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Biotransformation of terpenes by fungi Study of the pathways involved

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Abstract

The biotransformation of the pure terpene alcohols geraniol and nerol, the mixture of the alcohols, 'citrol', and the mixture of the aldehydes, citral, to 6-methyl-5-hepten-2-one by sporulated surface cultures of *Penicillium digitatum* was compared. It was found that citral was converted faster than the alcohols but gave a lower overall yield of ≈ 76%, whereas the pure alcohols and their mixture, 'citrol', gave a yield of ≈ 83%. It was also established that the bioconversion over prolonged periods was possible with an overall yield of 80–90% depending on the substrate used. The bioconversion of nerol to 6-methyl-5-hepten-2-one by a spore suspension was also shown. The pathways involved in the biotransformation of geraniol and citral to 6-methyl-5-hepten-2-one are also discussed.

Keywords: *Penicillium digitatum*; Biotransformation; Bioconversion; Fungal spores; Geraniol; Nerol; Citral; 6-Methyl-5-hepten-2-one; Pathway

1. Introduction

Over the last years, the biotechnological production of natural aroma chemicals (NACs) has been stimulated by the consumers demand for natural and healthy products [1]. This interest in natural flavours instead of 'synthetic flavours' has led to increasing research focused on the microbial production of the so-called 'bioflavours' [2–5]. Some of these processes are currently being exploited commercially. Nearly 80% of the flavours and fragrances used worldwide are produced chemically. However, about 70% of all food flavours used in Germany in 1990 were natural [6].

A natural flavour, according to the US FDA guidelines set down in 1958, must be produced from natural starting materials. The end-product must be identical to something already known to exist in nature. Thus, biocatalytic, but not chemical, transformation of natural substances and also microbiologically produced flavours can be legally labeled as 'natural' [7]. Terpenoids are inexpensive, readily available and renewable natural precursors. They constitute the largest group of natural products, with more than 22,000 individual structures now known [8]. Monoterpenes, widely distributed in nature (more than 400 structures), constitute suitable precursor substrates, which are ideal starting materials for the biotechnological production of NACs. Indeed, many data on terpenoid biotransformation by both fungi and bacteria are published. However, most of the monoterpene biotransformation studies described so

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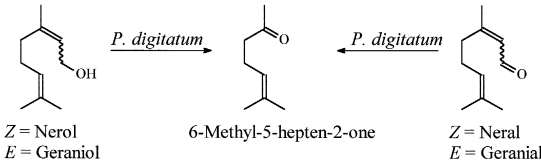


Scheme 1. Bioconversion of geraniol, nerol, geranial and neral to 6-methyl-5-hepten-2-one by sporulated surface cultures of *Penicillium digitatum.*

far have been of more academic than practical value, and no biotransformation process has been commercialised yet [6].

Previously, we reported on the biotransformation of geraniol and nerol [9] and citral [10] by spores of *Penicillium digitatum*. Both the mixture of the terpene alcohols nerol and geraniol, i.e. citrol, and the mixture of the terpene aldehydes neral and geranial, i.e. citral, were converted into 6-methyl-5-hepten-2-one ('methylheptenone' or MHO) by fungal spores (see Scheme 1).

In this article, the biotransformation of the pure terpene alcohols geraniol and nerol, and the mixture of the aldehydes neral and geranial, i.e. citral, is compared. Furthermore, the pathways involved in this biotransformation are discussed.

2. Results and discussion

2.1. Biotransformation of geraniol, nerol, citrol and citral by sporulated surface cultures

In a first experiment, the biotransformation of 300 μl geraniol, nerol, citrol (mixture of geraniol and nerol) and citral by sporulated surface cultures of *P. digitatum*, grown on 100 ml medium in conical flasks, was monitored over 3 weeks. One culture was treated with geraniol, one with nerol, two with citrol and two with citral.

A scheme with the bioconversion of geraniol, nerol, geranial and neral to 6-methyl-5-hepten-2-one is displayed in Scheme 1.

The results of the experiments carried out over the first 3-week period are shown in Fig. 1a–c. The final results and yields for the 3 weeks are shown in Table 1.

