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A bioprocess for the production of high concentrations of *R*-(+)-α-terpineol from *R*-(+)-limonene

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ABSTRACT

A bioprocess with a high conversion rate of limonene to α-terpineol was described. The enzyme hydratase involved in this process was found as being cofactor independent, non-inducible and able to perform the transformation of both *R*-(+) and *S*-(−)-limonene. The system used consisted of a biphasic medium in which the aqueous phase contained a concentrated resting cells of *Sphingobium* sp. and the organic phase was sunflower oil. After 30 h at 30 °C ca. 25 g of *R*-(+)-α-terpineol per liter of organic phase were obtained from *R*-(+)-limonene in Erlenmeyer flasks. Performance of the bioconversion in a bioreactor increased the production rate with no changes in yield and maximal *R*-(+)-α-terpineol concentration, which demonstrated that experiments in flasks were limited by liquid–liquid transport phenomena. A mathematical model able to explain the fact that the reaction always stopped before the precursor became exhausted has also been proposed and validated. Finally, the process reported was the most promising alternative for the biotechnological production of natural *R*-(+)-α-terpineol published so far and up to ca. 130 g L^{-1} metabolite could finally be obtained.

1. Introduction

R-(+)-Limonene is the most widespread monocyclic monoterpene in nature, accounting for more than 90% of orange peel oil [1]. It is thus an abundant and inexpensive precursor for the synthesis of fine chemicals. Biotechnological conversions of *R*-(+)-limonene for the production of bioflavors have been considered in the past few years [2]. However, the low yields and high production costs usually discourage their commercial adoption [3]. α-Terpineol, one of its oxygenated derivate, is a stable alcohol typically applied in household products, cosmetics, pesticide and flavor preparations [1,4]. This monoterpenoid is an important commercial product commonly produced by acid-catalyzed chemical synthesis from α-pinene or turpentine oil. Nevertheless, the biotransformation of limonene to α-terpineol as main product has already been described for the fungi *Cladosporium* sp. [5], *Penicillium digitatum* [6–10] and *Fusarium oxisporum* [11,12]. For bacteria, this conversion pathway has been reported in *Pseudmonas gladioli* [13,14], a recombinant *Escherichia coli* [15] and *P. fluorescens* [16]. The bioproduction of α-terpineol as main product has also been shown as possible using α- and β-pinenes as substrates [17–21].

The main drawback of monoterpenes biotransformation processes are the chemical instability, high volatility and high cytotoxicity of both precursors and products, the low solute solubility, and the low transformation rates [22]. The use of biphasic systems has been demonstrated to be an efficient technique since it eases the product recovery and increases yields by reducing the substrate and product toxicity and their losses by volatilization [23]. The organic phase is usually hydrocarbon solvents (*n*-decane, *n*-hexadecane) with an octanol:water partition coefficient (log *P*) higher than 4, a commonly accepted requisite for a good tolerance by whole microbial cells [23]. This strategy has already been applied for the production of isonovalal from α-pinene oxide by *Pseudomonas rhodesiae* [24] and patented for the bioconversion of some terpenes [25]. The use of vegetable oils as organic phase, already used for the biotransformation of sterols [26], has not, up to now, been considered for the bioconversion of monoterpenes, although they could be considered as an environment friendly alternative to organic solvents.

Production of 0.7 g L^{-1} [5], 1 g L^{-1} [13] and 2.4 g L^{-1} [12] of α-terpineol from limonene have already been reported, while the highest recovery of α-terpineol reported so far, about 3.2 g L^{-1}, has been obtained using sequential substrate feedings [7]. However, these values appear to be too low to allow an economic development of these processes. This work reports a methodology for the production of high amounts of *R*-(+)-α-terpineol from *R*-(+)-limonene in biphasic medium using vegetable oils as organic phase. It seems to be the most efficient alternative for the

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commercial production of natural *R*-(+)-α-terpineol reported so far.

