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PRODUCTION OF AROMAS AND FRAGRANCES THROUGH MICROBIAL OXIDATION OF MONOTERPENES

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Abstract - Aromas and fragrances can be obtained through the microbial oxidation of monoterpenes. Many microorganisms can be used to carry out extremely specific conversions using substrates of low commercial value. However, for many species, these substrates are highly toxic, consequently inhibiting their metabolism. In this work, the conversion ability of *Aspergillus niger* IOC-3913 for terpenic compounds was examined. This species was preselected because of its high resistance to toxic monoterpenic substrates. Though it has been grown in media containing R-limonene (one of the cheapest monoterpenic hydrocarbons, which is widely available on the market), the species has not shown the ability to metabolize it, since biotransformation products were not detected in high resolution gas chromatography analyses. For this reason, other monoterpenes (alpha-pinene, beta-pinene and camphor) were used as substrates. These compounds were shown to be metabolized by the selected strain, producing oxidized compounds. Four reaction systems were used: a) biotransformation in a liquid medium with cells in growth b) with pre-grown cultures c) with cells immobilized in a synthetic polymer network and d) in a solid medium to which the substrate was added via the gas phase. The main biotransformation products were found in all the reaction systems, although the adoption of previously cultivated cells seemed to favor biotransformation. Cell immobilization seemed to be a feasible strategy for alleviating the toxic effect of the substrate. Through mass spectrometry it was possible to identify verbenone and alpha-terpineol as the biotransformation products of alpha-pinene and beta-pinene, respectively. The structures of the other oxidation products are described.
Keywords: Bayer-Villiger; Cytochrome P450; Oxidation; Verbenone; Alpha-terpineol.

INTRODUCTION

Aromas and fragrances are used everywhere in the modern world, where they are considered essential for the final quality of, for example, foods and drinks. The main sources of these substances are the essential oils and their monoterpene compounds (Krasnobajew, 1984; van der Werf, Bont & Leak, 1997). Substances with organoleptic characteristics can be obtained directly from nature. However, these compounds occur in very low concentrations, and a variety of factors, such as seasonal variations in concentration, plant diseases, the complexity of the vegetable extracts and economic and environmental restrictions, can affect their yield or the extraction process. Common ways of obtaining chemical aromas are chemical syntheses using compounds found in abundance in nature, like terpenic hydrocarbons.

Presently, however, consumer prefers to avoid synthetic products, and there has been a corresponding upsurge in demand for products that

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can be labelled "natural" (Welsh, Murray & Williams, 1989). Biotechnology has the potential to generate these products through biotransformation carried out by microorganisms and their enzymatic systems (Santos et al., 2003; Santos et al., 2004). Other advantages of biotransformation include the fact that a single stage in such a process can encompass a series of steps in chemical synthesis and results in the formation of the desired products (Ribbons, 1990; Krings & Berger, 1998).

As part of our continuous work in microbial oxidation of natural products (Santos et al., 2003; Santos et al., 2004; Toniazzo et al., 2005), the objective of the present work is to investigate the capacity of *Aspergilus niger* (previously selected strain) in oxidizing some monoterpenes.

MATERIALS AND METHODS

Monoterpenes

Four hydrocarbons (R-limonene, S-limonene, alpha-pinene and beta-pinene) and one ketone (camphor), from Sigma-Aldrich, were used in this study. They were analyzed by high resolution gas chromatography. The purities of monoterpenes were over 98 %. All the substrates in the experiments were added at 1% v/v, although in the case of camphor, a 0.02 mg/mL solution of camphor in ethyl acetate was used.

Microorganism

The *Aspergillus niger* IOC-3913 strain used in this work was kindly supplied by FIOCRUZ (Coleção de Culturas de Fungos of the Fundação Oswaldo Cruz, Brazil).

