl-1-propanol allowing for the production of (*S*)-(+)-2-phenylpropanoic acid with a high enantiomeric excess. A limited number of experiments, guided by a simplex optimization program, gave reaction conditions able to furnish an efficient process. It can be concluded that the potential of enantioselective bio-oxidation of racemic alcohols with microbial whole cells is technically and economically interesting as a production method for optically active carboxylic acids.

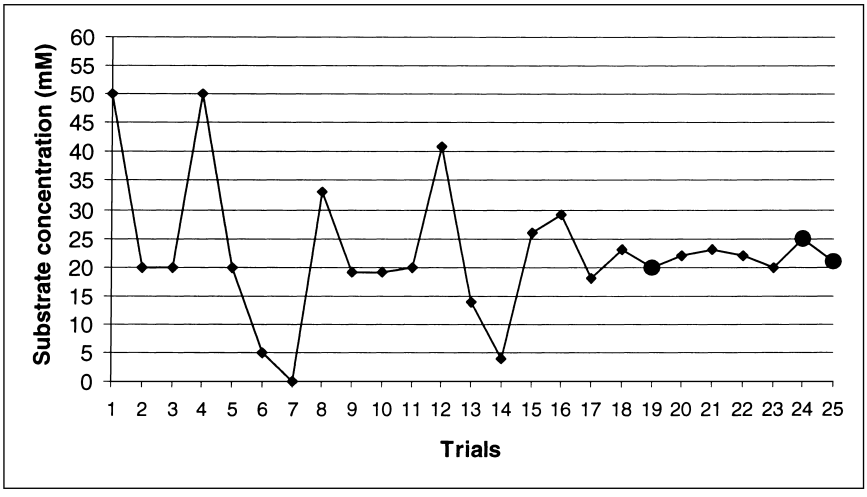

Figure 5. Oxidation of 2-phenyl-1-propanol by *Gluconobacter oxydans* DSM 50049: variation of substrate concentration as function of sequential trials. Circles correspond to the experiments giving the best enantioselective transformation

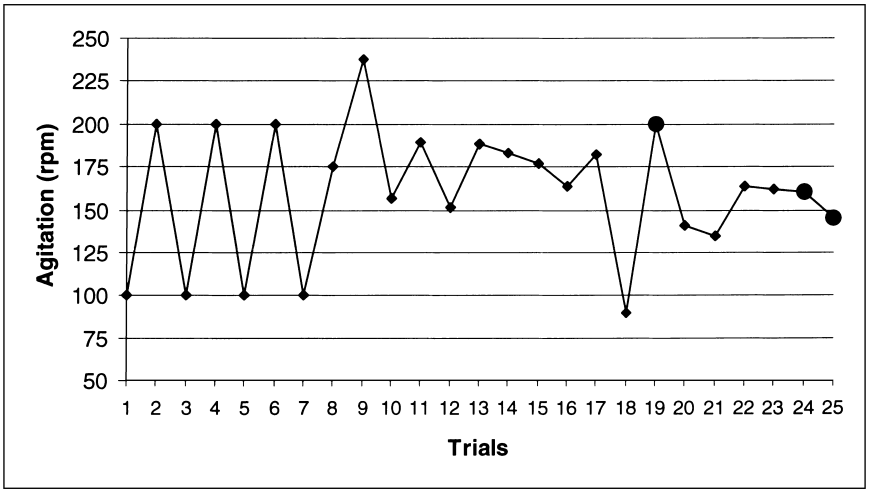

Figure 6. Oxidation of 2-phenyl-1-propanol by *Gluconobacter oxydans* DSM 50049: variation of the speed of agitation as function of sequential trials. Circles correspond to the experiments giving the best enantioselective transformation

4. Experimental

4.1. Microorganisms, growth and biotransformation conditions

Acetic acid bacteria were 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 strains, grown on GYC slants for 24 h at 28°C, were inoculated into 500 mL Erlenmeyer flasks containing 50 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). *Gluconobacter oxydans* DSM 50049 (Deutsche Sammlung von Mikroorganismen) was employed in optimization studies accomplished using cultures grown in a 1 L reactor with 200 mL working volume, agitation speed 250 rpm, air flow rate 1 vvm. Biotransformations were accomplished using bacteria grown directly inside the reaction vessel. Neat substrate was directly added onto suspensions. The control

