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Fungal Biotransformation of Monoterpenes Found in Agro-Industrial Residues from Orange and Pulp Industries into Aroma Compounds: Screening Using Solid Phase Microextraction

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Abstract The biotransformation of monoterpenic agro-industrial wastes (turpentine oil and essential orange oil) was studied. More than 40 fungal strains were isolated from Brazilian tropical fruits and eucalyptus trees and screened for biotransformation of the waste substrates. Solid phase microextraction was used to monitor the presence of volatile compounds in the headspaces of sporulated surface cultures. The selected strains were submitted to submerged liquid culture. The biotransformation of R-(+)-limonene and α-, β- pinenes from the oils resulted in α-terpineol and perillyl alcohol, and verbenol and verbenone, respectively, as the main products. The selected strains were also placed in contact with α- and β-pinenes standards. It was confirmed that verbenol, verbenone, and α-terpineol were biotransformation products from the terpenes. A concentration of 90 mg/L of verbenone was achieved by *Penicillium* sp. 2360 after 3 days of biotransformation.

Keywords: biotransformation, fungi, industrial residue, monoterpene, solid phase microextraction (SPME)

Introduction

Essential oils are dominated by monoterpene hydrocarbons, which are regarded as process waste mainly because of their low sensory activity, low water solubility, and tendency to autooxidize and polymerize (1), turns terpene hydrocarbons, such as limonenes, pinenes, and terpinenes, into ideal starting materials for use in microbial transformations. Limonene, α-pinene, and β-pinene are ideal starting compounds for bioconversions because of their cheap, widespread availability (2).

Currently, an increasing trend towards efficient utilization and value addition of agro-industrial residues is emerging. The application of agro-industrial residues in bioprocesses provides alternative substrates for production of important chemicals, helps to solve pollution problems (3), may prove to be economically advantageous.

Pinenes (α- and β-) are major components of turpentine, a by-product of the pulp making industry. Of the turpentine produced in the United States, about 90% is derived from the pulp and paper industry (4). Limonene is present at concentrations between 90-96% in essential orange oil, a by-product from the orange juice industry.

Microbial enzymes have been proven to be effective in the production of flavor compounds in aqueous media (5, 6). Furthermore, microorganisms can be used as catalysts for the biotransformation of terpenoids. As enzymes are more stable inside living cells, this property extends the life of the biocatalysts, and the addition of co-factors is not needed, since they are already present in the cells (7).

A great advantage of biotransformation as compared to chemical synthesis is that according to European and USA legislations, the compounds originated via biotransformation or bioconversion are considered as 'natural' compounds (8). In this way, flavor compounds obtained through such processes adhere to the increasing consumer demand for natural products (9).

Solid phase microextraction (SPME) is a simple and effective adsorption and desorption technique that eliminates the need for solvents or complicated apparatus. SPME can be used for concentrating volatile or non volatiles compounds in liquid samples or head spaces and is compatible with gas chromatography (10).

In this study, the screening of fungal strains by SPME for the biotransformation of monoterpenes contained in residues from orange citrus and pulp industries is described. The monoterpenes from the waste oils were biotransformed into aroma compounds used in food and flavor industries.

Material and Methods

Agro-industrial residues The industrial residues, essential orange oil and turpentine oil were obtained, respectively, from an orange juice industry and from a pulp plant, both situated in the State of São Paulo, Brazil. The turpentine oil was analyzed by gas chromatography-mass spectrometry (GC-MS) and the main compounds (over 0.1%, w/w) present in each residue are shown in Table 1. The composition of the essential orange oil was presented in a previous paper (11).

Chemicals R-(+)-Limonene (ca. 98%; Sigma, St Louis, MO, USA), α-pinene (ca. 98%; Aldrich, Milwaukee, WI, USA), and (1S)-(-)-β-pinene (Aldrich), S-*cis*-verbenol (ca. 95%; Aldrich), (1S)-(-)-verbenone (ca. 94%; Aldrich), R-(+)-α-terpineol (ca. 96%; Aldrich), were used. All the chemicals and solvents were of the best available commercial grade.

