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Screening of microorganisms for bioconversion of (−)β-pinene and R-(+)-limonene to α-terpineol

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ABSTRACT

This work is focused on the bioconversion of (−)β-pinene and R-(+)-limonene to α-terpineol. To carry out the present study, 400 microorganisms were tested for their ability to bioconvert the substrates. From the microorganisms, no one was able to convert R-(+)-limonene and 4 were able to bioconvert (−)-β-pinene to oxygenated monoterpenes. The metabolites recovered were α-terpineol (2856.54 ± 50.23 mg/L) and fenchol (traces) for *Aspergillus niger* ATCC 16404, α-terpineol (688.13 ± 41.27 mg/L) for *A. niger* ATCC 9642, α-terpineol (172.07 ± 32.94 mg/L) for *A. niger* ATCC 1004 and α-terpineol (24.38 ± 2.78 mg/L) and *trans*-pinocarveol (traces) for *Penicillium camembertii* ATCC 4845. After screening and optimization experiments, the best experimental condition for bioconversion of (−)β-pinene to α-terpineol was established using *A. niger* ATCC 16404 at 35 °C without addition of vitamin solution, yielding a conversion in α-terpineol of 15494.34 ± 193.87 mg/L.

1. Introduction

Flavors and fragrances are extremely important in food, cosmetic, chemical and pharmaceutical industries. Most part of available flavor compounds is obtained by chemical synthesis or extraction. The growing aversion of the consumer towards chemicals added to food, cosmetics and other household products has induced the flavor companies to direct their attention towards flavors compounds of biological origin, so called natural or bio-flavors (Adams, Demyttenaere, & De Kimpe, 2003; Maróstica & Pastore, 2007).

Terpenes and especially their oxygenated derivatives are extensively used in flavor industries. Limonene (4-isopropenyl-1-methylcyclehexane), a monocyclic monoterpene, is a low priced monoterpene and in most cases the major compound in essential oils of citrus fruits (De Conti, Rodrigues, & Moran, 2001; Onken & Berger, 1999; van der Werf, de Bont, & Leak, 1997). Pinenes are bicycle monoterpenes of low price and commonly used as substrates for biotransformation (De Conti et al., 2001). α-Terpineol is commonly used as fragrance compound. It is mainly produced chemically, starting from pinene or crude turpentine oil by acid hydration to terpine, followed by partial dehydration. In this way, α-terpineol is commercially available at relatively low price (Janssens, De Pooter, Schamp, & Vandamme, 1992; Tan, Day, & Cadwallader, 1998). Based on this aspect, the implementation of a microbial process requires high yields of α-terpineol in order to be competitive compared to the chemical transformation (Tan et al., 1998).

Screening of microorganisms, plants or animal cells represent a natural way to obtain new biotechnological products at relative low cost. The microorganisms, in this case, are of particular interest due to the large diversity of metabolic processes and enzymes involved, and the unlimited number of microorganisms in nature that can be tested. They modify and degrade a variety of organic molecules and complexes, and so, it can be expected that one of them may be responsible for catalyzing a specific reaction (Tan & Day, 1998).

The bioconversion of limonene to α-terpineol as the main end product has been described using a wide range of microorganisms as catalyst: *Pseudomonas* sp. (Yoo & Day, 2002), *Cladosporium* strain (Mattison, McDowell, & Baum, 1971), *Penicillium* sp. isolated from orange peel (Mattison et al., 1971), *Penicillium digitatum* (Mattison et al., 1971; Tan et al., 1998; Van Resburg, Moleleki, van der Walt, Botes, & Van Dyk, 1997) and *Pseudomonas gladioli* (Cadwallader & Braddock, 1992; Demyttenaere, Belleghem, & De Kimpe, 2001). However, the bioconversion of β-pinene to α-terpineol has been

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hardly described in the literature. Van Dyk, van Resburg, and Moleleki (1998), using *Hormonema* sp., obtained pinocamphone from (−)β-pinene. Yoo and Day (2002), using *Pseudomonas* sp., obtained limonene, ρ-cymene, α-terpinolene, α-terpineol and endo-borneol as the five major compounds from β-pinene. *Aspergillus niger* ATCC 9642 was also used as catalysts for this biotransformation, and the compound obtained was α-terpineol with low conversion (up to 4%) (Toniazzo, Oliveira, Dariva, Oestreicher, & Antunes, 2005).

In this context, this work describes a detailed study related to the bioconversion of limonene and β-pinene to the corresponding oxygenated compounds. From 400 tested of microorganisms, a pre-fermentation in a minimal medium containing limonene or β-pinene as sole carbon source was carried out. In a second step, a fermentative screening with all isolated microorganisms was performed so as to verify the process conversion. The best strain selected in the screening was used in a last step, with the main objective of verifying the influence of some fermentation variables on the reaction conversion.

2. Experimental

2.1. Chemicals

(−)-β-pinene (97%) and R-(+)-limonene (97%) from Aldrich were used as substrates without any pre-treatment. Other used chemicals were ethyl acetate (99.5%, Quimex), sodium sulphate (99%, Quimex) and ethanol (99.8%, Vetec).

