Biotechnology Letters (2005) 27: 149–153

A novel psychrotrophic fungus, *Mortierella minutissima*, for D-limonene biotransformation

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Received 12 October 2004; Revisions requested 27 October 2004; Revisions received 27 November 2004; Accepted 2 December 2004

Key words: biotransformation, D-limonene, *Mortierella*, perillyl alcohol, psychrotrophs

Abstract

Of 98 strains of moulds, isolated from arctic soils, *Mortierella minutissima* 01, grew the best on agar plates with limonene vapor. Perillyl alcohol and perillic acid were the main products of limonene biotransformation. Maximal yield of perillyl alcohol (125 mg l^{-1}) occurred in medium containing 0.8% substrate, at 15 °C, pH 6 and after 4–5 d.

Introduction

Terpenes, widely distributed in nature, constitute suitable precursors for potential production of valuable natural flavor and fragrance compounds. Monoterpenes, as substrates of microbial transformations, have led to a great variety of oxyfunctionalized compounds (Cadwallader *et al.* 1989, Chatterjee & Bhattacharyya 2001, Duetz *et al.* 2003). Allylic hydroxylations of monoterpene hydrocarbons are interesting reactions because of the multiple bioactivities of many of the resulting aroma compounds. Among various monoterpenes, limonene is cheap and readily available and can be biotransformed into more valuable compounds, such as carvone, perillyl alcohol and α-terpineol. *R*-(+)-Limonene, (*p*-mentha-1,8-diene) can be obtained from orange peel oil at up to 50 million kg per annum. It is widely distributed in many volatile oils, in some as the main constituent, especially in citrus oils, and so widely employed for the flavoring of cosmetics, soap, and many kinds of technical goods (Cadwallader *et al.* 1992).

A number of microorganisms can transform limonene to other monoterpenoid compounds (Noma *et al.* 1992, Tan *et al.* 1998, Chatterjee & Bhattacharyya 2001, Adams *et al.* 2003), though the amounts obtained (a few milligrams per litre range) were insufficient for industrial applications. In addition to the lack of effective biotransformation systems for the production of valuable natural flavor and fragrance compounds, terpene transformations generally suffer from volatility of the substrate and toxicity of terpenes towards microorganisms.

Cold-adapted microorganisms have a considerable potential in biotechnology (such as waste treatment at ambient temperatures), enzymology, food industry, and medicine (Margesin & Schinner 1994, Horikoshi 1995). The use of psychrotrophic microorganisms offers numerous advantages: high microbial growth rates, as well as high enzymatic activities and catalytic efficiency, from 10 °C to 25 °C which then prevents microbial contamination (especially in continuous systems) and may also shorten process times as well as decreasing costs of heating/cooling systems. Psychrophilic microorganisms may also be useful for transformation of terpenes but do not appear to have been investigated for this property.

In a previous study, a novel method for enzymatic biotransformation of D-limonene to carvone was developed (Trytek & Fiedurek 2002). In the present study, biotransformation of D-limonene by

psychrotrophic fungi has been examined and optimized to improve the yield of this process.

Materials and methods

Chemicals

(−)-Perillic acid was purchased from Sigma. (−)-Linalool (>97%), (+)-perillyl alcohol, (+)-perillaaldehyde, R-(+)-limonene, (−)-carveol and (−)-carvone were obtained, in purities of >99%, from Fluka. All reagents and solvents were of analytical grade.

Microorganisms and media

Fungi used in this study were isolated from soils (Fiedurek *et al*. 2003) in Spitsbergen (lat 77° 33′ N long 14° 30′ E).

The composition of media (BM) used for biotransformation of limonene is presented in Table 2.

Biotransformations analysis

After a specified period of time, 200 μl of 0.05 % (v/v) linalool in hexane was added to the medium. Subsequently, the biomass was harvested by centrifugation and the liquid was extracted by equal volume of diethyl ether. The ether fraction was separated, dried over anhydrous Na_2SO_4, and evaporated in a rotary vacuum evaporator at 30 °C. The residues was dissolved in 2 ml hexane and used for GC and GC-MS analyses, as reported previously (Trytek & Fiedurek 2002).

For determining the perillic acid content, acid methanolysis was used (Hollingsworth & Dazo 1988) with prior addition of cumic acid as an internal standard. Biotransformations were performed on two replicate samples and analyses carried out in duplicate. The data given here are the average of the measurements. The means standard error of terpenes estimate was ±0.18 and ranged from ±0.01 to ±9.8.

Results and discussion

Of 98 strains of moulds, 71 grew well with limonene as a sole carbon and energy source. Twelve of these were subsequently grown at 4 °C and 30 °C, and 86 at 4 °C. Only those giving the highest conversions of limonene are shown in Table 1.

Table 1. Preliminary evaluation of bioconversion activity of psychrotrophic moulds.