Microrganisms and cultivation The filamentous fungal strains used in this study belonged to the genera:

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Aspergillus sp., *Penicillium* sp., *Fusarium* sp., *Mucor* sp., and *Chrysosporium* sp. The fungi were isolated from soil samples, from eucalyptus trees and from spoiled Brazilian fruit.

Optimization of SPME analysis Twenty mL SPME vials were filled with 5 mL YM agar medium [YM, bacteriological peptone (Becton Dickinson and Company Sparks, Le Pont de Claix, France) 0.5%(w/v); glucose (Merck, Darmstadt, Germany) 1.0%(w/v); malt extract (Difco Laboratories, Le Pont de Claix, France) 0.3%(w/v); yeast extract (Merck) 0.3%(w/v); agar (Merck) 2.0% (w/v) pH 6.0] and autoclaved. Ethanolic solutions (10 μL, ethanol from Synth, Diadema, Brazil) of the orange and turpentine oils, containing the expected biotransformation products (α-terpineol, perillyl alcohol and verbenol, verbenone, α-terpineol, respectively) were sprayed over the agar surface, and the vials covered with silicone septa. The concentrations of the oils in the ethanolic solutions and the theoretical biotransformation products were 20% (v/v) and 0.1%(v/v) each. The SPME fiber used was CAR/PDMS (Supelco, Bellfonte, PA, USA). The adsorption parameters used for optimization were temperature (20, 30, and 40°C) and time (10, 20, and 30 min). Desorption occurred in a gas chromatograph at 250°C for 2 min.

During the past decade, SPME has been widely applied to extract volatile and semi volatile compounds from biological, environmental, and food samples. Some suitable fibers for monoterpene recovery are: PDMS (polydimethylsiloxane), CAR/PDMS (Carboxen/PDMS), PDMS/DVB (PDMS/divinylbenzene), and DVB/CAR/PDMS (12).

In the present work we used a CAR/PDMS SPME fiber and considered the influence of different temperatures and exposure times in the recovery of terpenes by an ethanolic solution sprayed into the SPME vials. For the turpentine oil - verbenone - verbenol system, the chosen temperature/time was: 20°C/20 min, and for the essential orange oil-perillyl alcohol-α-terpineol system: 40°C/20 min. For the turpentine oil system all the conditions tested were satisfactory regarding compound recovery and so the milder/faster condition was preferred. For the orange oil system 40°C showed more suitable results, considering the recovery of the oxygenated compounds. Chemical oxidations of the monoterpenes were not detected in the SPME conditions analyzed.

SPME biotransformation experiments For the SPME biotransformation experiments, 20 mL SPME vials (Supelco) were filled with 5 mL YM agar medium, plugged with cotton wool, autoclaved, and inoculated with fresh spores. They were incubated at 30°C for 6-7 days until complete sporulation, after which 10 μL of each essential oil in ethanol (20%, v/v) was sprayed onto the agar medium and the vials covered with silicone septa. The SPME fibers were then exposed to the headspace. The exposition temperature/time was determined from the optimization of the SPME analysis for each substrate. The fiber was desorbed in the same way as described above.

Liquid culture biotransformation experiments The strains selected using SPME were cultivated [2.5 mL of an aqueous spore ($1\text{-}7\times10^7$ spore/mL) suspension] in 250 mL conical flasks containing 50 mL of YM liquid medium (same composition as YM agar medium, but without the agar) and incubated in a rotary shaker at 160 rpm and 30 °C (all in triplicate). After 3 days of growth, biotransformation was initiated by the addition of 50 μL of the same oils (or α-pinene, β-pinene, limonene standards) in an alcoholic solution (under sterile conditions). Two subsequent additions of 50 μL every 24 hr were made. Total incubation time was 6 days. Each 24 hr, samples of 5 mL were taken and extracted with ethyl acetate. The samples were analyzed directly by GC/FID [decane (Aldrich) was the internal standard]. Similarly, chemical blanks at pH 3.0, 4.0, and 5.0 (the pH adjustments were done with HCl prior to autoclaving) were performed, without mycelium, to ensure the absence of chemical transformation reactions.