2. Materials and methods

2.1. Microorganisms and chemicals

The strain employed in this work, formerly known as *Pseudomonas fluorescens* NCIMB 11671, has been re-assigned as *Sphingobium* sp. This was done by the Pasteur Institute (Paris, France), using the classical method of partial sequencing of the 16S RNA gene. The terpene compounds *R*-(+)-limonene (Fluka, ~98% purity), *S*-(−)-limonene (Fluka, ≥95% purity), (+)-limonene-1,2-oxide (Aldrich, mixture of *cis*/*trans* isomers, 97% purity), (+)-carvone (Acros Organics, 98% purity), *R*-(+)-α-terpineol (Fluka, ~99% purity) and α-terpineol (Aldrich, ~98% purity, $[\alpha]^{19} = -30^{\circ}$) were kept under refrigeration temperature. *n*-Hexadecane (SDS, 99% purity), sunflower oil (commercial grade), rapeseed oil (commercial grade), heptadecane (Fluka, ≥98% purity) and 1-pentanol (Acros Organics, 99% purity) were kept at ambient temperature.

2.2. Biocatalyst production

Three full loops of a 24 h old culture on Petri dish were transferred to a 500 mL-conical flask containing 1.0 g glucose, 0.25 g $(NH)_4SO_4$, 5 mL Hutner solution [27], 10 mL of solution A and 235 mL distilled water. Solution A was made of 6.5 g K_2HPO_4 and 8.28 g KH_2PO_4 in 250 mL distilled water. The flasks were incubated at 30 °C and 200 rpm for 24 h, time at which the optical density at 600 nm (OD_{600}) was close to 4.0. The resulting cultivation medium was referred to as pre-culture.

Twenty milliliters of the pre-culture were aseptically transferred to a 500 mL-conical flask with the same culture medium as described above except that glucose was replaced by an organic solution containing 500 μg of limonene in 12.5 mL of *n*-hexadecane (final concentration of 40 g per liter of organic solvent). The flasks were left at 30 °C and 200 rpm for 24 h, time at which the OD_{600} of the aqueous medium reached 0.8.

Growth experiments in the bioreactor (4.8 L working volume) were performed using 170 mL of the above pre-culture as inoculum, 4 L distilled water, 170 mL solution A, 85 mL Hutner solution, 4.25 g $(NH)_4SO_4$, 212.5 mL hexadecane and 15 g limonene (70 g L^{-1} organic solvent). Temperature, agitation and aeration were kept at 30 °C, 500 rpm and 0.3–0.5 slpm, respectively. The CO_2 and O_2 in the air exiting from the bioreactor were monitored by a gas analyzer (Servomex 4100, Servomex, Zoetermeer, The Netherlands), the pH was controlled at 6, the OD_{600} of the liquid phase and the composition of organic and aqueous phases (GC-FID, see Section 2.4) were monitored.

After centrifuging the culture medium at 2600 × *g* for 10 min, the resulting biomass was resuspended in phosphate buffer 20 mM pH 7.5 in order to achieve a 10-fold concentration. This biomass was either used directly for the biotransformation with fresh cells (biomass from conical flasks) or frozen (−18 °C) before its use (biomass from bioreactor). The biomass concentration was determined using the formula:

$$\text{Biomass}\,(\text{g L}^{-1}\ \text{medium}) = 0.35 \times \text{OD}_{600}.$$

The residual ammonium content was determined using the Patton and Crouch [28] colorimetric method. For this purpose, samples were previously membrane filtered (pore size 0.45 μm).

2.3. Biotransformation procedure

Twenty-five milliliters of the concentrated biomass (OD_{600} = 7 for fresh cells and OD_{600} = 13 for frozen and thawed biomass) and the same volume of organic phase were transferred to a 250 mL-conical flask. The substrate was added to reach a final concentration of 40 g per liter of organic phase. The flasks were incubated at 30 °C and 200 rpm. The performance of anaerobic bioconversion involved the use of 250 mL-conical flasks with two entries (one at the base and another at the neck) plugged with a rubber stopper instead of polyurethane plugs. At the beginning of the conversion, as well as after each sampling, the medium was flushed by bubbling N_2 (~0.5 L min^{-1}) from the base entry for 5 min. For the bioconversion in bioreactor (0.5 L), 150 mL of concentrated frozen cells and the same volume of organic phase with 40 g L^{-1} of *R*-(+)-limonene were maintained at 30 °C and 800 rpm.

Samples were periodically taken from the organic phase to follow the substrate consumption and the formation of products. When *n*-hexadecane was used as organic phase, it was directly injected (1 μL) in the gas chromatograph while the vegetable oils had to be extracted (1 min vortexing) with the same volume of 96 vol% ethanol which was also assayed by GC (see Section 2.4).

