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Biotransformation of Citronellol in Rose Oxide by *Pseudomonas* Spp.

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Rose oxide, a flavour-impact component, occurs in traces in some essential plant oils, such as Bulgarian rose oil, and it is considered one of the most important fragrance materials in creating rosy notes for perfumery. The biotechnological production of rose oxide by using citronellol as a precursor in biotransformation processes has called particular attention as an alternative to chemical synthesis and extraction from natural sources. The biotransformation of citronellol by strains of *Pseudomonas* spp. was reported in this paper. The main bioconversion products were *cis*- and *trans*-rose oxides, reaching the yield of 29.67 mg/L. In citotoxicity tests, the strain showed impressive terpene resistance, growing under high concentrations of citronellol. Auto-oxidation products were not detected in the control experiments. Thus, rose oxide production by *Pseudomonas* spp. appeared as a promising alternative for commercial production of this bioflavour.

1. Introduction

The flavour industry is definitely moving more towards the natural arena, which is growing faster than synthetic market space, mainly due to the preference of consumers for products or ingredients bearing the label ‘natural’. Therefore, the biotechnological production of flavours appears as an interesting alternative to overcome the problems associated to chemical synthesis and extraction from natural sources (Ibdah et al., 2010). Biotransformation has emerged as an advantageous method for production of bioflavors since they proceed under mild conditions, do not generate toxic wastes, and the products can be labelled as ‘natural’ (Berger, 2009). In addition, bioconversion processes are able to produce compounds not easily prepared by chemical methods (Bicas et al.2009). Besides, the enzymes are capable of regio- and enantioselective hydrolysis producing enantiomerically pure compounds, which is especially important when optical isomers of a specific aroma compound exhibit different sensorial properties (Serra et al., 2005, Ibdah et al., 2010).
In this sense, the genus of *Pseudomonas* spp. appears to have great potential as biological agents in microbial transformation of terpenes, since they have few restrictions regarding environmental and nutritional conditions and have been subject of pioneering studies on terpene bioconversion. First studies on biotransformation of terpenes started 40-50 years ago, when Seubert (1960) reported the ability of a strain isolated from soil, *Pseudomonas citronellolis*, to use citronellol as sole carbon source. Strains of *Pseudomonas* including *P. mendocina*, *P. aeruginosa* and *P. fluorescens* were also reported to degrade citronellol (Cantwell et al., 1978; Förster-Fromme and Jendrossek, 2006; Tozoni, 2010). In addition, the catabolic pathway of citronellol in *Pseudomonas* species was investigated and elucidated

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by several research groups (Seubert et al. 1963; Seubert and Remberger 1963; Seubert and Fass 1964a,b; Cantwell et al., 1978; Förster-Fromme and Jendrossek, 2010).
Citronellol (3,7-dimethyl-6-octen-1-ol) is a linear monoterpene alcohol naturally ocurring in about 70 essential oils, with prevalence in bulgarian rose oil and geranium oil (Rao et al., 2004; Demyttenaere et al., 2004). This monoterpene is an interesting flavour compound for food and flavour industries due to the presence of floral notes (Schrader and Berger, 2001). It is also used as flavor agent in perfumes, cosmetics, detergents, and "biosafe" insect repellents. Citronellol is a natural compound which occurs as two isomeric optical forms. The *R*-(+)-isomer is commonly found in plant essential oils, while *S*-(-)-isomer is a natural constituent of geranium oil and citronella oil (Sousa et al., 2006).
The bioconversion of citronellol led to production of several metabolites (Demyttenaere et al., 2004, Onken and Berger, 1999; Tozoni et al., 2010). However, there is particular interest in the biotechnological production of rose oxide, a high value flavour compound. Rose oxide ((4-methyl-2-(2-methyl-1-propenyl)-tetrahydropyran) is a chiral monoterpenoid ether found in traces in some essential oils of plants, such as Bulgarian rose, Geranium and Damask rose, and contributes immensely to the unique bloomy-green top notes of these essential oils (Onken and Berger, 1999; Wüst et al., 1998; Babu et al., 2008). Thus the biotechnological production of rose-oxide is especially interesting to flavour industry, since it is one of the most important components in creating rosy notes in perfumery.
The biotransformation of citronellol into rose-oxides, has already been reported and studied for several other microorganisms (Onken and Berger, 1999, Demyttenaere et al., 2004, Maróstica and Pastore, 2006). To the best of our knowledge, bacterial biotransformation from citronellol into high value flavor compounds such as rose oxides by *Pseudomonas* spp. strains has not been published before.
This study investigated the production of rose oxide derived from biotransformation of citronellol by strains of *Pseudomonas* spp.