Analysis of the samples by GC and GC-MS GC-analyses were performed with a Chrompack CP9001 gas chromatograph (Middelburg, The Netherlands) equipped with a split/splitless-injector, a FID-detector, and a WCOT Fused Silica column. The stationary phase was CP-Sil CB (60 m length × 0.25 mm i.d.; coating thickness of 0.25 μm) (Middelburg). The working conditions were: injector 220°C, detector 250°C (make-up gas He - 99.999%, White Martins, Novasco, Brazil - 1 mL/min). The oven temperature was programmed to increase in temperature from 40 to 210°C at 5°C/min with an initial holding time of 2 min and a final holding time of 5 min. Quantification was performed by calibration according to the internal standard decane.

GC-MS-analyses were performed using a Varian Saturn gas chromatograph (Walnut Creek, CA, USA) equipped with an EM-IT mass selective detector, a CP-Sil 8CB Low bleed/MS capillary column (30 m × 0.25 mm × 0.25 μm) (Middleburg) and split/splitless CP1177 injector. The working conditions were: injector 280°C, transfer line to MSD 260°C. The oven temperature was programmed from 40 (1 min) to 160°C (3 min) at 5°C/min and from 160 to 250°C with a final holding time of 5 min, scan range *m/z* 40-500, carrier gas (He, 99.999%, White Martins) 1 mL/min; split 1/50; ionization EI 70 eV; scan rate, 1/sec. Positive identifications were made by matching sample retention indices (RI) and mass spectra of the samples with those of the standards, analyzed under identical conditions.

Results and Discussion

Chemical composition of essential orange and turpentine oils The turpentine oil used in this study was analyzed by GC-MS. The major compounds identified and their abundance (reported as a percentage of the total composition) are shown in Table 1.

The results show that α-pinene (71%) is the major compound in the turpentine oil, followed by β-pinene (16 %). Camphene is also found in considerable amonts (4%). The comparison of the composition of turpentine oil used in this work with other turpentine oils is difficult because it can varie from region to region, but, to the best of our known, α- and β-pinenes are the major compounds found

Table 1. Composition of the turpentine oil as determined by GC-MS

Compound	Turpentine oil (%)
α-Pinene	71.0
β-Pinene	16.0
Myrcene	0.5
Terpinene	1.4
Limonene	1.1
Carvone	0.1
Camphene	4.0
Unknown 1	0.7

in turpentine oils. In several regions of the world, different parts of the turpentine tree are exploited for various purposes, but little is known about the composition of the essential oil of some turpentine trees. Some investigations have characterized certain turpentine species. The major components of the essential oil of Chios turpentine resin were α-pinene, β-pinene, sabinene, and terpinen-4-ol (13). Similarly, the most prominent components in the oils from the different parts of *Pistacia terebinthus* were α-pinene, β-pinene, limonene, and germacrene D (14).

The composition of the essential orange oil used in this work is the same as presented previously (11) and is not presented and will not be further discussed here. For further details, please, check our previous paper. But, it should be emphasized here that limonene was the major compound present in the oil (more than 94% in the oil). One point should be emphasized: the presence of carvone (more than 1%) in essential orange oil was detected in the biotransformation analyses. Carvone was recovered mainly in the chemical blanks of liquid cultures (medium with essential orange oil, but without the culture), but was not identified as a biotransformation product by the strains used in this work. The chromatographic profiles obtained from the biotransformation experiments were compared to those from the diluted essential orange oil (to confirm that α-terpineol and perillyl alcohol were really biotransformation products and did not come from the orange oil) and to those from the chemical blanks (to ensure that the compounds were not chemically transformed).

Screening experiments using surface cultures and headspace analysis by SPME More than 40 strains were tested for their capacity to transform the terpenes contained in the waste oils. The recovery of volatiles from the headspace was done by SPME. Six strains were selected for their ability in converting monoterpenes into oxyfunctionalized compounds. Table 2 shows the strains used in this investigation and respective origins.