Culture Media

The medium used for microbial growth was prepared as described in the literature (Asakawa et al., 1991). Its composition (g/L) consisted of sucrose (15), glucose (15), hydrolyzed soya peptone (5), K_2HPO_4 (1), $MgSO_4.7H_2O$ (1), KCl (0.5) and $FeSO_4.7H_2O$ (0.01) in distilled water. pH was not controlled.

Biotransformation in Petri Dishes

The Petri dishes were prepared with 20 mL of medium [Czapeck (g/L): glucose (20), $NaNO_3$ (2), $MgSO_4.7H_2O$ (1.025), KCl (0.5), $(NH_4)_2SO_4$ (0.01), agar-agar (20)]. After inoculation with a loop full of fungi spores with seven days' growth, they were inverted so that the substrate could be put on the lid, coming into contact with the microorganism via the gas phase. This way, it was possible to add the substrate a number of times, after 0, 24, 48, 96 and 144 hours for the limonene isomers and after 0, 24, 72, 120 and 168 hours for the pinenes and camphor. All the dishes used in the experiments were incubated in a microbe culture incubator at 30°C. The growth on the dishes containing R- and S-limonene was interrupted after 168 hours, and that on for the dishes containing alpha-pinene, beta-pinene and camphor after 216 hours. Then, solid-phase extraction was undertaken by grinding the material contained in the dish in 20 mL of PA ethyl acetate 99.5% (VETEC) using a mortar and pestle.

Biotransformation in a Liquid Medium

Conical flasks (250 mL) were used for all the tests in the liquid medium. One hundred mL of culture medium and the inoculum were added to each flask. The inoculum consisted of suspensions of filamentous fungal spores in saline solution (NaCl 0.9%), with $1x10^8$ spores per 100 mL of medium.

The terpenic substrate was added to the medium together with a surfactant agent (Tween 80) at 1%v/v each, when microbial growth was being tested. When the biotransformation was not associated with growth, the transformation medium consisted only of the terpenic substrate and the surfactant agent, each at a 1% v/v concentration.

The experiments were done in a mechanical rotary shaker at 28°C and 150 rpm. The only exceptions were the flasks intended for immobilizing the fungus in a synthetic polymer network, which consisted of 20 cubes with an average volume of 1 cc each. In this case the biotransformation reaction was done at 28°C and 100 rpm.

The biotransformation not associated with growth took place in two ways. In the first case, the pellets formed during the fungal growth in the liquid medium were filtered after 74 hours, using a vacuum pump under unsterilized conditions, and immediately incubated in a transformation medium at 28°C with stirring at 150 rpm. The second strategy consisted of entrapping mycelia in a synthetic network made up of small cubes of commercial sponge. After 74 hours the content of the flasks was inverted and the immobilized fungi were added to the transformation medium.

Every 24 hours samples were taken from all the flasks, including the control flasks, from the very beginning until the sixth day after addition of the

transformation medium. After the samples were removed from each flask, they were centrifuged and submitted to liquid-liquid extraction with ethyl acetate.

Analytical Methods

The organic extract obtained in the experiments was submitted to analysis using high resolution gas chromatography (HRGC) to assess the biotransformation and abiotic losses. The equipment used was a Hewlett-Packard chromatograph model HP5890 series II with a capillary column model HP-5 (crosslinked 5% phenylmethylsiloxane), 30 meters in length with an internal diameter of 0.32 mm and a film thickness of 0.25 μm. The temperature of the injector was kept at 250°C and that of the detector at 260°C. Carrier gas was N_2 (120kPa, 4 mL/min). The detector was supplied with air and hydrogen at 275 kPa and 100 kPa, respectively. The programming used was an initial temperature of 40°C, maintained for 3 minutes, a heating rate of 5°C/min and a final temperature of 220°C. Total analysis time was 39 minutes. The Chemstation program version A.03.34 HP 3365 series II was used to integrate the areas obtained on the chromatogram.