Organism	Bioconversion value (mg l^{-1})	
	Perillyl alcohol	Perillic acid
Chrysosporium pannorum	5.8	1.5
Mortierella alpina	15.8	4.7
Mortierella minutissima	22.9	12.5
Penicillium chrysogenum	2.2	0.3
Penicillium cyclopium	1.5	0.2
Penicillium islandicum	21.2	0.1

Moulds were grown in 250 ml conical flasks, each containing 50 ml of medium M-I at 20 °C and 150 rpm for 5 days. *R*-(+)-Limonene (200 μl) was placed into a glass substrate reservoir, and then applied through the gas phase. The media were inoculated with 2 ml spore suspensions (about 2×10^6 spores).

Among the main products analyzed after transformation (perillyl alcohol and perillic acid), there were limonene oxide, carveol and carvone (Figure 1). This may indicate that *M. minutissima*

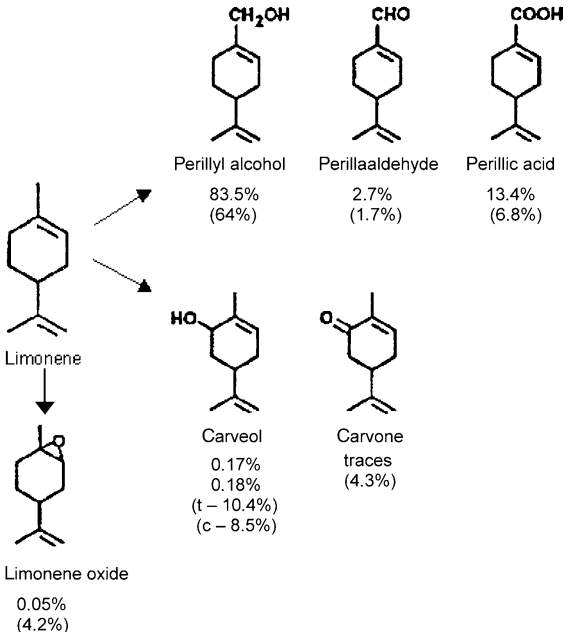

Fig. 1. Products of the biotransformation of D-limonene by *Mortierella minutissima* 01. Growth conditions of *Mortierella minutissima* 01 were the same as shown in Table 3. Conversion conditions: pH 6; temp. 20 °C or 15 °C (values given in the brackets); conversion time 96 h; volume of limonene: 400 μl.

01 utilises three different pathways of limonene bioconversion. It is worth noting that hydroxylation of C-6-position of limonene to give carveol was favored at 30 °C in comparison with 15 °C, the latter being the optimum temperature for hydroxylation of C-7-position (perillyl alcohol, perillaaldehyde and perillic acid).

The best of the tested media leading to the highest biomass production was medium M-II, which also proved to be the best for bioconversion (Table 2). We obtained the highest yields of perillyl alcohol and perillic acid from limonene after 120 h, accompanied by the highest biomass accumulation.

Among two main bioconversion products, perillyl alcohol is of particular importance due to its high bulk prices which are in the range of US $300–600/kg. It has chemopreventive properties against liver, mammary and lung carcinogenesis, which suggests a great market potential (Reddy *et al.* 1997, Bardon *et al.* 1998). In further studies, optimization was done to improve only the yield of this product.

Generally, high concentrations of organic solvents are toxic to biological systems (Laane *et al.* 1987). The optimum substrate concentration for perillyl alcohol production was about 0.8% (v/v). Increasing limonene concentrations (to 1.2%) brought a decrease in perillyl alcohol production and accumulation of dry weight (over 1.8-fold) (Figure 2). The optimum concentration of limonene for α-terpineol production by *Pseudomonas gladioli* was less than 1% (v/v) (Cadwallader *et al.* 1989).

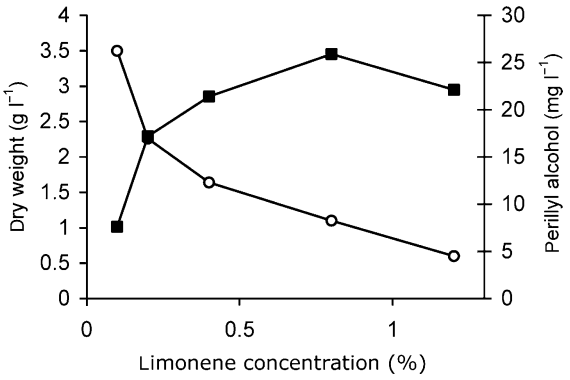

Fig. 2. Effect of substrate concentration on D-limonene bioconversion by *Mortierella minutissima* 01. Substrate was added directly to the medium after inoculation with 2 ml of spore suspensions (about 2×10^6 spores) and cultured at 20 °C for 4 days on rotary shaker (150 rpm). ■ – Perillyl alcohol; ○ – dry weight.