We found that the strains *Mucor* sp. 2276, *Penicillium* sp. 2319, and *Mucor* sp. 2288 were able to bioconvert the terpenes from the turpentine oil mainly into verbenol, while *Penicillium* sp. 2327, and *Penicillium* sp. 2360 produced verbenone after 1 day of contact with the alcoholic solution of turpentine oil sprayed onto the sporulated surface cultures. Previous investigations also

Table 2. Strains selected for their ability in converting monoterpenes from waste oils into oxyfunctionalized aroma compounds

Strain	Isolated from
Mucor sp. 2276	Southeast Brazilian *Araucaria* sp.
Penicillium sp. 2319	South Brazilian soil sample
Mucor sp. 2288	Southeast Brazilian eucalyptus tree
Penicillium sp. 2327	Southeast Brazilian eucalyptus tree
Penicillium sp. 2360	Northeast Brazilian sugar apple
Aspergillus sp. 2357	South Brazilian grape

reported that the α-pinene underwent two distinct biochemical reactions: hydroxylation and dehydrogenation, producing verbenol and verbenone, respectively (15, 16). Such compounds have previously been described as the major biotransformation products of α-pinene formed by fungi (15, 16). Figure 1 shows the structures of the compounds reported in this investigation.

Similarly, we also found that the strains *Mucor* sp. 2276, *Aspergillus* sp. 2357, *Penicillium* sp. 2319 could biotransform the R-(+)-limonene present in the essential orange oil into α-terpineol, and that the strain *Penicillium* sp. 2360 could transform this substrate into the valuable monoterpene perillyl alcohol (Fig. 1). These results are certainly in agreement with the literature. The production of oxygenated compounds originating from the transformation of the inexpensive limonene such as carvone, carveol, α-terpineol, and perillyl alcohol has already been described (17). These compounds are among the most commercially important biotransformation products derived from limonene (17).

Liquid culture biotransformation The strains selected in the SPME experiments were studied in submerged liquid cultures. After 3 days of growth in YM medium, the strains were placed in contact with the terpenic solutions. The same compounds found in the SPME analyses could be recovered by solvent extraction from the liquid cultures. The SPME technique is a fast and simple technique, but

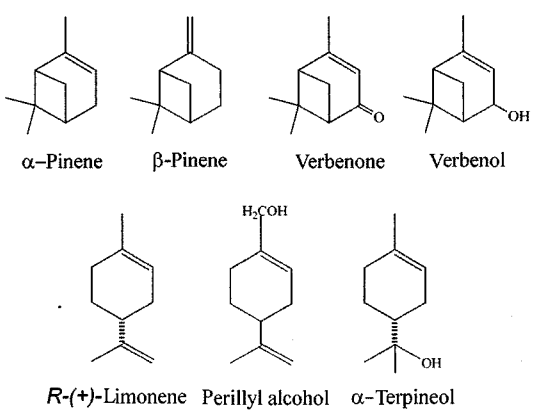

Fig. 1. Structures of α-, β-pinenes, verbenol, verbenone, lionene, α-terpineol, and perillyl alcohol.

cannot be used for quantification. Solvent extraction is more reliable for quantification. Table 3 and 4 show the concentrations of the biotransformation products in liquid medium 24, 48, and 72 hr after the first additions of turpentine and orange oils, respectively. No oxidation products could be recovered from the chemical blanks, indicating that the compounds recovered originated from the biotransformation. Of the bioconversion products obtained from limonene, perillyl alcohol is of particular interest because it has been reported that this terpenoid has chemopreventive properties against liver, mammary, and lung carcinogenesis (7). In general however, oxygenated monoterpenes are mainly used as food flavorizers.