2. Material and Methods

2.1 Reagents and Standards

(+/-)-Citronellol was supplied by the Sigma-Aldrich Chemical Company and the (+/-)-*cis*/*trans*-Rose oxides from Fluka. All other chemicals and solvents were of the best available commercial grade.

2.2 Microorganisms and cultivation

Isolation of microorganisms from soil and fruit samples was carried out in selective-differential agar medium for *Pseudomonas*. Samples of soil, fruits and vegetables were incubated in liquid yeast-malt medium (YM: 1.0 % glucose, 0.5 % bacteriological peptone, 0.3 % malt extract, 0.3 % yeast extract) at 30 °C for 24 h. One full loop of 24-h-old cultures were transferred to petri dishes with solid medium agar containing 0.14 % $MgCl_2$, 1.0 % bacteriological peptone, 0.16 % $K_2HPO_4 \cdot 3H_2O$, 1.5 % bacteriological agar and 0.2 % TTC solution (5 % of 2,3,5-triphenyltetrazolium chloride added to distilled water). The TTC was sterilized by filtering through 0.22 µm membrane filters (Millex® - Millipore) and added aseptically to the sterilized medium. The incubation temperature was 30 °C for 24 h. Colonies with purple coloration were selected and inoculated on petri dishes containing selective-differential King`s B medium for *Pseudomonas* (KING: 2 % proteose peptone n°3, 0.15 % $MgSO_4.7H_2O$, 1 % glycerol, 2 % bacteriological agar, pH 7.2, adapted from (King et al., 1954) and incubated at the same conditions. Single well isolated colonies were picked up for determination of morphology and correct isolation by gram coloration method.

2.3 Cell culture preparation

Three full loops of a 24-h-old culture on a petri dish were transferred to a 500 mL conical flask containing MBP medium: 0.8 g glucose as carbon source, 0.2 g $(NH_4)_2SO_4$, 4 mL Hutner solution (Fontanille, 2002), 8 mL of solution A (6.5 g K_2HPO_4, 8.28 g KH_2PO_4 in 250 mL distilled water) and 188 mL distilled water (Bicas et al., 2008). The incubation conditions were 30 °C and 150 rpm for 24 h.

2.4 Evaluation of terpene citotoxicity

A 5 mL aliquot of 24-h-old culture grown on liquid YM medium was transferred to 250 mL conical flasks containing 45 mL of YM liquid medium and citronellol was added at different concentrations (in %) of 0.1, 0.5, 1.0, 3.0, 7.0, 10.0, and incubated at 30 °C and 150 rpm. After 24 h, a 100 µL aliquot of each

culture grown in different concentrations of citronellol were transferred to petri dishes with YM medium agar, and incubated at the same conditions.

2.5 Biotransformation experiments

Screening experiments were performed in order to evaluate the potential of microorganisms in terpene bioconversion. The biotransformation procedure was done by using phosphate buffer with addition of citronellol as carbon source. The cell culture was produced as described above, and the biomass was recovered by centrifuging the culture at 10,000 rpm and 5 °C for 10 min. The resulting biomass was resuspended in 20 mL of phosphate buffer (20 mmol·L^{-1}, pH 7.5), and biotransformation was initiated by adding 100 µL citronellol directly in the culture flasks, under sterile conditions. Similarly, chemical blank (without microorganism) was realized to ensure the absence of chemical transformation reactions. After adding citronellol, samples of 500 µL of each culture and blank were periodically taken and extracted with 500 µL of Et_2O (decane was the internal standard), in order to follow the substrate consumption and product formation. This procedure was repeated at 24, 48, 72 and 96 h of experiment.

