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RESEARCH ARTICLE

Biocatalytic oxidative kinetic resolution of (±)-4-(chlorophenyl)phenylmethanol by *Nocardia corallina* B-276

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Abbreviations: ATCC: American Type Culture Collection
DMF: *N*,*N*-dimethylformamide
ee: enantiomeric excess
GC: gas chromatography
HPLC: high pressure liquid chromatography
IR: infrared spectroscopy
NMR: nuclear magnetic resonance
OD: optical density
TLC: thin layer chromatography
vvm: aereation rate

Chiral diarylmethanols are versatile building blocks for the preparation of biologically active substances, but they are difficult to obtain in enantiopure form. We used *Nocardia corallina* B-276 for the oxidative kinetic resolution of (±)-4-(chlorophenyl)phenylmethanol, 1. Two experimental methods were used: 1) Suspension of cells in a phosphate buffer solution and 2) Cells in the culture media, in a 3-L bioreactor. After 36 hrs using the first method, the ketone/alcohol ratio was 56/44 and the unoxidized alcohol had an enantiomeric ratio of 93/7, predominating the *R*-alcohol.

Optically active diarylmethanols are important aromatic compounds (Fontes et al. 2004), useful as versatile building blocks for the synthesis of biologically active substances (Pérez et al. 2000). In recent years there has been a strong focus on sustainable processes,in the academic community and in the industrial research groups. Biocatalysis is a valuable alternative to traditional organic synthesis, with advantages such as higher enantioselectivity, milder reactions conditions, and lower energy requirements (Coward-Kelly and Chen, 2007).

In this context, the recent successful applications of biocatalysis for the preparation of active pharmaceutical ingredients are promissory (Tao et al. 2007), in particular whole cell catalyzed processes applied to the commercial synthesis of fine chemicals (Ishige et al. 2005; Woodley, 2006). Compared with isolated enzymes, whole cell catalysts can be much readily accessible and inexpensive. Besides in an oxidative process, an isolated enzyme requires cofactors and is sensitive to product inhibition, whereas whole cells do not need the addition of cofactors since they are provided by the cell (Whitesides and Wong, 1985). Oxidation of natural products, like terpenic substrates, is another important *via* to fine chemicals, in this case the use of plant cell suspension cultures is an important approach (Limberger et al. 2007).

Deracemization of secondary alcohols through the stereoinversion of one alcohol enantiomer, has been observed in the presence of growing or resting cells, using one (Comasseto et al. 2004; Nie et al. 2004; Padhi et al. 2006; Vaijayanthi et al. 2007; Yamada-Onodera et al. 2007) or two different microorganisms (one for oxidation and the other for reduction) (Fantin et al. 1995). Then, *Sterigmatomyces elviae* DSM 70852; *Candida parapsilosis* CCTCC M203011; *C. parapsilosis* ATCC 7330; *Aspergillus terreus* CCT 4083; *A. terreus* CCT 3320 were usedrespectively. Recently Voss et al. (2008) resolved the problem of the limited application of these methods to specific substrates, with only moderate substrate concentrations, through a concurrent tandem biocatalytic oxidation and reduction using a microorganism (*Alcaligenes faecalis* or *Rhodococcus erythropolis*) and alcohol dehydrogenases from micoorganisms. This concept is very interesting and promissory.

However, based in our previous work on microbial oxidations using growing cells of *Nocardia corallina* (Pérez et al. 2000; Pérez et al. 2001), we selected it to carry out the oxidative kinetic resolution of *rac*-4-(chlorophenyl)phenylmethanol (Figure 1), a precursor of important active pharmaceutical ingredients (Kleemann and Engel, 2001; McCalmont et al. 2004), by two methods: 1) With a suspension of whole cells in a phosphate buffer solution and 2) With cells suspended in the culture media, in a bioreactor of 3-L. Considering that prochiral diaryl ketones usually contain two sterically and electronically similar aryl groups, the asymmmetric reduction is a serious problem to resolve. Then the possibility of a concomitant reduction of the ketone formed by the same microorganism needs to be explored too.