The application of the monoterpenic oils in ethanolic solution was an attempt to decrease the toxicity and increase the solubility of the substrate in the medium. A parameter expressing the toxicity of the solvent for the microrganisms is the log P_{OW} (logarithm of the partition coefficient of the substance in an *n*-octanol/water system). The greatest toxicities were observed for compounds with a log P_{OW} between 1 and 5 (18). Limonene (log P_{OW} = 4.8), α-pinene (log P_{OW} = 4.9), and β-pinene (log P_{OW} = 4.8) are toxic for microorganisms (19). Ethanol was shown to be a suitable solvent for the biotransformation of limonene by *Penicillium digitatum* strains (2). Removing the terpenoids from contact with the aqueous phase is expected to improve the stability of these compounds while preventing their toxicity towards the biocatalyst (19).

Limonene, α-pinene, and β-pinene were not detectable at the end of the experiments (perhaps due to their high volatility at the temperature selected for the experiment). The addition of increased volumes of the substrate should be carefully considered. Recently, Agrawal and Joseph (15) reported the influence of the α-pinene concentration on the yields of verbenone produced by a strain of *Aspergillus niger*. The authors concluded that the optimum concentration was 20 mg of α-pinene in 100 mL of medium. A concentration of 40 mg/100 mL was inhibitory, confirming the toxicity caused by terpenes to microorganisms.

Verbenol, verbenone, and α-terpineol were obtained from the biotransformation of pinenes from turpentine oil. However, it was impossible to know from which monoterpene (α- or β-pinene) each biotransformation product originated from as the turpentine oil used was basically a mixture of both pinenes. Furthermore, α-terpineol could also result from the biotransformation of the limonene present in the turpentine oil. The same strains were also capable of transforming limonene from essential orange oil into α-terpineol. For this reason, the biotransformations of α- and β-pinenes were performed separately. α-Pinene was mainly transformed into verbenol by *Mucor* sp. 2276, *Penicillium* sp. 2319, and *Mucor* sp. 2288 and into verbenone by *Penicillium* sp. 2319, *Penicillium* sp. 2327, and *Penicillium* sp. 2360 (Fig. 2). On the other hand, β-pinene was transformed into α-terpineol by *Penicillium* sp. 2319 and *Penicillium* sp. 2327 (Fig. 3). These findings are

Table 4. Concentration of biotransformation products in liquid medium 24, 48, and 72 hr after the first addition of essential orange oil

Strain	Perillyl alcohol			α-Terpineol		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Mucor sp. 2276	n.d.[1)]	n.d.	n.d.	8.6	5.2	9.0
Penicillium sp. 2319	n.d.	n.d.	n.d.	50.3	63.7	70.2
Penicillium sp. 2327	tr[2)]	tr	tr	37.4	31.5	45.1
Penicillium sp. 2360	12.3	8.5	9.7	tr	tr	tr
Aspergillus sp. 2357	n.d.	n.d.	n.d.	42.6	83.1	88.4

[1)]Not detected. [2)]Trace amounts (<1.0 mg/L). Concentrations are the average of triplicate experiments expressed in mg/L.

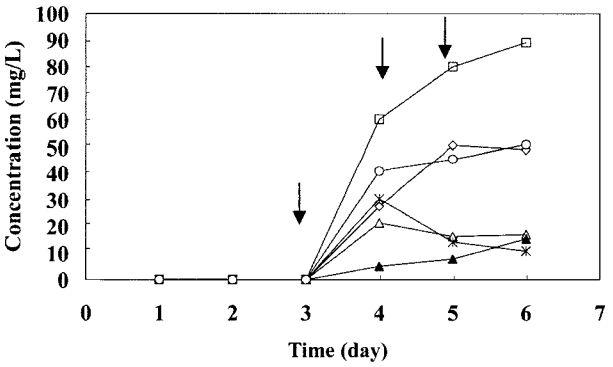

Fig. 2. Biotransformation of α-pinene in liquid cultures by the screened strains. (◇ verbenol, *Mucor* sp. 2276); (△ verbenol, *Penicillium* sp. 2319); (▲ verbenone, *Penicillium* sp. 2319); (✳ verbenol, *Mucor* sp. 2288); (○ verbenone, *Penicillium* sp. 2327); (□ verbenone, *Penicillium* sp. 2360); (↓ indicates the additions of α-pinene).