2.4. Analytical conditions

The products obtained were analyzed with a HP 5890 gas chromatograph with flame ionization detector (GC-FID). A SBP-5 (Supelco) capillary column of 30 m × 0.32 mm i.d. × 0.25 μm film thickness was employed. Nitrogen was used as gas carrier with a constant pressure at the head of the column of 0.8 bar and the injection split ratio was 1:5. The oven temperature program used involved an initial temperature of 80 °C hold for 5 min, then rise at 20 °C min^{-1} to 200 °C, value maintained for 5 min. The injector and detector temperatures were both 250 °C. Solute quantification was performed after adding 1% (v/v) heptadecane in *n*-hexadenane samples or 0.2% (v/v) 1-pentanol in ethanol samples as internal standards and concentrations were expressed as mass of product per liter of organic phase.

The identification of enantiomers was carried out by comparison of the retention time of standards (*R*-(+)-α-terpineol and α-terpineol ($[\alpha]^{19} = -30^{\circ}$; majority of *S* isomer)) with samples. A Beta Dex™ 120 fused silica capillary column (Supelco; 30 m, 0.25 mm i.d., 0.25 μm film thickness) was mounted in a HP 6890 gas chromatograph with flame ionization detector (GC-FID) with an oven temperature maintained at 120 °C for 20 min, then rise to 200 °C at 50 °C min^{-1}, value hold for 5 min. The other conditions were the same as described above. The enantiomeric excess (ee) was estimated by the ratio of the *R*/*S* areas as obtained after GC analysis.

Unknown products were analyzed by a HP 6890 gas chromatograph coupled to a HP 5973 mass selective detector (GC–MS). The carrier gas was helium and the injection split ratio was 1:5. The capillary column was the already mentioned SBP-5 used with the same conditions as above. The MS system was operated with an electron impact of 70 eV, an acceleration tension of 1.1 kV and an emission current of 35 μA. The temperatures of quadrupole, ionic source and interface were 150 °C, 230 °C and 280 °C, respectively.

Results were expressed with respect to the organic layer, and they were found to be always obtained with a relative error less than 10%.

3. Results and discussion

3.1. Use of limonene as sole carbon source for the biocatalyst production

The culture of *Sphingobium* sp. with limonene as sole carbon source was monitored in a 4.8 L bioreactor coupled with a gas analyzer (see Section 2). The results obtained revealed the presence of a long adaptation phase characterized by a very slow growth rate (0–25 h). The true active growth took place after this time and biomass production stopped near 40 h, before the carbon source became exhausted. Ammonium assay at this time revealed that no residual mineral nitrogen was present, and it could be assumed that nitrogen source was the limiting substrate. At this stage, the culture reached a concentration of 1.4 g of biomass per liter of aqueous phase (OD_{600} = 4). The global mass yield was 67% in biomass (Fig. 1) and the carbon balance was close to 100% at the end of the active growth period phase.

It is worth noticing that trace amounts of limonene-1,2-diol were detected throughout the growing phase (<40 h) while traces of α-terpineol were evidenced after, during the stationary phase (data not shown). These observations were consistent with an already reported hypothesis, where this microorganism presents

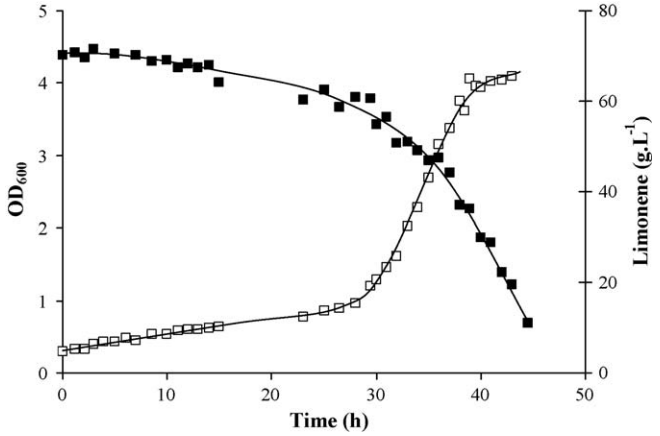

Fig. 1. Growing curve of *Sphingobium* sp. (□) in *R*-(+)-limonene (■) as sole carbon source. Culture developed in a 4.8 L bioreactor at 30 °C/500 rpm and aeration of 0.3–0.5 slpm using *n*-hexadecane as organic phase. Limonene concentration is expressed in g L^{-1} of organic phase, while the OD_{600} was determined in the aqueous phase.