MATERIALS AND METHODS

Compounds 1 and 2 were purchased from Aldrich and were analyzed by IR, ^{1}H NMR, ^{13}C NMR and TLC. IR spectra were recorded on a Perkin-Elmer Paragon 1600 FT, as KBr discs. Hydrogen and Carbon nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR), were recorded on a Varian 400 MHz instrument, in $CDCl_3$ and tetramethylsilane as internal reference. TLC was developed on silica gel 60 GF_{254} plates (Merck). HPLC analysis was performed on an Agilent 1100

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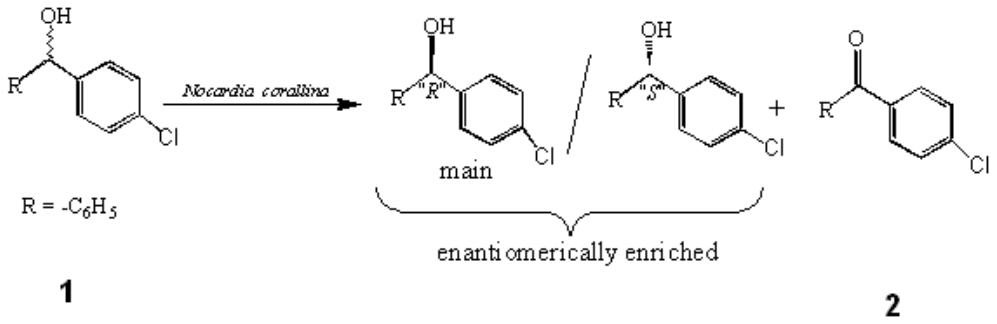

Figure 1. Biotransformation of (±)-4-(chlorophenyl)phenylmethanol, 1.

liquid chromatograph, equipped with a diode array detector and a Chiracel OB-H column (25.0 x 0.46 cm L X ID), the mobile phase was hexanes:isopropyl alcohol (90:10), at 0.8 mL min^{-1}, 24ºC, detected at λ = 230 nm. The GC analysis was performed on a Hewlett-Packard 6890 gas chromatograph, equipped with a flame ionization detector, a HP-5 column (30 m x 0.32 mm L x ID), T = 180ºC, N_2 as carrier gas, with a flow of 0.8 mL min^{-1}. Optical rotations were measured in a Perkin-Elmer polarimeter model 341 and the OD in a Genesys 20 spectrophotometer at 660 nm.

The growth experiments and biotransformation with *Nocardia corallina* were performed in a stirred 3-L bioreactor (2.2 L working volume), equipped with an ADI 1030 Bio Controller (Applikon, Schiedam, The Netherlands), at atmospheric pressure and at 226 rpm with an aeration rate of 0.9 vvm (air only). The pH and oxygen percentage were monitored continuously in the fermentor. To determine the correlation between OD and the quantity of the dry cells (g L^{-1}), samples were withdrawn every hour, until the end of the exponential growth.

Organism and growth

Nocardia corallina B-276 (ATCC 31338) was grown at 28-30ºC on agar plates (15 g agar L^{-1}; 3 g beef extract L^{-1}; 5 g peptone L^{-1}).

Liquid cultures were incubated in an orbital shaker, the broth composition was: 0.05 g $FeSO_4 \cdot 7H_2O$ L^{-1}; 1.74 g K_2HPO_4 L^{-1}; 2 g $(NH_4)_2SO_4$ L^{-1}; 1 g yeast extract L^{-1}; 1.5 g $MgSO_4$ L^{-1}; 2 g glucose L^{-1}; the solution was sterilized, and the pH adjusted to 8.0 (± 0.5).

Biotransformation using a suspension of cells in a phosphate buffer solution

A 500 mL Erlenmeyer flask containing 200 mL of sterile culture medium was inoculated from an agar plate (three days old) and incubated at 28-30ºC on an orbital shaker (170 rpm) for 48 hrs. The cells were collected by centrifugation at 4500 rpm for 15 min. Cells were washed twice with a potassium phosphate buffer (0.1 M, pH 7.0).