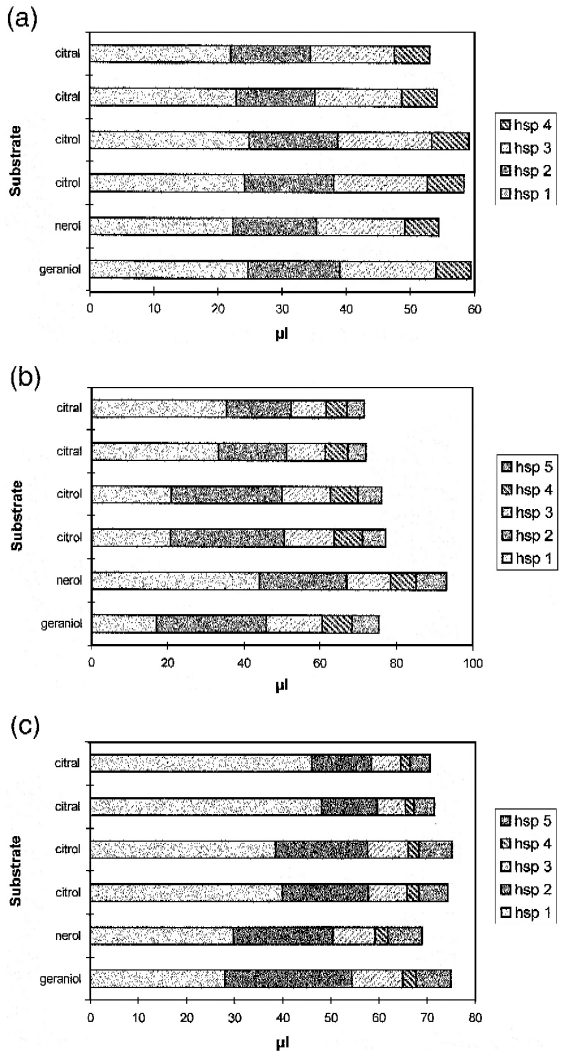


Fig. 1. (a) Cumulative production of 6-methyl-5-hepten-2-one in μl from various substrates during week 1. Hsp 1–4 represent the total amount of 6-methyl-5-hepten-2-one produced during sampling intervals of 24, 24, 52 and 65 h, respectively, as judged by continuously collected headspace samples of the total gas phase from an aerated experimental setup. (b) Cumulative production of 6-methyl-5-hepten-2-one in μl from various substrates during week 2. Hsp 1–5 represent the total amount of 6-methyl-5-hepten-2-one produced during a sampling interval of 16–67 h as judged by continuously collected headspace samples of the total gas phase from an aerated experimental setup. (c) Cumulative production of 6-methyl-5-hepten-2-one in μl from various substrates during week 3. Hsp 1–5 represent the total amount of 6-methyl-5-hepten-2-one produced during a sampling interval of 24–66 h as judged by continuously collected headspace samples of the total gas phase from an aerated experimental setup.

Table 1
Production of 6-methyl-5-hepten-2-one by six batches of fungal spores over 3 weeks

Substrate[a]	Yield[b]					
	Flask 1 — Geraniol	Flask 2 — Nerol	Flask 3 — Citrol	Flask 4 — Citrol	Flask 5 — Citral	Flask 6 — Citral
Week 1 (μl)	59.4	54.4	58.4	59.2	54.2	53.1
Week 2 (μl)	75.4	93.1	77.2	76.2	72.1	71.8
Week 3 (μl)	74.9	69.0	74.4	75.3	71.7	70.9
Total (μl)	209.7	216.5	210.0	210.7	198.0	195.8
Yield (%)	83.1	86.0	83.3	83.6	76.7	75.8

[a] One hundred microliters added at the beginning of each week.
[b] Total amount in μl of 6-methyl-5-hepten-2-one formed during the specified week.

It was found that citral was converted to 6-methyl-5-hepten-2-one faster than the other substrates and gave an overall yield of ≈ 76%, whereas the pure alcohols and the mixture of alcohols (citrol) were converted slower but gave a yield of ≈ 83%.

