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BIOTRANSFORMATION OF GERANIOL AND NEROL BY SPORES OF *PENICILLIUM ITALICUM*

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Abstract—A simple and efficient method was developed to carry out biotransformation reactions on volatile terpenoid compounds. Both geraniol and nerol were transformed into 6-methyl-5-hepten-2-one by sporulated surface cultures of *Penicillium italicum* over prolonged periods (up to 2 months). The bioconversion was followed by dynamic headspace techniques.

INTRODUCTION

In recent years there has been an increasing tendency to replace 'synthetic' flavouring substances by 'natural' ones [1, 2]. These products can be obtained by extraction from plant material, but they can also be produced by microorganisms [3]. *De novo* synthesis of 'flavours', such as volatile esters [4], and the bioconversion of monoterpenoids are fields of investigation that gain a growing interest: these reactions are performed by bacteria (e.g., *Pseudomonas* [5, 6]), *fungi* (e.g., *Aspergillus niger* [7–9], *Penicillium* [10], *Cladosporium* [11], *Botrytis cinerea* [12–14]), yeasts and even algae [15].

Although most of the biotransformation reactions are carried out by whole fungal cells, for some bioconversion reactions fungal spores are used, even in industrial applications [16]. The best known and most intensively studied example is the bioconversion of octanoic acid to 2-heptanone by spores of *Penicillium roqueforti* [17–19].

Another important application for which fungal spores are used, is the biotransformation of steroids [20]: a well known example is the 11α-hydroxylation of progesterone by spores of *Aspergillus ochraceus* [21–23].

The biotransformation of monoterpenoids by fungal spores is a new area. Indeed, most literature deals with the bioconversion of monoterpenoids by whole fungal cells in submerged shaken cultures. An important example is the biotransformation of monoterpene alcohols (geraniol, nerol, linalool, citronellol) by *Aspergillus niger* [24, 25]. In these articles, the regiospecific hydroxylation of the acyclic monoterpene alcohols and their acetates by cultured cells of *A. niger* is described, using shaken culture flasks. Another example is the biotransformation of these monoterpene alcohols by *Botrytis cinerea* [12, 26]. Using a grape must, be the predominant conversion of geraniol and nerol to their ω-hydroxylated products was observed. When using synthetic growth medium the ω-hydroxylation reactions were again observed, but 6-methyl-5-hepten-2-one was also identified as a major bioconversion product of geraniol.

This paper reports the bioconversion of geraniol and nerol to pure 6-methyl-5-hepten-2-one by fungal spores of *Penicillium italicum* over prolonged periods (up to 2 months).

RESULTS AND DISCUSSION

The biotransformation of volatile terpenoid alcohols by fungi was examined. In a first experiment the bioconversion of pure geraniol by one batch of fungal spores of *Penicillium italicum* was followed. Conical flasks were used, the bottom of which was coated with 100-ml solid medium. Two weeks after inoculation the surface was completely covered with spores; geraniol (100 μl) was then sprayed onto the sporulated surface culture. Immediately after the addition of the substrate, the headspace of the conical flask was concentrated on Tenax tubes. The adsorbed substances were recovered from the Tenax tubes by elution with diethyl ether, and analysed by GC-MS. Quantification was performed by comparison with added standards. In a period of 4 days, four headspace samples were taken. After this period 63 μl methylheptenone (MHO) was obtained from the headspace of the conical flask. In the final headspace sample, the volume of MHO was 3 μl, whereas in the other samples it was *ca* 20 μl. This means that the geraniol was transformed with a molar yield of 76%.

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To verify if MHO was further metabolized by the spores a control experiment was undertaken. Two conical flasks were used. One flask contained a sporulated surface culture. The other flask contained 100 ml sterile agar and was used as a blank test without *P. italicum*. MHO (100 μl) was sprayed onto both the sporulated surface culture and the pure agar layer. The headspaces of the conical flasks were concentrated on Tenax tubes, in the same way as described above. After a period of 4 days 74 μl MHO was recovered from the conical flask with the sporulated surface culture: in the first headspace 55 μl MHO was found, in the last 6 μl. From the blank flask 84 μl MHO was recovered, 60 μl in the first headspace, 10 μl in the last. The two experiments with MHO were repeated twice. When 100 μl MHO was sprayed onto a sporulated surface culture 79 μl was recovered the first time, 77 μl the second time. When 100 μl MHO was sprayed onto a pure agar layer 79 μl was recovered the first time, 80 μl the second time. It appears likely that *ca* 20 μl of MHO is retained by the agar layer.

In a next step the bioconversion of pure nerol by one batch of fungal spores was followed. The same procedure was followed as described above. It was found that after 4 days 100 μl nerol yielded 62 μl MHO.

As both geraniol and nerol are transformed to methylheptenone it apeared worthwhile to test if a mixture of geraniol and nerol would also be transformed. A mixture of 50 μl geraniol and 50 μl nerol was sprayed on to a sporulated surface culture of *P. italicum*. During a period of 4 days four headspace samples were taken. After this period 57 μl of MHO was recovered.