The cells were incubated in 50 mL phosphate buffer, (0.1 M, pH 7.0), for 30 min at 28-30ºC on an orbital shaker (170 rpm), then a solution of *rac*-4-(chlorophenyl)phenylmethanol, 1, (0.09 g, 0.41 mmol) in DMF (0.6%, v/v) was added to the cells suspension, at a substrate:dry cells ratio of 1:18 (m/m), the mixture was shacked under the same conditions for 32-34 hrs. Then, the reaction mixture was centrifuged at 4500 rpm for 15 min. The supernatant liquid was extracted with dichloromethane (3 x 10 mL), the combined organic phase was dried over Na_2SO_4 and filtered. Evaporation of the solvent in vacuo gave a mixture of the secondary alcohol, 1, and the ketone 2.

To determine the enantiomeric excess (ee) of the alcohol, a sample of the crude product was dissolved in 0.5 mL of isopropyl alcohol and analyzed by HPLC, retention time: $t_{(R)}$ = 18.6 min and $t_{(S)}$ = 27.9 min. The conversion degree was determined by GC. Retention time: $t_{(ketone)}$ = 4.03 min and $t_{(alcohol)}$ = 4.5 min.

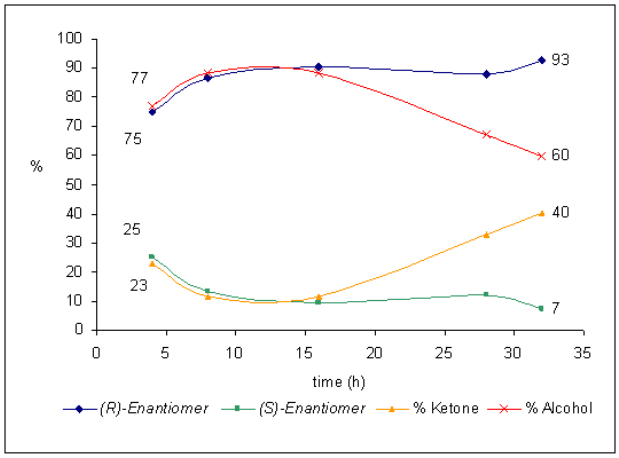

Figure 2. Biotransformation of 1 with 1:18 substrate:dry cells ratio.

Table 1. Oxidative kinetic resolution of 1, with resting cells.

Substrate:dry cells ratio	Reaction time (hrs)	Enantiomeric ratio *R*/*S*	Ketone/Alcohol ratio
1:3	36	56/44	10/90
	48	57/43	10/90
	60	56/44	10/90
	72	56/44	8/92
1:9	24	67/33	19/81
	48	68/32	28/72
	72	72/28	37/63
1:18	36	93/7	56/44
	48	65/35	47/53
	60	66/34	23/77

Control experiments

In all these experiments, a sample of resting cells without substrate was added to the same reaction system, as control, to determine potential impurities in the analytical background.

On the other hand a solution of *rac*-4-(chlorophenyl)phenylmethanol, 1, with potassium phosphate buffer (0.1 M, pH 7.0), was shacked for 72 hrs without the microorganism under the same conditions, after work-up the alcohol 1 was quantitatively recovered. A similar experiment with 4-chlorobenzophenone, 2, was studied, and 2 was also quantitatively recovered.

Biotransformation using a bioreactor

The cells of *Nocardia corallina* B-276 (ATCC 31338) were grown as indicated above.

Two 500 mL Erlenmeyer flask containing sterile culture medium (200 mL each), were inoculated from an agar plate (three days old) at 28-30ºC on an orbital shaker, at 170 rpm for 40-44 hrs.

The content of both flasks (400 mL) was aseptically poured into a 3-L bioreactor, containing 1800 mL of culture medium at 28-30ºC, stirred at 226 rpm and 0.9 vvm. Cellular growth was monitored every hour by OD.

Biotransformation. After 6 hrs, *rac*-4-(chlorophenyl)phenylmethanol, 1, (0.25 g, 1.14 mmol) in DMF (13.2 mL) and 100 mL of *n*-octane, were added to the bioreactor and incubated under the same conditions for 72 hrs. Samples of 20 mL were taken at 0, 24, 48 and 72 hrs. Each sample was saturated with NaCl and centrifuged at 4500 rpm for 15 min. The supernatant was extracted with dichloromethane (3 x 10 mL), the organic phase was dried over Na_2SO_4, filtered and evaporated to dryness, and then analyzed by GC and HPLC.