In the second part of the experiment (weeks 4–6), the continuous production of 6-methyl-5-hepten-2-one by sporulated surface cultures over prolonged periods was tested. For this test, the same six batches of sporulated surface cultures were used with the same substrates as in the previous test.

The results obtained from the second 3-week period for continuous production of 6-methyl-5-hepten-2-one are shown in Table 2.

From these data, it can be concluded that again citral was converted faster than the alcohols (higher yield during the first week, lower yield during the third week), but gave a lower overall yield (approximately 80%). In all experiments, it was noticed that only after a few days maximum conversion occurred. The alcohols were converted slower but gave an overall yield of approximately 90%. It is also clear that the spores retained their biotransformation capacity over a period of at least 6 weeks.

2.2. Biotransformation of nerol by a spore suspension of P. digitatum

A spore suspension of *P. digitatum* was prepared from sporulated surface cultures. To this spore suspension, nerol was added and at different time intervals, samples were taken and extracted. It was found that the maximum conversion of nerol to 6-methyl-5-hepten-2-one was reached after 10 days (43.7%) and that, from then on, the yield dropped. After repeated substrate additions, large amounts of non-converted nerol were found, meaning that the substrate accumulated and no more bioconversion took place, probably due to the high concentration of nerol in the suspension, causing substrate inhibition.

Table 2
Continuous production of 6-methyl-5-hepten-2-one by six batches of fungal spores over 3 weeks

Substrate[a]	Yield[b]					
	Flask 1 — Geraniol	Flask 2 — Nerol	Flask 3 — Citrol	Flask 4 — Citrol	Flask 5 — Citral	Flask 6 — Citral
Week 4 (μl)	25.6	29.2	37.1	35.7	39.8	41.4
Week 5 (μl)	90.0	82.1	81.9	85.5	72.3	74.4
Week 6 (μl)	100.9	90.5	95.4	93.5	75.7	82.4
Total (μl)	216.6	201.8	214.4	214.7	187.8	198.2
Yield (%)	92.0	85.9	91.1	91.3	77.9	82.2

[a] Eighty microliters added at the beginning of week 4 and 100 μl added at the beginning of weeks 5 and 6.
[b] Total amount in μl of 6-methyl-5-hepten-2-one formed during the specified week.

It can be concluded that spores in suspension without any nutrients are also able to transform nerol to 6-methyl-5-hepten-2-one.

2.3. Study of the pathways involved

The biotransformation of geraniol and geranial by the bacterial strain *Pseudomonas incognita* has been described [11]. Two pathways were proposed for the degradation of geraniol (see Scheme 2). Pathway A involves an oxidative attack on the 2,3-double bond resulting in the formation of an epoxide, which then yields the triol, which is further oxidised to the ketodiol. This ketodiol is then converted to 6-methyl-5-hepten-2-one by an oxidative process. Pathway B is initiated by the oxidation of the primary alcoholic group to geranic acid which is then further metabolised via β-oxidation [11]. Nerol is metabolised following the same pathways. However, judging from our experiments, the bioconversion by *P. digitatum* seemed to follow a different pathway. Because it was observed that citral is converted faster than the alcohols geraniol and nerol, it was assumed that citral is an intermediate in the bioconversion of the terpene alcohols to 6-methyl-5-hepten-2-one. The complete study of the enzymatic pathways involved is in progress and will be published in due time.