In the course of further studies, limonene was added directly to cultures after 96 h of growth to decrease the time of exposure of the cells to the toxic substrate; cultures were continued for another 144 h. The maximum bioconversion of limonene by *Mortierella minutissima* 01 was 120 h after substrate addition, when the highest amount of perillyl alcohol was obtained (Table 3). The decrease in product concentration, having reached its maximum, may be caused by the inactivation of the initial enzymatic oxidation step of limonene, while further metabolism of perillyl alcohol continues to unknown compounds. Some substrate may be lost through evaporation. Only trace amounts of limonene were left in the cultures of

Table 2. Biotransformation of D-limonene by *Mortierella minutissima* 01 in various growth media.

Media	Composition (g l^{-1})	Mycelia dry weight (g l^{-1})	Bioconversion value (mg l^{-1})	
			Perillyl alcohol	Perillic acid
M-I	Yeast extract (2), $NaNO_3$ (5), KH_2PO_4 (1), $MgSO_4$ (1), $CaCl_2$ (1)	1	22.6	13.6
M-II	Malt extract (10), peptone (5), glucose (10), yeast extract (5)	2.3	29.1	22.6
M-III	Yeast extract (1), sucrose (30), urea (3), $(NH_4)_2SO_4$ (0.5), KH_2PO_4 (4)	0.2	12.7	18.4
M-IV	Yeast extract (1), sucrose (50), $NaNO_3$ (2), $MgSO_4 \cdot 7H_2O$ (0.5), K_2HPO_4 (1), KCl (0.5)	1.5	24.2	12.4
M-V	Urea (0.3), raffinose (10), KH_2PO_4 (2), $(NH_4)_2SO_4$ (1.4), Tween-80 (1)	0.6	7.4	5.3

Growth conditions of *Mortierella minutissima* 01 were the same as shown in Table 1.

Table 3. Factors affecting biotransformation of D-limonene to perillyl alcohol by *Mortierella minutissima* 01 in various growth conditions.

Facto varied	Perillyl alcohol formed (mg l^{-1})	Limonene remaining (mg l^{-1})
Conversion time (h)		
24	5.8	40.9
48	56.7	14.8
72	71.5	7.1
96	104.1	2.8
120	112.3	0.4
144	70	0
pH		
3	6.3	2.7
4	7.5	9.9
5	63.5	2.2
6	124	7.8
7	95.5	16.2
Temperature (°C)		
5	8.3	31.7
10	20.5	16.3
15	125.6	0.5
20	94.7	0.5

Moulds were grown in 250 ml conical flasks, each containing 50 ml of medium. After 4 days of mycelium cultivation, 400 μl of limonene was added directly to the medium and flasks were closed with Teflon stoppers. Conversion conditions: 1. pH 6; temp. 20 °C; 2. temp. 20 °C; conversion time 120 h; 3. pH 6; conversion time 96 h.

Penicillium digitatum NRRL 1202 after 48 h biotransformation, as observed by Tan *et al.* (1998). The time of this bioconversion is shorter than that of *Pleurotus sapidus* (12 d) (Onken & Berger 1999). The time of biotransformation of limonene to perillyl alcohol by the *Pseudomonas putida* was reported to be 5 d (Chatterjee & Bhattacharyya 2001).

Mycelial cultures of *Mortierella minutissima* 01 showed a maximum bioconversion at 15 °C (Table 3), which was similar to that of its growth optimum (20 °C) (Fiedurek *et al.* 2003). Higher optimum temperatures were reported for bioconversion of limonene to oxygenated products by *Pen. digitatum* (Tan *et al.* 1998). The temperature optimum for the bioconversion of D-limonene by *Pseudomonas gladioli* and *Ps. putida* was 30 °C (Cadwallader *et al.* 1992, Chatterjee & Bhattacharyya 2001). Bioconversion activity occurred from pH 3–7. One peak of activity was identified with the most rapid bioconversion occuring at pH 6 (Table 3), which is the growth optimum pH (data not shown). This pH profile was different from that for bacterial bioconversion (Cadwallader *et al.* 1992).

As far as we know, reports on limonene bioconversion by psychrotrophic fungi have not been published. Therefore, our results in this respect can be compared with the data concerning mesophilic fungi. Bioconversion yield of perillyl alcohol gained by *Mortierella minutissima* 01 was either lower than values reported for mesophiles, such as *Pseudomonas putida* (Chatterjee & Bhattacharyya 2001), or significantly higher in comparison with the product obtained by another fungal strain *Aspergillus cellulosae* M-77. It, furthermore, displayed a higher degree of regiospecificity than mentioned before (Noma *et al.* 1992).

The results reported here point to the possibility of obtaining an active strain able to transform limonene to perillyl alcohol and perillic acid from psychrotrophic microorganisms. The advantage gained in utilizing with psychrotrophic microorganisms is a significantly reduced loss of terpenens by evaporation. The main disadvantage concerns the longer cultivation time of this fungus, which can be avoided by using an immobilized mycelium of *Mortierella minutissima* 01 or resting cells. Here, we show that the final concentration of perillyl alcohol in the culture medium can be increased to over 120 mg l^{-1} by optimizing biotransformation conditions.

Acknowledgement

This work was financially supported by research program No.I/BiNoZ/statut/.

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