RESULTS AND DISCUSSION

Due to the use of two different experimental methods for this oxidation, the results will be presented and discussed separately.

Method 1

The oxidative resolution of 1 was carried out working with a suspension of cells of *Nocardia corallina* in a phosphate buffer solution, with three different substrate:dry cells ratios (m/m), 1:3, 1:9 and 1:18 for 60-72 hrs. As can be observed in Table 1, an increase in the cell ratio improved substantially the enantiomeric enrichment of 1, 93% of (*R*)-1 and 7% of (*S*)-1; in contrast with the enantiomeric ratio 56/44, reached using a 1:3 substrate:dry cells ratio after 36 hrs reaction. Also the proportion of the ketone, 2 was the highest (56%) using the 1:18 ratio; in contrast with the 1:3 ratio, where the conversion to the ketone was only 10%.

In some reports of oxidative kinetic resolution of alcohols, when the highest conversion (approximately 50% of the alcohol) has been reached, there is no racemization or

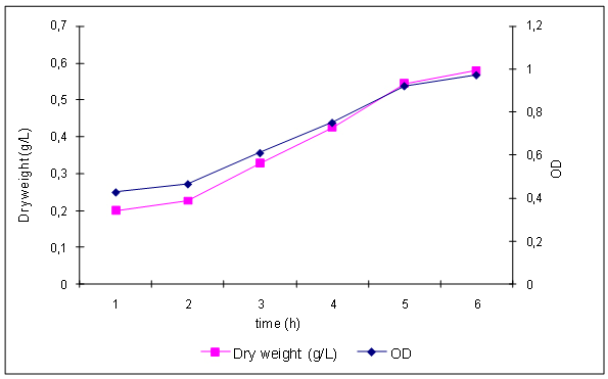

Figure 3. Growth curve of *N. corallina* in the 3-L bioreactor.

further consumption of the oxidized product in the reaction system (Su et al. 2004). But contrary to what was expected, using the ratio 1:18 at longer biotransformation times (60 hrs), the amount of ketone and the enantiomeric ratio of 1 decreased. One possible explanation to these facts is the presence of a slow reduction of the ketone, 2, mainly to (*S*)-1, under the reaction conditions studied.

Since the 1:18 ratio gave the best conversion to 2 (56%) and a good enantiomeric ratio (93/7), (Table 1), we carried out an experiment to observe the evolution of the reaction (Figure 2), after 32 hrs the enantiomeric ratio was similar to that obtained at 36 hrs (Table 1), but the conversion to the ketone was 40%.

The main alcohol has *R* configuration (Pérez et al. 2000). The physical and spectroscopic data (IR, ^{1}H and ^{13}C NMR spectra) were compared with authentic samples and were in accordance with the literature values (Lerebours and Wolf, 2006).

We observed that after the reaction has reached the highest yield of the oxidation of 1, the ketone produced, 2, could be reduced under this reaction conditions (Table 1). Consequently, we designed an experiment to demonstrate this hypothesis. Ketone 2 was added to the enzymatic equipment of *Nocardia corallina* in the indicated buffer solution, and after 73 hrs the ketone was quantitatively recovered. But when we used a 50:50 mixture of 1 and 2 in the same reaction system, after 48 hrs, the ratio of 1/2 changed to 63/37, and the enantiomeric ratio *R/S* of 1 was 40/60, this means that the ketone, 2, was reduced to the *S*-1 alcohol. To our knowledge, this is the first report of bioreduction of a prochiral benzophenone derivative with *Nocardia corallina* B-276.

Method 2

Since it was necessary to obtain enough biomass of *Nocardia corallina* for our experiments, we studied the growth kinetics of the microorganism under batch cultivation in a 3-L fermentor, the best conditions were: agitation rate 226 rpm, aeration rate 0.9 vvm (air only), pH = 8.4 and temperature between 28-30ºC.

