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Article

Biotransformation of (S)-*cis*-verbenol with *Nocardia corallina* B-276

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A biotransformação de (S)-*cis*-verbenol com *Nocardia corallina* foi investigada usando-se dois procedimentos: Suspensão de células em tampão de fosfatos (pH 7), com várias relações substrato: células e bioreator de 3-L com células em meio de cultura. (1S)-(-)-verbenona foi obtida com excelentes rendimentos que variaram de >99% a 98%, em escala de 0,7 e 7 mmol respectivamente.

The biotransformation of (S)-*cis*-verbenol with *Nocardia corallina* was investigated using two methods: Suspension of cells in a phosphate buffer (pH 7) with various substrate:cells ratios; and bioreactor of 3-L with cells in the culture media. Both gave (1S)-(-)-verbenone with excellent yields ranging from >99 to 98%, at scale of 0.7 and 7 mmol respectively.

Keywords: biocatalysis, (1S)-(-)-verbenone, oxidations, *Nocardia corallina*

Introduction

Oxidation is one of the most frequently used processes in organic synthesis and it is an important reaction from the academic and industrial points of view. Although, a big number of methods has been developed to achieve this transformation, most of them have the inconvenient of using highly corrosive acids or toxic metallic compounds that generate undesirable waste materials.

The use of biotransformations offers significant advantages over chemical reactions and in the last ten years has been increasing constantly. Then biocatalysts have attracted much attention from the viewpoint of green chemistry.

As an alternative to chemical methods to oxidize alkanes, alkenes, aromatic compounds, aldehydes or alcohols, microbial oxidations have been used, among the oxidizing microorganisms are: *Acinetobacter*, *Candida*, *Corynebacterium*, *Cunninghamella*, *Nocardia*, *Pseudomonas* and *Rhodococus.*[1]

Due to our interest in microbial oxidations, we have been studying this biotransformation using *Nocardia corallina*, previously we reported the oxidation of aldehydes, and allylic and benzylic alcohols[2,3] to produce carboxylic acids or ketones with this microorganism, in a process using whole cells in the culture medium, at a 1 mmol scale. Besides we developed an important tool for the enantioselective oxidation of secondary alcohols in an ecofriendly way, with good yields at 28-30 °C.[4,5]

We focused our attention in (S)-*cis*-verbenol, (1*S*,2*S*,5*S*)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol, **1**, a bicyclic secondary allylic alcohol, to continue the study of this biotransformation because the oxidation product, (1S)-(-)-verbenone, (1*S*,5*S*)-4,6,6-trimethylbicyclo [3.1.1]hept-3-en-2-one, **2**, is a valuable and versatile compound, as well as an essential oil component. Which has been used in flavours and fragrances;[6] for the prevention and treatment of cancer;[7] as the antimicrobial agent, mastic, a natural resin obtained from the mastic tree, *Pistacia lentiscus*;[8] as an antioxidant agent in active packing for the food industry;[9] as an important semiochemical for the control of the tree-killing bark beetle (*Coleoptera: Scolytidae*);[10] and as anti-aggregation signal in *Ips typographus* used to protect spruce plantations.[11] Besides, ketone **2** is a relevant starting material in asymmetric synthesis, as a chiral precursor to cyclobutane carbocyclic nucleosides and oligopeptides[12] and cyclobutyl GABA analogues.[13]

Recent US[14] and European Community regulations[15] labelled as 'natural' flavour products prepared by enzymatic or microbial processes, allowing them to be included in products for human consumption, especially processed foods. These regulations have stimulated the research and development of new biotechnological

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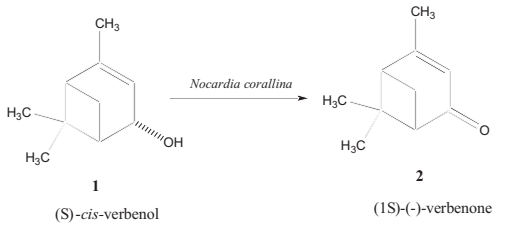
processes for these valuable compounds,[16] because the food industry shows a strong interest in unexpensive processes for the production of 'natural' products.

Herein we describe the results on the oxidation of (S)-*cis*-verbenol, **1**, carried out with *Nocardia corallina* to produce **2**, by a clean oxidation. Two methods were explored: oxidation using resting cells in a phosphate buffer and biotransformation in a 3-L bioreactor.



Scheme 1.

Results and Discussion

Our first approach was to carry out the oxidation of **1,** (Scheme 1), working with resting cells of *Nocardia corallina* in four different substrate: wet cells ratios, (m/m), 1:20, 1:30, 1:50 and 1:100 for 24 hours, at a 0.7 mmol scale. As can be observed in Figure 1, an increase in the cell ratio improved substantially the oxidation of **1**, then **2** was obtained in high proportion, 73, 89 and 81% using the ratios 1:30, 1:50 and 1:100 respectively, but with a ratio of 1:20, the conversion was lower, only 30%.