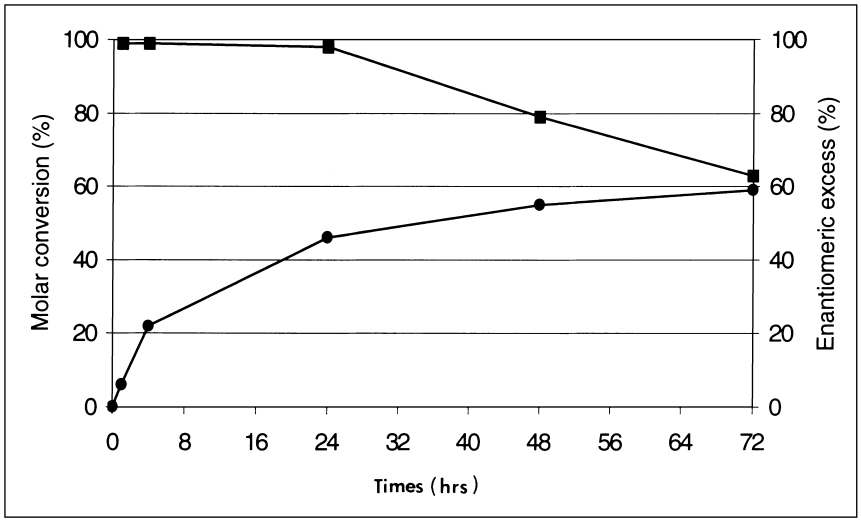

Figure 7. Time course of (*S*)-2-phenylpropanoic acid production catalyzed by *Gluconobacter oxydans* DSM 50049 (● molar conversion, ■ enatiomeric excess)

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).

*4.2. Preparation of (*S*)-(+)-2-phenylpropanoic acid*

The work-up of the biotransformation corresponding to trial **24** is reported as an example. Biotransformation was carried out starting from 680 mg of racemic alcohol in 200 mL of cultural broth and after 24 h the reaction mixture was centrifuged (15 000*g*, 10 min) to remove the bacterial cells, the surnatant was brought to pH 2 with aqueous HCl and extracted with ethyl acetate. The organic extracts were dried over Na_2SO_4 and the solvent was removed; the crude product was purified by flash chromatography (hexane:ethyl acetate:acetic acid, 20:10:1) to give 280 mg of (*S*)-(+)-2-phenylpropanoic acid (37%, e.e.>98%).

4.3. Determination of dry weight

After centrifugation of 100 mL of cultures, cells were washed with distilled water and dried at 110°C for 24 h.

4.4. Analytical methods

The absolute configuration of the obtained acid was determined by comparison with the optical rotation of authentic samples of the enantiomerically pure enantiomers. 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 $CHCl_3$. Phenylpropanoic acid concentrations were routinely determined by GLC analysis after conversion to the corresponding methyl ester after treatment with CH_2N_2; the organic extracts were then dried and dissolved in a $CHCl_3$ solution containing an internal standard (phenethyl alcohol). GLC was carried out on a Carlo Erba Fractovap G1 gas chromatograph equipped with a hydrogen flame ionization detector. The column (3×2000 mm) was packed with Carbopack B-DA (4% CW 20M) with the column temperature kept

at 200°C. The enantiomeric composition was routinely determined by gas chromatographic analysis of the methyl ester using a chiral capillary column (diameter 0.25 mm, length 25 m, thickness 0.25 μ, DMePeBeta-CDX-PS086, MEGA, Legnano, Italia). The stereochemical outcome of the transformations was expressed as enantiomeric excess (e.e.) of the major enantiomer or as the enantiomeric ratio (E).[6,7] The upper threshold for the numerical value of E was 200, since higher values of E cannot be accurately determined.[11]

4.5. Optimization by sequential simplex method

The simplex optimization method was based on sequential experimental trials guided by the systematic search strategies of the Multisimplex® 2.0 program (Multisimplex AB, Karlskrona, Sweden). The five starting experiments were selected with levels of each control variable (temperature, pH, substrate concentration and agitation) within the following ranges: pH 3–7, temperature 22–32°C, substrate concentration 25–35 mM, speed of agitation 100–200 rpm.

The control responses to be optimized were the initial rate (expressed as mmol of product formed in 1 h by 1 g of dry cells) and the enantiomeric ratio E. Each experiment was carried out in triplicate. Control using the conditions of trial **2** was performed together with each of the subsequent iterative trials and results were normalized to the initial response obtained for trial **2**.

Acknowledgements

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