To determine the correlation between optical density and dry cell weight, samples were withdrawn every hour, the optical density (at 660 nm) was measured and the cells were dried and weighted to determine the concentration of dry cell (g L^{-1}) in the culture. The highest concentration of biomass was achieved in 6 hrs (Figure 3).

In this method the oxidative kinetic resolution of 1 was then performed using *N. corallina* cells suspended in the culture media, in the 3-L bioreactor. By OD measurement and using Figure 3, we estimated a 1:5 ratio of substrate:dry cells in the bioreactor, then 1.14 mmol (0.5 mM) of *R/S*-1 dissolved in DMF (13.2 ml), were added to the bioreactor. It is important to note that in the resting cells method a 8.2 mM solution of 1 was used, 0.41 mmol, and in this second method (semi-preparative scale) an important dilution was present. Samples were analyzed during 24 hrs, and the results are shown in Table 2.

Due to the fact that alcohol enrichment and the conversion

Table 2. Oxidative kinetic resolution of 1, in the 3-L bioreactor.

Substrate:dry cells ratio	Reaction time (hrs)	Enantiomeric ratio R/S	Ketone/Alcohol ratio
1:5	0	53/47	6/94
	19	61/39	13/87
	21	67/33	9/91
	22	73/27	8/92
	23	67/33	8/92
	24	56/44	13/87

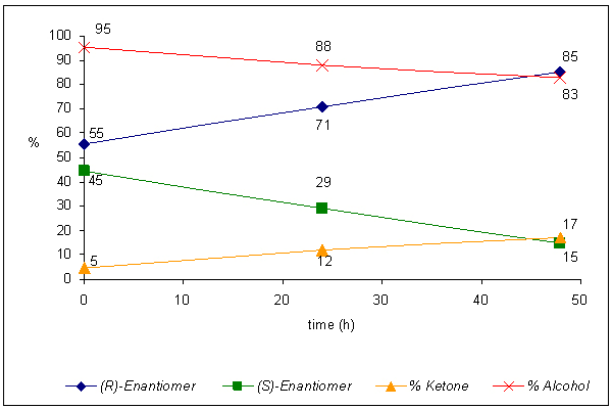

Figure 4. Evolution of the biotransformation of 1 in the 3-L bioreactor.

to the ketone were low after 24 hrs, we extended the biotransformation time, and the results are summarized in Figure 4.

From Figure 4 we can observe that after 48 hrs of biotransformation, the oxidative kinetic resolution provided a moderate enantiomeric ratio, 85/15 (*R*/*S*). The low amount of the ketone observed, 17%, can be due to two factors: dilution by the nature of the method or by a bioreduction of the 4-chlorobenzophenone, 2, formed.

CONCLUDING REMARKS

The (*S*)-4-(chlorophenyl)phenylmethanol was enantioselectively oxidized to 4-chlorobenzophenone (56%) using a suspension of cells in a phosphate buffer solution, allowing an excellent optical enrichment of the residual alcohol, after 32-36 hrs the enantiomeric ratio *R*/*S* was 93/7 (86% ee). The reverse process (reduction) was only present at very long biotransformation time and when both chemicals were present.

In contrast, when the enantioselective oxidation of 1, was performed with cells in the culture media in the 3-L bioreactor, the ketone was produced in just 17%, and the enantiomeric ratio R/S, after 48 hrs, was 85/15 (70% ee). This is a complex biocatalyzed process in which more than one reaction can occur, then a biocatalytic conversion of *rac*-4-(chlorophenyl)phenylmethanol was accomplished by a combined enantioselective oxidation-reduction sequence using the same 'single' biocatalyst (microorganism) for both steps (Voss et al. 2007).

If we compare with new approaches in the literature, for example the enzyme-catalyzed enantioselective reduction of diaryl ketones (Truppo et al. 2007), the biocatalyzed reduction (ketoreductases) of 4-(chlorophenyl)phenyl ketone produces only 64% ee of the corresponding (*R*)-diarylmethanol, this result proves the significant challenge of this kind of compounds.

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