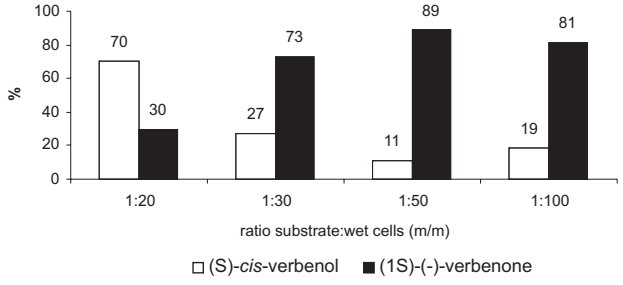


Figure 1. Oxidation activity of *Nocardia corallina* on various substrate:wet cells ratios (m/m). Measurement after 24 h of biotransformation by HPLC.

Contrary to what was expected, with the ratio 1:100 the conversion was lower (81%) than that observed using the ratio 1:50 (89%). As a possible explanation for these results could be the reduction of the formed ketone, **2,** to the starting alcohol **1**. In order to determine if this reversible process was taking place we subjected ketone, **2,** to the reaction using two different substrate:wet cells ratios, (m/m), 1:30 and 1:100, we detected by HPLC that after 24 and 48 hours, respectively, the ketone remained unchanged, and this compound was recovered in both cases. So we can say that *Nocardia corallina* did not reduce **2**, or the reduction was too slow to be considered, in the reaction conditions studied.

Since the ratio 1:30 gave good conversion to **2** after 24 h (73%), we continued the oxidation for a longer period of time, and at 48 h the alcohol **1** was quantitatively oxidized to the ketone (Figure 2)**.** In all these experiments, a sample of resting cells without substrate was subjected to similar conditions, as control.

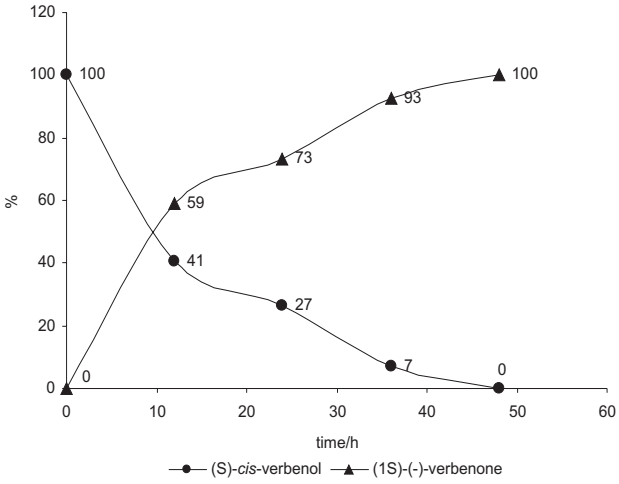


Figure 2. Progression curve with time for the oxidation of **1** to **2**. 1:30 substrate: wet cells ratio.

The isolation of the corresponding ketone **2**, from the suspended resting cells method, was very easy due to its low solubility in water and to the small working volume, in contrast with the difficulties found in the work up of the oxidation reaction from the culture medium.[2]

Since it was necessary to obtain enough biomass of *Nocardia corallina* to apply this methodology, we studied the growth kinetics of the microorganism under batch cultivation in a 3-L fermentor. The best conditions found were: agitation rate 226 rpm, aeration rate 0.9 vvm (air only), pH 8.4 and 28-30 °C. The cell growth was monitored by optical density (660 nm), previously we correlated the optical density with the quantity of the dry cells, data not shown. After 5 hours the bioreactor had enough biomass (wet cells 18.4 g L^{-1}) to carry out the biotransformation in the cell culture or to harvest the cells. This point was coincident with the end of the logarithmic growth, from the data in Figure 3 we can see that the % of dissolved oxygen after 5-6 h began to increase.

In a second method to obtain **2**, we carried out this biotransformation in the 3-L bioreactor. With respect to the scale, in the resting cells method were used 0.7 mmol of **1** and it was increased to 7.24 mmoles. After 48 h the product **2** was extracted with ethyl acetate and purified

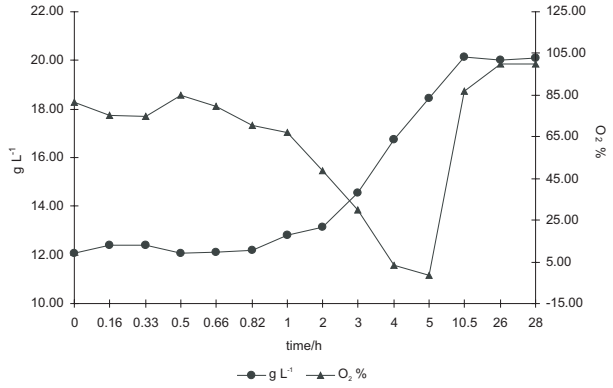


Figure 3. Biomass concentration and % of dissolved oxygen *versus* fermentation time.

by column chromatography, the yield was 98%, with a >99% purity, determined by GC.