From these experiments it is clear that fungal spores are able to convert both geraniol and nerol to methylheptenone with a high purity (97% as checked with GC) and a molar yield varying from 69% to 76% during a short period of 4 days. The reason why the yield is not higher is that part of the MHO remains adsorbed to the agar layer. This means that more headspace samples have to be taken during a longer period.

In a final experiment the transformation of geraniol and nerol over prolonged periods was tested. In this case instead of a conical flask a cylindrical flask was used, providing a large surface in a smaller volume. The inner wall of the flask had been coated with solid medium by rotating the flask horizontally while the medium was congealing. Once solid it was inoculated with fungi and incubated for a few days. After 2 weeks the surface was completely covered with spores and 100 μl geraniol was introduced onto the bottom of the flask. During a period of 2 months six more additions of 100 μl geraniol followed, and two additions of 100 μl nerol. After each addition the flask was closed for 2 days, after which headspace samples of 24 hr or 48 hr were taken.

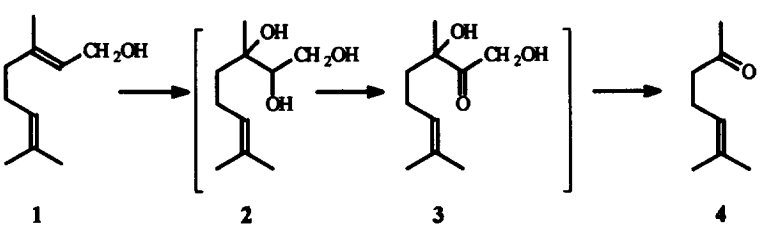
Both geraniol and nerol were biotransformed by the same batch of *P. italicum* spores to MHO. A headspace sample of 24 hr yielded 30–40 μl of MHO, while one of 48 hr yielded 40 to 95 μl MHO. In the final headspace only 5.3 μl nonconverted geraniol was found, and there was no more MHO. When all the collected headspace concentrates were added together, 670 μl methylheptenone was obtained with a purity of 96%. This means that the total yield of the experiment was 88.7%.

The fungal biotransformation of both geraniol and nerol to pure methylheptenone has not been published before. There are, however, two examples found in the literature where geraniol is transformed by bacteria. Devi and Bhattacharyya [27] described the complete degradation of geraniol (**1**) by *Pseudomonas incognita*. They isolated and characterized the triol (**2**) and the hydroxyketone (**3**) from the fermentation broth. As a further intermediate in the degradation of geraniol (**1**) they suggested methylheptenone (**4**), which is then decomposed by a yet unexplained pathway. The second example is the biotransformation of geraniol to methylheptenone by the bacterium *Pseudomonas putida* NRRL-B-18040 (PP U 2.9) [28].

Based on the latter data, and the degradation scheme proposed by Devi and Bhattacharyya [27], we suggest that the bioconversion of geraniol by the fungus *Penicillium italicum* follows the same pathway (Scheme 1).

It can be concluded that fungal spores have sufficient metabolic activity to perform biotransformations on monoterpenoids. The product obtained is of very high purity and is a natural compound as both the substrate and the biotransformation method are natural. In this method, no liquid cultures, but surface cultures with fungal spores are used. It offers many advantages; for example the danger of contamination of the fungal culture is limited.

The main problem most researchers encounter, i.e. the poor solubility of the terpenoid compounds in liquid aqueous media, is obviated because the precursor terpenoids evaporate slowly—the closed system is saturated with terpenoid vapors, which come in contact with the fungal spores. These spores perform the bioconversion into new compounds that are released in the gas phase,



Scheme 1. Proposed degradation of geraniol by *Penicillium italicum*

after which they are concentrated by a headspace sampling. There is one important condition for this method: namely both the precursor and the reaction product must be volatile.

The sporulated surface culture can keep its activity over prolonged periods, as the spores are immobilized on the agar layer. Higher substrate concentrations can be applied to spores than to viable cells. While terpenoids can cause toxicity and growth inhibition for whole viable cells, this does not appear to be the case for fungal spores.

Many different terpenoid compounds can be tested for bioconversion reactions with the same batch of fungal spores. It is clear that this method can be scaled up and applied for preparative purposes.

EXPERIMENTAL

Microorganism. Penicillium italicum was isolated from a spoiled tangerine. It was identified macroscopically and microscopically by comparison with literature data [29] with the help of Prof. Dr Ir. J. Poppe (Laboratory of Phytopathology and Phytovirology, Faculty of Agricultural and Applied Biological Sciences, Coupure Links 653; University of Gent, Belgium). It was conserved by periodic replications (every 2 weeks) on malt extract agar (MEA).

Growth medium. For the isolation, growth and conservation of the fungus the same medium was used: MEA (malt extract 2%, bacteriological peptone 0.1%, glucose 2% and agar 2%). The fungus was stored in Petri dishes, filled with 20 ml sterile medium.