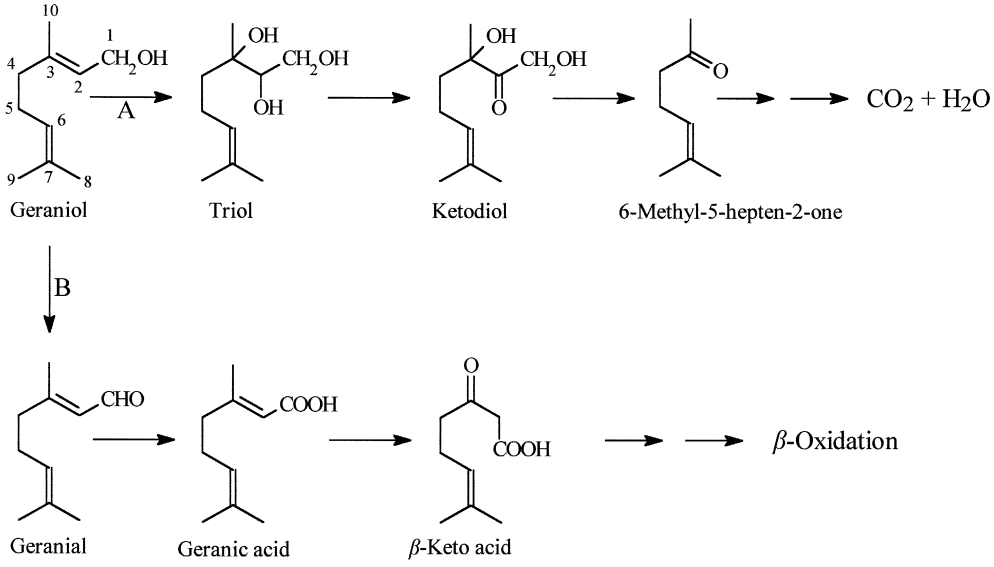
3. Experimental

3.1. Microorganism

A strain of *P. digitatum* was isolated from a spoiled tangerine. It was identified by the MUCL (Mycothèque de l'Université Catholique de Louvain, Laboratoire de Mycologie Systématique et Appliquée) as *P. digitatum* Saccardo. It was assigned by the ATCC (American Type Culture Collection) as *P. digitatum* ATCC 201167.

3.2. Growth medium and conditions

For the isolation, growth and conservation of the fungi in Petri dishes one and the same solid medium was used: Malt Extract Agar (MEA) (OXOID®): Malt Extract (2%), Bacteriological Peptone (0.1%), Glucose (2%) and Agar (2%). The biotransformation experiments on larger scale were carried out in 500-ml conical flasks, filled with 100 ml of MEA: the



Scheme 2. Pathways of degradation of geraniol by *Pseudomonas incognita* (after Ref. [11]).

solid agar medium was inoculated with spores of *P. digitatum*. The flasks were incubated at 30° for 24 h during which germination of the spores and mycelial growth took place and were then stored at room temperature. After 2 weeks, the surfaces were completely covered with spores and biotransformation reactions were started.

3.3. Biotransformation of terpenes by sporulated surface cultures in conical flasks

In the first part of the experiment (3 weeks), the biotransformation of pure terpene alcohols, the mixture of the alcohols and the mixture of the aldehydes was compared.

For this test, six batches of sporulated surface cultures in 500-ml conical flasks were used. Two batches were treated with pure terpenoid alcohols: one (flask 1) with geraniol, one (flask 2) with nerol; two batches (flasks 3 and 4) were treated with a mixture of the alcohols, i.e. citrol; and two batches (flasks 5 and 6) were treated with the mixture of the aldehydes geranial and neral, i.e. citral. The biotransformation capacity of the six cultures was followed over prolonged periods (3 weeks) with dynamic headspace sampling. During this period, three substrate additions were carried out (100 μl/week), each time followed by four or five headspaces of 24 h or more.

The first addition of 100 μl substrate was carried out 3 days before the start of the first headspace (day 1 of the first week). After the addition, the flasks were closed during 3 days and the first headspace sample was started on the first day of week 1 and taken until day 2, during 24 h. Two more headspace samples were taken during this week, during 24 and 52 h, respectively. The fourth and final headspace was taken from day 5 of week 1 to day 1 of week 2, during 65 h.

The second addition of 100 μl substrate took place on day 1 of week 2. After this addition, the flasks were closed during 8 h, after which four headspace samples were taken, during 16, 24, 24 and 34 h, respectively. The fifth and final headspace was taken from day 5 of week 2 to day 1 of week 3, during 67 h.