In general, enzymatic oxidations work best at elevated pH (8-9) where, in contrast, nicotinamide cofactors are unstable.[1] But, in our hands, when the pH was kept between 8.0-8.5 during the whole process, the alcohol **1** was no oxidized and was quantitatively recovered.

We found that if we add the alcohol, **1,** after 6 h of cell growth, the initial pH was 7.44 and after the end of the process (54 h) the final pH was 7.63 (Figure 4), the yield was almost quantitative (98%). So the pH in this biotransformation was crucial.

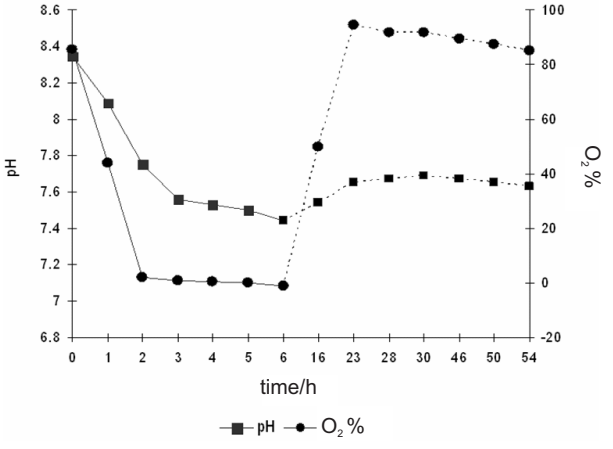


Figure 4. Variation of pH and the % of dissolved oxygen in the growth phase (bold line) and during the biotransformation (dotted line).

The variation of pH and the % of dissolved oxygen in the growth phase and during the complete biotransformation are presented in Figure 4.

Experimental

Compounds **1** and **2** were purchased from Aldrich and they were characterized by infrared spectra, recorded on a Perkin-Elmer Paragon 1600 FT, as liquid films; Hydrogen and Carbon nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR), were recorded on a Varian 400 MHz instrument, in $CDCl_3$ using tetramethylsilane as internal reference; and by TLC on silica gel 60 GF_{254} (Merck). HPLC analysis was performed on an Agilent 1100 liquid chromatograph, equipped with a diode array detector, using a Hypersil BDS-C18 (5 μm, 250 × 4.6 mm) column; the mobile phase was methanol-water (90:10), 0.8 mL min^{-1} and at 24ºC. The GC analysis was performed on a Hewlett-Packard HP 6890 gas chromatograph, equipped with a flame ionization detector, a HP-5 column (30 m × 0.33 mm), at 100 °C, N_2 as carrier gas, at 0.6 mL min^{-1}. Optical rotations were measured in a Perkin-Elmer polarimeter model 341.

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 of fermentor were monitored continuously, samples were withdrawn every hour and the optical density (at 660 nm) and the dry cell weight (g L^{-1}) were measured.

Organism and growth

Nocardia corallina B-276 (ATCC 31338)[17] was grown at 28-30 °C on agar plates (15 g L^{-1} agar; 3 g L^{-1} beef extract; 5 g L^{-1} peptone L^{-1}). Liquid cultures were incubated in an orbital shaker, the broth composition was: Solution A: 0.05 g L^{-1} $FeSO_4 \cdot 7H_2O$; 1.74 g L^{-1} K_2HPO_4; 2 g L^{-1} $(NH_4)_2SO_4$; 1 g L^{-1} yeast extract L^{-1}; Solution B: 1.5 g L^{-1} $MgSO_4$; Solution C: 2 g L^{-1} glucose; each solution was sterilized separately, later combined and the pH adjusted to 8.0 (± 0.5).

Biotransformation using resting cells[18]

Pre-culture

A 500 mL Erlenmeyer flask containing 200 mL of sterile culture medium was inoculated from an agar plate (three days old) and incubated at 28-30 °C on an orbital shaker (170 rpm) for 48 h. The cells were collected by centrifugation at 4500 rpm for 15 min. Cells were washed twice with potassium phosphate buffer (0.1 mol L^{-1}, pH 7.0).