The products were identified using a gas chromatograph coupled to a mass spectrometer (GC/MS) to determine the molecular weights of the products and ions formed by the fragmentation of their molecules. The equipment used in this stage was a Coupled GC/MS Hewlett Packard model 5973 HP, with the temperature of the mass spectrometer injector at 270°C and that of the interface at 280°C. The helium flow rate was 1 mL/min and chemical ionization took place at 70 eV. The other conditions were the same as those already given for the HRGC.

RESULTS AND DISCUSSION

In the tests Petri dishes, inhibition caused by the terpenic substrate was found by the ratio of the diameters of the colonies formed. It was found that the two limonene isomers interfere more in fungal growth, with inhibition rates greater than 50%, while alpha-pinene, beta-pinene and camphor yielded values lower than 40%. Figures 1 and 2 show the effect of the monoterpenic substrate on microbial growth.

Chromatographic analyses of the extracts obtained did not reveal any biotransformation product in the media containing R-limonene or S-limonene, showing that the strain of *Aspergillus niger* used in this work was not capable of metabolizing them. However, a number of products were found in the extracts obtained after microbial growth in the media containing alpha-pinene, beta-pinene and camphor.

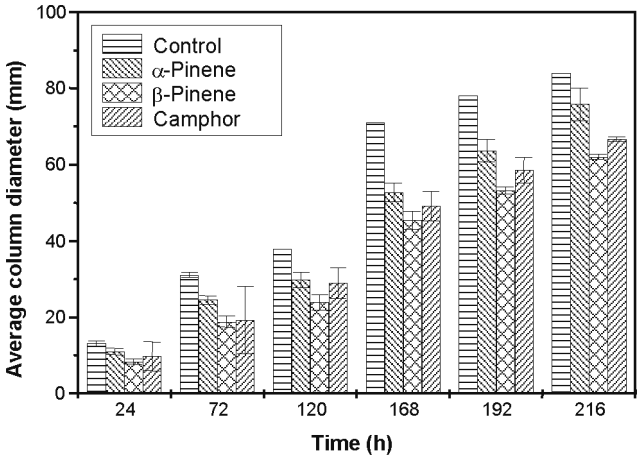

Figure 1: The effect of the presence of R- and S-limonene on the growth of *Aspergillus niger* in solid medium.

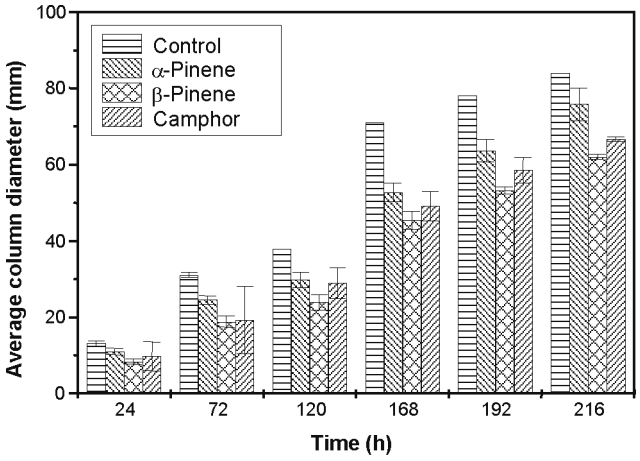

Figure 2: The effect of the presence of alpha-pinene, beta-pinene and camphor on the growth of *Aspergillus niger* in solid medium.

The tests carried out in the liquid medium, where the substrate was added together with the growth medium indicated that the consumption of sugars is affected to a greater degree by the R-limonene. Moreover, no biotic consumption of R-limonene or S-limonene was noted.

By monitoring the concentrations of the other monoterpenic substrates in the inoculated flasks associated with growth and in the controls it was possible to calculate the biotic consumption of each substrate. The maximum values of biotic consumption for alpha-pinene, beta-pinene were approximately 80 mg/100 mL of medium, while for camphor, which was added as a more dilute solution, biotic consumption was approximately 8 mg/100 mL.