Cultivation of surface cultures in conical flasks. The fungi were cultivated in 500-ml conical flasks, filled with 100 ml of solid medium (MEA). This surface was inoculated with spores, using an inoculation needle. The flasks were incubated for 24 hr at 30° during which germination of the spores and mycelial growth took place and was maintained for 15 days at room temp., after which complete sporulation had occurred.

After this period test substrates were added: 100 μl geraniol, nerol or methylheptenone was sprayed onto the surface of the sporulated cultures. Immediately after addition of the test substrate the reaction vessel was equipped with an air inlet and outlet. To the outlet was attached a Tenax adsorption tube (length 22 cm, i.d. 13 mm, o.d. 14 mm). The system was continuously aerated from an air cylinder at 30 ml min^{-1}. The volatiles were carried away and the headspace of the flask was concd on the Tenax tubes [30, 31]. During a period of 4 days four dynamic 24-hr headspace samples were taken.

As control a conical flask was filled with 100 ml pure agar (2% agar dissolved in 100 ml H_2O) and sterilized. This agar layer was not inoculated. The same procedure of adding test substrates was then applied to this flask.

Cultivation of a surface culture in a cylindrical flask. The fungus was cultivated in a cylindrical washer (5 cm diameter, 15 cm height), the inner wall of which had been coated with 20 ml MEA (leaving the bottom free), providing approx. 200 cm^2 of contact surface. This surface was inoculated with spores, using an inoculation needle. The flask was incubated for 24 hr at 30° and 15 days at room temp., after which complete sporulation had occurred. Test substrates were then introduced onto the bottom of the flask, which was closed for 2 days. After this period the headspace of the flask was concd on Tenax tubes [30, 31]. Dynamic headspace samples were taken over 24 or 48 hr. During a period of 2 months 7 additions of 100 μl geraniol and two additions of 100 μl nerol were made. The last headspace sample was taken 2 weeks after the last addition of 100 μl nerol and over 30 hr.

Chemical compounds. Geraniol was obtained from Fluka, nerol from Aldrich and methylheptenone from Janssen Chimica (Belgium).

Recovery of the adsorbed volatiles and their quantification. The adsorbed volatiles were desorbed from the collector tubes by application of 3 × 5 ml Et_2O to the Tenax tube. After addition of 1 ml of a standard soln of 0.1% (v/v) *n*-undecane in Et_2O, the eluate was directly analysed by GC and GC-MS.

Efficiency of the desorption method. When 10 μl MHO was injected in a Tenax tube and the Tenax tube was eluted by the method described, 99.9% of the MHO was recovered (analysis by GC). When the same tube was eluted for a second time the other 0.1% of the added MHO was recovered. When the same test was carried out after addition of 100 μl MHO to a Tenax tube, the first elution recovered 99.8% and the second 0.1–0.2% of the added MHO.

Analysis of the samples with GC and GC-MS. GC-analyses were performed with a Delsi-200 instrument, equipped with a DB-5 FSOT (fused silica open tubular) column (30 m × 0.32 mm i.d.; coating thickness 1 μm) and FID. Injector: cold on-column injector, detector temp. 230°; oven temp. programmed from 50–160° at 5° min^{-1} and from 160–200° at 10° min^{-1}; carrier gas (He) 1.9 ml min^{-1}; air 250 ml min^{-1}; H_2 25 ml min^{-1}. Peak areas were calculated by a computer equipped with Nelson-software. GC-MS analyses were performed with an HP 5890 gas chromatograph, equipped with a DB-5 FSOT column (50 m × 0.32 mm i.d.; coating thickness 0.2 μm) and an HP 5970A mass selective detector (quadrupole type). Working conditions: injector: 220°; oven temp. programmed from 50–150° at 5° min^{-1} and from 150–200° at 10° min^{-1}; carrier gas (He) 1 ml min^{-1}; ionization: EI 70 eV; acquisition parameters: scanned *m/z*: 40–250. Substances were identified by comparison of their mass spectra and retention indexes (Kováts Indexes) with those of reference substances. Response factors (Delsi): methylheptenone: 1.22; geraniol: 1.16, nerol: 1.08. retention indexes (Kováts Indexes [32]) as calculated from ref. substances: methylheptenone: 988, nerol: 1237, geraniol: 1262.

Mass spectra. Methylheptenone *m/z*: (rel. int.): 126 $[M]^+$ (1), 43 (100), 41 (54), 55 (28), 69 (19), 108 (18), 58 (11), 67 (10), 111 (8), 53 (7), 68 (7). Geraniol *m/z* (rel. int.): 41 (100), 69 (56), 93 (15), 68 (14), 43 (12), 67 (12), 53 (11), 55 (10), 84 (8), 80 (7). Nerol *m/z* (rel. int.): 41 (100), 69 (67), 93 (15), 68 (14), 67 (13), 53 (12), 43 (11), 55 (8), 80 (7), 83 (7).

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