The cells were incubated in 50 mL phosphate buffer, 0.1 mol L^{-1}, pH 7.0, for 30 min. at 28-30 °C on an orbital shaker (170 rpm), then (S)-*cis*-verbenol was added to the whole cells, with a substrate:cells ratio (m/m) 1:30, using 0.6 % (v/v) of *N,N*-dimethylformamide, and were shaked under the same conditions for 48 h. Then, the reaction mixture was centrifuged at 4500 rpm for 15 min. The (1S)-(-)-verbenone

was extracted with ethyl acetate (4×15 mL) and purified by column chromatography, SiO_2, (*n*-hexane: diethyl ether = 4:1). The product was dissolved in 0.5 mL of methyl alcohol, HPLC grade, and analyzed by HPLC using a C18 column, detected at 215 nm, and methyl alcohol-H_2O (90:10) as the eluent, to determine the conversion degree, $t_{r(alc)}$= 5.672 min and $t_{r(ketone)}$= 5.007 min. The product, isolated as a yellow oil, was identified by $[\alpha]_D^{20}$= –121.8° (*c* 1.9, $CHCl_3$) [lit.[19] $[\alpha]_D^{20}$= –126.5° (*c* 0.29, $CHCl_3$)]; IR(neat): ν_{max}/cm^{-1} 1679; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.73 (1H, m), 2.79 (1H, m); 2.64 (1H, dt, *J* 5.7, 1.8 Hz), 2.41 (1H, t, *J* 5.1 Hz), 2.08 (1H, d, *J* 9.0 Hz), 2.01 (3H, s); 1.49 (3H, s); 1.01 (3H, s): ^{13}C NMR (100 MHz, $CDCl_3$): δ 203.6, 169.9, 120.9, 57.4, 53.9, 49.5, 40.7. 26.5, 23.5, 21.9 ppm; and thin layer chromatography against an authentic sample, the data were in full accordance with the literature values.[19,20]

Control experiments

The (S)-*cis*-verbenol, (**1**), in potassium phosphate buffer (0.1 mol L^{-1}, pH 7.0), was shaked without the microorganism under the same conditions for 48 h, after working-up the alcohol **1** was quantitatively recovered.

(1S)-(-)-verbenone, (**2**), was subjected to the same conditions for 48 h, **2** was also quantitatively recovered.

Biotransformation using a bioreactor

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

Pre-culture I: A 500 mL Erlenmeyer flask containing sterile culture mediun (200 mL), was inoculated from an agar plate (three days old) at 28-30 °C on an orbital shaker 170 rpm for 40-44 h (for duplicated). The content of both pre-culture I flasks (400 mL) was aseptically poured into a 3-L bioreactor (Pre-culture II) containing 1800 mL culture medium at 28-30 °C, 226 rpm and 0.9 vvm. Cellular growth was monitored every hour and analyzed by optical density (660 nm). After 6 hours, (S)-*cis*-verbenol (1.10 g, 7.24 mmol) in *N,N*-dimethylformamide (13.2 mL), were added and incubated in the same conditions for 48 h. The reaction mixture was saturated with NaCl and centrifuged at 4500 rpm for 15 min, the (1S)-(-)-verbenone was extracted with ethyl acetate, and purified by column chromatography, SiO_2, (*n*-hexane: diethyl ether = 4:1), producing 1.06 g (7.07 mmol, 98% yield). A sample was analyzed by HPLC in the conditions indicated above to determine conversion percentage. The purity determined by GC was >99%, t_r= 3.886 min. The (1S)-(-)-verbenone obtained was compared with an authentic sample by IR, ^{1}H NMR, ^{13}C NMR, optical rotation and thin layer chromatography.

Conclusions

Nocardia corallina B-276 oxidize in a convenient and ecofriendly way (S)-*cis*-verbenol, **1**, to (1S)-(-)-verbenone, **2**. In the two methods studied the yields were excellent. The method using resting cells has been shown to be an alternative to study these biocatalyzed oxidations, because the low working volume and the possibility to use different substrate: wet cells ratios. But in both methods the yields were excellent, more than 98% in 48 h, in contrast with the process of bioconversion of (-)-α-pinene to (1S)-(-)-verbenone using *Catharanthus roseus*, *Psychotria brachyceras* and *Raufolfia sellowii* [21] and immobilised *Picea abies* cells,[11] these biotransformations need 5 to 15 days to complete. *Nocardia corallina* did not show reductase activity over the ketone or in the C=C bond of the enone under the conditions studied, in contrast to *Nicotiana tabacum* that is able to reduce verbenone to verbenol and verbanone.[22] The preparation of substrates by enzymatic or microbiological processes will permit their use as 'natural' products, for example they can be added to essential oils and reinforce their characteristics or attributes.

Acknowledgments

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