The chromatographic analyses of the extracts indicated that the biotransformation products obtained using alpha-pinene, beta-pinene and camphor are basically the same as those obtained in solid media. These products were detected in the samples after 71 h.

The tests done in liquid medium, where the substrate was added after microbial growth (noassociated with growth), showed that biotic consumption in the flasks containing pinenes varied between 200 and 400 mg/100 mL, while the consumption of camphor was approximately 20 mg/100 mL. Moreover, the maximum consumption for each substrate occurred more quickly than in the cases where microbial growth had not previously taken place, with the maximum accumulated consumption taking place in less than 72 hours after addition of the substrate in all cases.

Samples obtained by extraction and submitted to chromatographic analysis allowed not only the measurement of the substrate concentration, but also the opportunity to check for the presence of biotransformation products.

For alpha-pinene, the characteristics of three products, with retention times in the chromatography column of 12.9, 14.2 and 14.8 min, were monitored (Figure 3). Of these, only the substance with the 14.8-min retention time had been observed in previous experiments and identified as verbenone. However, the product that disappeared at the start of the run (Rt=12.9 min) seemed to be directly related to the formation of this product, which suggests it could be verbenol, an intermediate alcohol in verbenone synthesis.

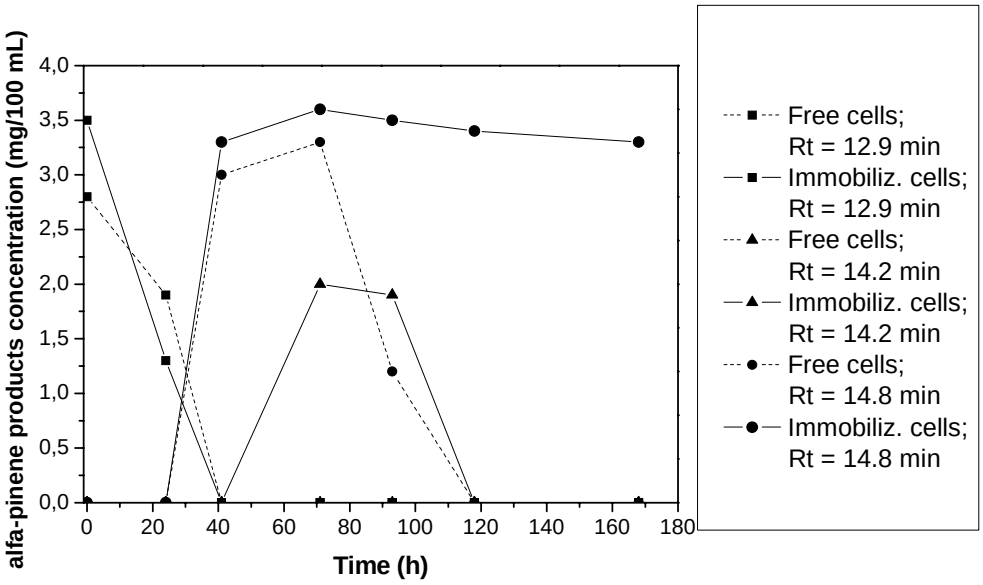

Figure 3: Kinetic profile of the formation of biotransformation products obtained with alpha-pinene, not associated with fungal growth.