The third addition of 100 μl substrate was done on day 1 of week 3 and was immediately followed by three headspace samples of 24 h each. The fourth headspace sample was taken from day 3 to day 5 during 30 h and the fifth and final headspace was taken from day 5 of week 3 to day 1 of week 4, during 66 h.

In the second part of the experiment, the continuous production of MHO by sporulated surface cultures over prolonged periods was tested. For this test, the same six batches of sporulated surface cultures were used with the same substrates as in the previous test. The first week, four additions of 20 μl substrate took place, each day during the first 4 days. After each addition of precursor, the flasks were closed for 8 h after which headspace samples were taken during 16 h. Only the fourth headspace sample was taken during 24 h.

In the second week, four substrate additions took place, 30, 20, 30 and 20 μl, respectively, were sprayed onto the cultures on the first 4 days. After each addition, the flasks were closed for 8 h after which headspace samples were taken during 16 h. Only the fourth headspace sample was taken during 24 h. Two additional headspace samples were taken, during 20 h (days 5–6) and during 44 h (days 6–8).

In the third week again, four substrate additions took place, 30, 30, 20 and 20 μl, respectively, on the first 4 days. Immediately after each addition, headspace samples of 24 h each were taken. Only after the fourth substrate addition, a headspace sample of 30 h was taken. After day 5, the flasks were closed for 4 days after which 2 headspace samples were taken, (hsp 5) 48 h and (hsp 6) 24 h. Then again the flasks were closed for 6 days after which the seventh and final hsp sample was taken, 48 h.

3.4. Experiments with spore suspensions

Spores were recovered from nine 2-week-old surface cultures of *P. digitatum* grown in Petri dishes on MEA. This was done by adding 10 ml of a sterile Tween 80 solution (0.1%) onto each culture, bringing the spores into suspension, filtrating the spore suspension through cotton wool to remove cells and mycelium and combining the suspensions. A total

spore suspension of 50 ml was obtained, which was shaken in a 250-ml conical flask.

To this spore suspension, 0.5 ml of a solution of 5% nerol in Ethanol absolute (Merck) was added, i.e. 25 μl nerol. After 7 days, a 5-ml sample of this suspension was taken and extracted with 2×2 ml Et_2O, after which 1 ml of the solution of 5% nerol in Ethanol absolute was added to the suspension, i.e. 50 μl nerol. During an additional period of 20 days more samples were taken at different time intervals.

3.5. Chemical compounds

The substrates used for the biotransformation experiments were: nerol (Aldrich), geraniol (Fluka), citral (36.8% neral, 63.2% geranial) (Janssen Chimica). The control compound used was 6-methyl-5-hepten-2-one (MHO) (Janssen Chimica).

3.6. Headspace analysis

The solid surface cultures were sampled by the dynamic headspace technique. Therefore, the conical flasks were 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 for conical flasks; i.d. 9 mm, o.d. 10 mm for Petri dishes) filled with 1.5 g Tenax. The system was continuously aerated from an air cylinder at 30 ml min^{-1}. The volatiles were collected and the headspace of the flask was concentrated on the Tenax tubes. After each addition of substrate to the sporulated surface culture, dynamic headspace samples of 24 to 67 h were taken.

3.7. Recovery of the adsorbed volatiles and their quantification

The adsorbed volatiles were desorbed from the collector tubes by application of three times 5 ml diethylether to the Tenax tube. After addition of 1 ml of a standard solution of 0.1% (v/v) of *n*-undecane in diethyl ether, the eluate was directly analysed by GC.

3.8. Analysis of the samples with GC

GC-analyses were performed with a Delsi-200 instrument, equipped with a DB-5 fused silica open tubular (FSOT) column (30 m × 0.32 mm i.d.; coating thickness 1 μm) and FID. Working conditions: injector: cold on-column injector, detector temperature 230°C; oven temperature programmed as follows: from 50°C to 160°C at 5°C min^{-1} and from 160°C to 200°C at 10°C 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, taking into account the response factor of 6-methyl-5-hepten-2-one (1.22). Retention indexes as calculated from reference substances: 6-methyl-5-hepten-2-one, 988; nerol, 1237; neral, 1243; geraniol, 1262, geranial, 1274.

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