2.6 Analysis of the samples by GC-MS

The samples were analyzed in a gas chromatograph HP-7890 coupled with a mass spectrometer HP-5975 (GC-MS) (Agilent Technologies) equipped with split-splitless injector and a HP-5 MS capillary column of 30 m x 0.25 mm x 0.25 µm (J&W Scientific, Folsom, Califórnia, USA). Helium was the carrier gas, with a constant flow of 1.0 mL·min^{-1} and split ratio of 1:10. The oven temperature settings used was as follow: initial temperature of 80°C for 2 min, rising at 20 °C min^{-1} until 220 °C, then held for 6 min. The temperature in the injector was constant at 250 °C. The temperatures of the quadrupole, the ionic source and the GC-MS interface were 150 °C, 230 °C and 250 °C, respectively. The MS system operated with an electron impact of 70 eV, an acceleration voltage of 1.1 kV, and scan range of m/z 40-500. The products were identified by comparison of their mass spectra and retention indexes with those of reference substances and by comparison with the US National Institute of Standards and Technology (NIST) mass spectral library (Version 2008). Product quantification was performed with a calibration curve at different concentrations of standard.

3. Results and Discussion

More than 34 strains of *Pseudomonas* were isolated from petroleum, soil, fruits and vegetables. All strains selected for biotransformation processes of citronellol were first developed on growth media with indicator and, selected strains produced a red color as a result of the precipitation of formazan following the reduction of TTC. Then the isolates were able to grow in selective-differential KING medium for Pseudomonas detection, and identified as gram-negative bacilli bacterium through gram coloration method.

The terpene-resistance of selected strain was evaluated in order to investigate the potential of such microorganism to surpass the terpene citoxicity and eventually low transformation rates by the addition of higher concentrations of substrate. It is known that the toxicity of these terpenoid compounds for the microorganisms is one of the main problems in monoterpene bioconversions (Demyttenaere, 2001). The results demonstrated that the bacteria was able to grow under terpene concentrations ranging from 0.1 % to 10 %, showing small differences between them and high resistance against terpene toxicity (Figure 1). This is an important variable for further process otimization.

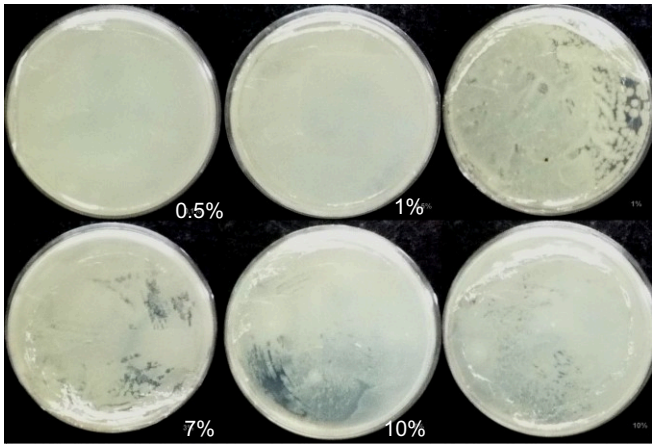


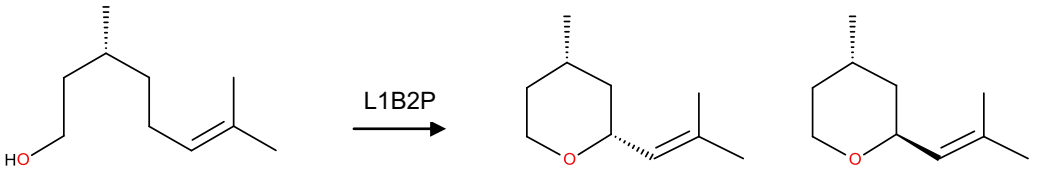
Figure 1: Terpene citotoxicity for L1B2P strain under different concentrations of citronellol, ranging from 0.1 % to 10 %, in YM medium agar

The selected strains were screened for their ability to convert the substrate (*R*)-(+)-*β*-citronellol in aroma compounds, under liquid phase fermentation. From the GC-MS analysis of liquid phase extracts of bacterial cultures, it was observed that four strains of *Pseudomonas* spp. were able to grow on citronellol as sole carbon and energy source. Hence, it could be concluded that the given substrate was able to support bacterial growth when using terpene as sole carbon source.