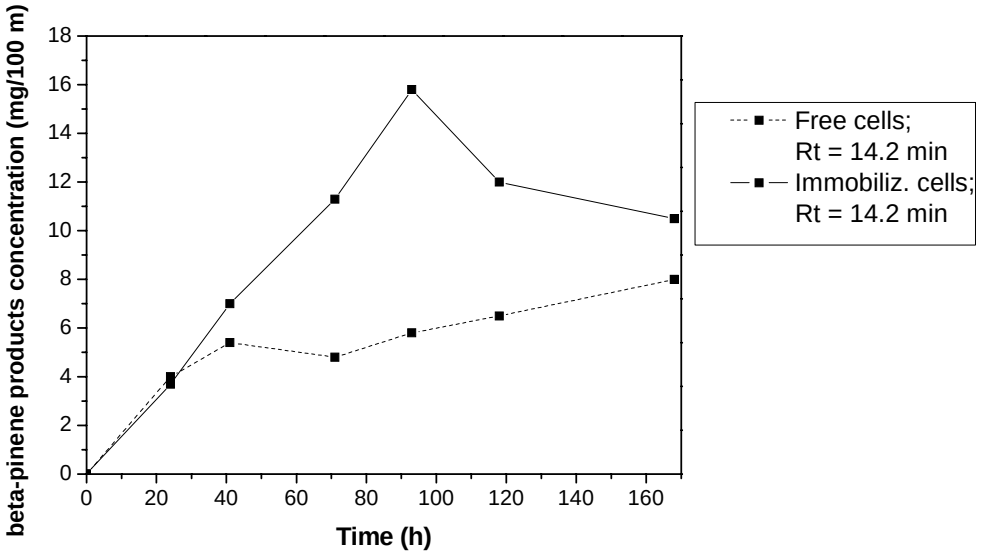

Figure 4: Kinetic profile of the formation of biotransformation products obtained with beta-pinene, not associated with fungal growth.

In the biotransformation of beta-pinene (Figure 4), a product with a retention time of 14.2 min, α-terpineol, reached its highest concentrations between 72 and 120 hours.

For camphor, three main products were observed in all the tests done. The kinetic profile of these biotransformation products not associated with growth is shown in Figure 5. The proposed structures of these products, based on an analysis of the ions formed by fragmentation in a mass spectrometer, are of two isomers of a keto acid derivative and one lactone formed in a Baeyer-Villiger reaction.

Figure 6 shows the substrates utilized and their biotransformation products.

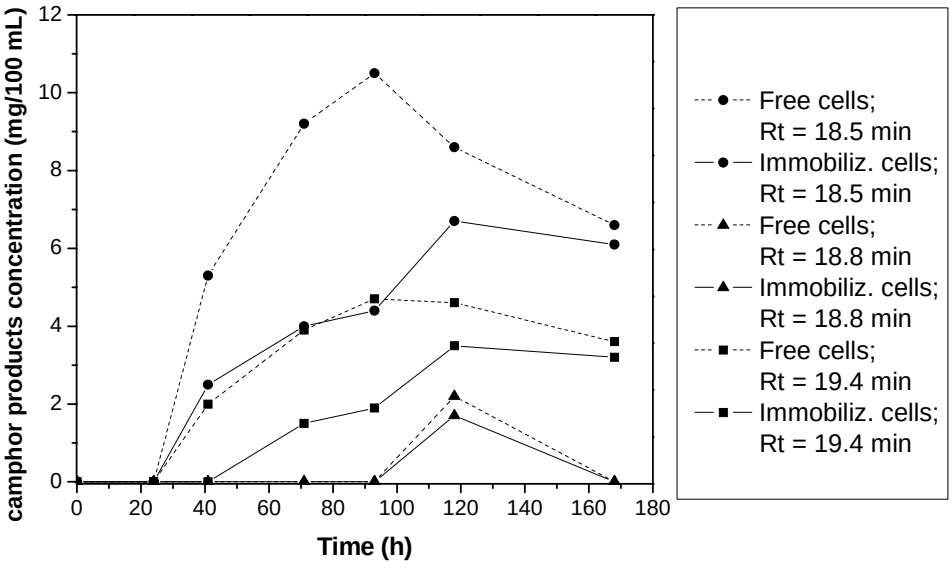

Figure 5: Kinetic profile of the formation of biotransformation products from camphor, not associated with fungal growth.