Table 3. Concentration of biotransformation products in liquid medium 24, 48, and 72 hr after the first addition of turpentine oil

Strain	Verbenol			Verbenone			α-Terpineol		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Mucor sp. 2276	37.1	46.0	51.3	n.d.[1)]	n.d.	n.d.	tr[2)]	tr	tr
Penicillium sp. 2319	25.4	11.4	17.0	3.7	6.7	11.0	5.1	7.4	12.9
Mucor sp. 2288	28.8	17.5	6.1	tr	tr	tr	n.d.	n.d.	n.d.
Penicillium sp. 2327	tr	tr	tr	35.3	41.9	45.7	7.4	5.1	6.9
Penicillium sp. 2360	tr	tr	tr	41.5	77.4	72.2	tr	tr	tr

[1)]Not detected. [2)]Trace amounts (<1.0 mg/L). Concentrations are the average of triplicate experiments expressed in mg/L.

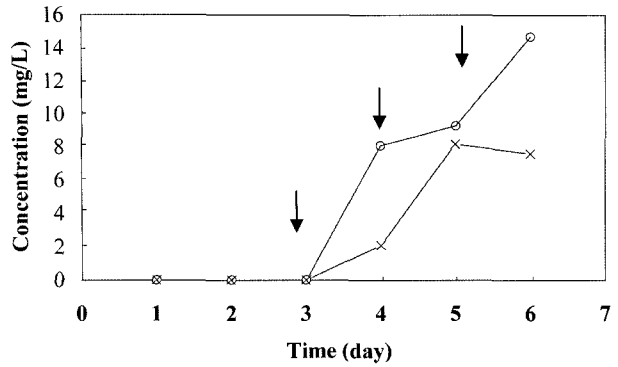

Fig. 3. Biotransformation of β-pinene in liquid cultures by the screened strains. (×, α-terpineol *Penicillium* sp. 2319); (○ , α-terpineol *Penicillium* sp. 2327); (↓ indicates the additions of β-pinene).

similar to those reported in previous investigations (15, 16, 20, 21). Higher yields of each product were expected since the relative amounts of each pinene were higher than in the experiments with the turpentine oil. Unfortunately, the yields were similar to those obtained before. Even in the presence of the co-solvent, high volatility of the pinenes was observed.

The low final concentrations of the biotransformation products are a great drawback for a direct application of this process on an industrial scale. Higher final yields of flavor compounds from biotransformation are a new challenge. In a recent investigation, improvement of the *Aspergillus* sp. and *Penicillium* sp. strains for the production of verbenol/verbenone from α-pinene was proposed. The authors employed UV radiation, ethyl methanesulphonate, and colchicines to mutate the strains, and for some of the mutant strains, substantial increases in the ability to biotransform α-pinene into verbenol were found, but the production of verbenone decreased (22).

In plants, there is evidence of further modification of monoterpenes mediated by P450 cytochromes and redox transformations. The oxygenated compounds are the result of allylic oxidations. One example is the use of *Picea abies* culture suspensions to transform α-pinene, β-pinene, and limonene into *trans*-verbenol, *trans*-pinocarveol or carveol, and perillyl alcohol respectively (20). Some authors have proposed that the cloning and expression of the P450 cytochrome in suitable organisms for use in fermentation processes is one of the most promising solutions for terpene biotransformation (17, 23).

In this study, the use of SPME was shown to be a fast and simple technique for the screening of potential fungal strains for the biotransformation of monoterpenes from two agro-residues. The productions of verbenol and verbenone as the main biotransformation products from α-pinene were verified. In addition, the production of α-terpineol and perillyl alcohol from limonene was detected. Unfortunately, the yields presented in this study were not great enough for direct industrial application. Substrate and biotransformation product inhibition is a major obstacle in the biotransformation of α-, β-pinenes and limonene. The use of industrial wastes as inexpensive substrates is certainly one step more towards the application of the biotransformation of terpenes on an industrial scale, as it makes the process economically advantageous.

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