The L1B2P strain was selected for continuing studies due to high resistance against citronellol toxicity (Figure 1) and higher bioconversion product concentration (Figure 2). It was observed that L1B2P strain converted *(R)*-(+)-*β*-Citronellol into (+)-*cis*-Rose oxide and (+)-*trans*-Rose oxide, in a *cis*/*trans* ratio of 65/35. Other derivative products such as dihydrocitronellol and *β*-Citronellal were identified as minor compounds (Figure 3). The *cis*/*trans* ratio in favor of *cis*- isomer can be explained by the enzymatic bacterial conversion, but also due to the fact that the *cis*-forms of rose oxide are thermodynamically more stable than the *trans*-forms (Demyttenaere et al., 2004).

The product concentration peaked 1 day after addition of citronellol and then decreased. The oxidation of citronellol started right after the L1B2P strain was supplemented with citronellol, increased considerably over a period of 24 h, and then decreased rapidly. The *cis*- and *trans*-rose oxide yield reached the concentration of approximately 29.67 mg/L after addition of citronellol. The final pH in the end of biotransformation process was 6.5.

Previous investigations also demonstrated the conversion of citronellol into rose oxide by *Penicillium* sp., at concentrations varying from 30 to 70 mg/L for the *cis*- isomer, and 12 to 31 mg/L for the *trans*-isomer, approximately (Maróstica et al., 2006). The same bioconversion of citronellol was performed by the basidiomycete *Cystoderma carcharias* in an aerated-membrane bioreactor, and rose oxide were detected in amounts of up to 10 mg day^{-1} (Onken and Berger, 1999).



(*R*)-(+)-*β*-Citronellol (+)-*cis*-Rose oxide (+)-*trans*-Rose oxide

Figure 2: Enantioselective bioconversion of (R)-(+)-β-citronellol to cis- and trans-rose oxide by the Pseudomonas spp. strain L1B2P

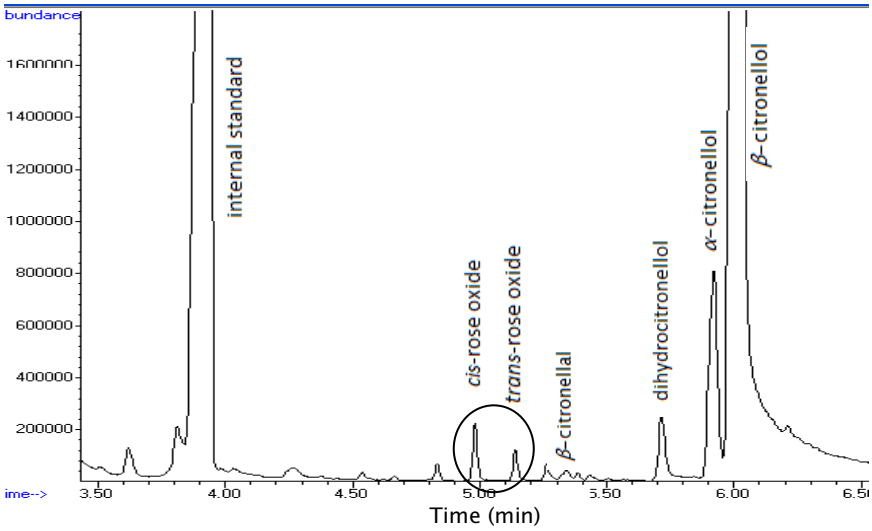


Figure 3: Chromatogram of citronellol degradation and rose oxide production by L1B2P. The biotransformation medium consisted of phosphate buffer and citronellol as sole carbon source.

4. Conclusion

The biotransformation of citronellol resulted in the production of *cis*- and *trans*-rose oxides as the main products. The utilization of the given terpene indicated that a metabolic pathway involving it was actually active in the corresponding bacterial strain. Besides, chemical blanks of the bioconversion experiments clearly indicated that the formation of the biotransformation products was not caused by chemical autoxidation but required an enzymatic step for the formation of *cis*- and *trans*-rose oxides. The selected *Pseudomonas* strain appeared to have impressive resistance in culture medium with high concentration of citronellol, showing great potential for further otimization of citronellol bioconversion. Rose oxide production by *Pseudomonas* spp. appeared as a promising alternative for commercial production of this bioflavour.

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