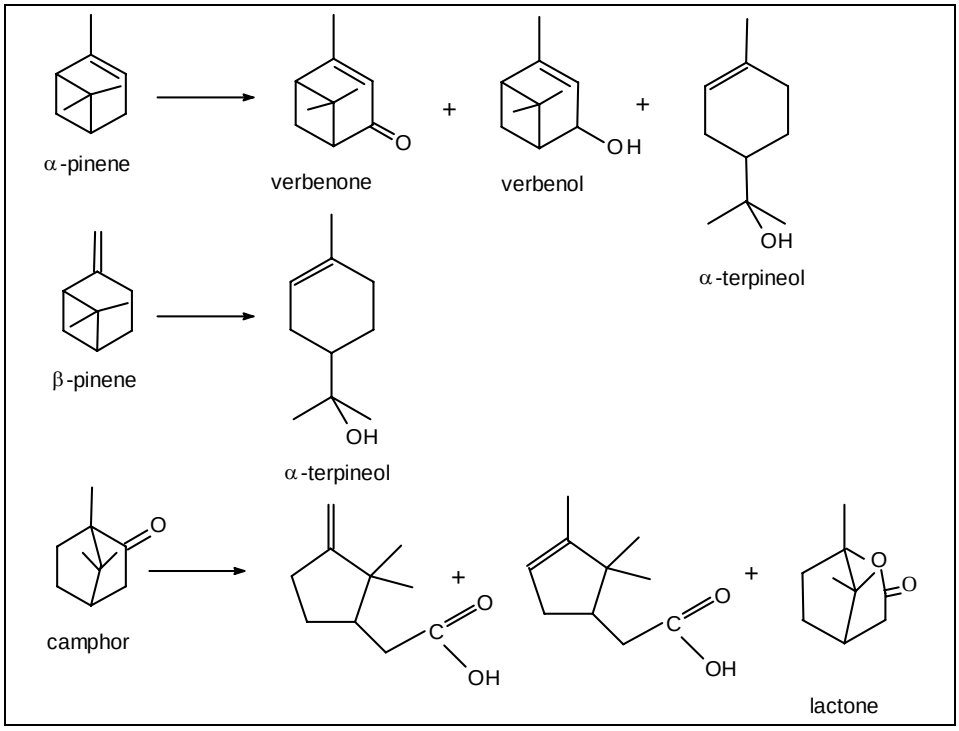

Figure 6: Monoterpenic substrates and their products of biotransformation with *Aspergillus niger* IOC-3913.

In the tests where cell growth and immobilization did not take place concomitantly, there was a greater formation of products. Additionally, products appeared earlier due to the use of previously grown cells. The immobilization of cells was shown to be suitable in the media containing the toxic substrates in higher concentrations, as was the case with the pinenes, bringing about an increased formation of products. Tan and Day (1998) have already observed the possibility of immobilizing the filamentous fungus *Aspergillus niger* in biotransformation studies on limonene, leading to the reutilization of the biomass. In the tests with camphor using a dilute solution, the separation of the growth and biotransformation stages also generally resulted in a greater formation of products. For camphor, the free cells had a better performance, probably due to a larger biomass capable of participating in the biotransformation, since the toxic effect of the substrate was reduced through dilution.

Importantly, it has been made possible to obtain verbenone from alpha-pinene and and alpha-terpineol from β-pinene (Toniazzo et al., 2005). These compounds are important in the flavor/perfumery industries.

CONCLUSIONS

The results obtained in this work indicate that the enzymes responsible for the biotransformation producing the metabolites found in all the reaction systems studied are probably not of an inductive nature, since the bioconversions are not related to the presence of the substrate in the initial stages of microbial growth and the biomass already formed anticipates the appearance of the biotransformation products. In general, the biotransformation of alpha-pinene, beta-pinene and camphor results in the formation of oxidized products through the insertion of oxygen into the substrate molecule. There are probably a number of mechanisms involved in the biotransformation of each monoterpene used in this study, suggesting the action of a variety of enzymes. Verbenone and alpha-terpineol were identified as biotransformation products of alpha-pinene and beta-pinene, respectively. These products have applications in the aroma and fragrance industry and as intermediaries in the synthesis of pharmaceuticals, adding considerable value to the substrates used.

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