2.2. Microorganisms

A. niger ATCC 16404, *A. niger* ATCC 9642, *A. niger* ATCC 1004 and *Penicillium camembertii* ATCC 4845 were used in this work. The microorganisms were inoculated in plates with Potato Dextrose Agar (PDA) medium and incubated at 28 °C for 5–7 days. After growth of several microorganisms, they were sub-cultured to PDA plates and incubated at 28 °C for 5–7 days. This procedure was performed until the complete microorganism isolation. Some microorganisms belonging to our culture collection (Laboratory of Biotechnology-URI-Campus de Erechim) were also tested in this step.

2.3. Pre-screening experiments

After the fungus filamentous growth in PDA medium (Acumedia Manofactures, In. Lansing, Michigan 48912), 5 mL of saline water (0.85 g/100 mL) were added and stirred for removing the spores. Bacteria and yeasts were grown in PD medium (Acumedia Manufactures, In. Lansing, Michigan 48912) in microtubes, centrifuged for cellular mass precipitation, the supernatant was discarded and 1 mL of water containing salt at the level of 0.85 g/100 mL were added.

After dilution, aliquots of 100 μL were removed and inoculated in microtubes with 1200 μL of minimal MS medium (3.0 g/L of $NaNO_3$, 1.0 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4$, 0.5 g/L of KCl and 0.01 g/L of $FeSO_4$) added of glucose (1 g/100 mL). Another aliquot of 100 μL was inoculated in microtubes containing 1200 μL of minimal MS medium added of limonene (1.5 g/100 mL). Then, the microtubes were electromagnetically stirred (60 Hz) for 10 days at 28 °C. The samples were analyzed in spectrophotometer at 490 nm at 0, 2, 6 and 10 days so as to evaluate the cell growth. All tests were carried out in triplicates runs.

2.4. Screening experiments

Screening experiments were performed to select the most promising microorganisms with ability to bioconvert the substrates β-pinene and limonene. For this, a loopfulls of cells stored in medium agar slants was used, which was aseptically transferred to Erlenmeyers containing 30 mL of the specific medium of each microorganism. Filamentous fungi were cultivated in PD medium (300 g/L infusion from potatoes and 20 g/L glucose), yeasts were cultivated in YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose) and bacteria in LB medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl). The cultures were aerobically incubated in shaker (150 rpm) at 25 °C for 72 h for fungi and for 24 h for bacteria and yeasts.

The biotransformation experiments were started in orbital shaker (150 rpm) 72 h after inoculation for filamentous fungi and 24 h for bacteria and yeasts, by adding the substrate directly into the culture flasks with 30 mL of medium. Substrate was added as solutions in absolute ethanol (1:1 v/v). In all experiments it was used 1 mL/100 mL of substrate in 1 mL/100 mL of EtOH, at 25 °C and 150 rpm during 6 days. Toniazzo et al. (2005) showed that EtOH had a positive effect on the bioconversion, when applied in concentration of 1 mL/100 mL. All experiments were carried out in parallel with controls, in the same conditions without the presence of microorganism. All runs of each experimental was performed in duplicate in closed stoppered glass flasks in order to avoid the substrate and product evaporation.

At the end of the experiments, the cells were removed by filtration for filamentous fungi and centrifugation for bacteria and yeasts. The product recovery was performed by liquid–liquid extraction using for three consecutive times of 25 mL of ethyl acetate. After extraction, the solution volume was completed to 25 mL of ethyl acetate. The final solution was dried over anhydrous sodium sulphate.

The reaction products were identified by GC/MS (Shimadzu QP5050A), using a capillary column DB-WAX (30 m, 0.25 mm, 0.25 μm). The column temperature was programmed to 50 °C for 3 min, increased at 5 °C/min at 130 °C and then increased at 15 °C/min at 210 °C by 5 min. Helium was the carrier gas, and the injection and detector temperatures were 250 °C. 0.5 μL of the solution was injected into the GC/MS system. The apparatus operated with a flow rate of 1 mL/min in electronic impact mode of 70 eV and in split mode (split ratio 1:10). The identification of the compounds was accomplished by comparing the mass spectra with those from the Wiley library, and by additional comparison of the GC retention time of standard compounds. The quantification was carried out by the standard curve of the interest compound, evaluating the relative area from the interest compound and the standard curve. (−)-β-pinene (99%, Fluka), *R*-(+)-limonene (97%, Aldrich) and α-terpineol (90%, Aldrich) were used as external standards.

After selecting the most promising microorganism to bioconvert the substrate (based on the higher product conversion), studies were carried out for evaluating the effect of some process variables on cell growth and bioprocess conversion.

2.5. Effect of process variables on cell growth

2.5.1. Influence of addition of vitamin solution

The influence of addition (15 μL) of vitamin solution (100 g nicotinic acid, 0.2 g ρ-aminobenzoic acid, 0.2 mg biotin, 50 mg pyridoxine, 100 mg riboflavin per liter in deionized water, aseptically filtered and stored at 4 °C) on cell growth was also investigated. Aliquots of 1 mL of spores suspensions (1.63×10^9 spores/mL) of *A. niger* ATCC 16404 were transferred aseptically to an Erlenmeyer with 30 mL of PD media as inoculum and incubated for 72 h at 28 °C and 120 rpm. In order to evaluate the influence of vitamin solution, the cultures were filtered and the dried mycelium (24 h, 105 °C) was weighted. All experiments were carried out in