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ORIGINAL RESEARCH PAPER

In situ recovery of the aroma compound perillene from stirred-tank cultured *Pleurotus ostreatus* using gas stripping and adsorption on polystyrene

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Abstract Supplementation of the key metabolite, α-(*Z*)-acaridiol, to stirred-tank cultured *Pleurotus ostreatus* was used to demonstrate that integrated in situ product recovery resulted in high conversion rates and quantitative separation of the target product perillene from the nutrient medium. The conversion of *β*-myrcene by *P. ostreatus* was scaled-up from shake-flasks into a controlled, stirred tank bioreactor equipped with gas stripping and adsorption on a polystyrene fixed bed. The formation of the attractive flavour compound perillene was measured daily using standard controlled capillary gas chromatography. The formation of α-(*Z*)-acaridiol was the metabolic bottleneck of the conversion of *β*-myrcene to perillene. Efficient in situ recovery of the volatile product enabled quantitative separation of the pure flavour compound. Appropriated bioprocessing, i.e. in situ separation of product, steadily shifted the metabolic equilibria and thus accomplished high conversion rate and pure product.

Keywords α-(*Z*)-acaridiol · Adsorption · In situ recovery · Myrcene · Perillene · *Pleurotus ostreatus*

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Introduction

The growing market of flavoured and fragranced products (convenience food, beverages, cosmetics, detergents) requires novel strategies towards the production of aroma chemicals. The conventional routes of chemical synthesis or isolation from plants are still viable, but the biotechnological generation of aroma compounds is gaining increasing attention (Krings and Berger 1998). Commercial production of aroma compounds by de novo microbial biosynthesis has often been limited by the low productivity (Rito-Polomares et al. 2001). The feeding of chemically related and readily available natural compounds, so called precursors, as well as the application of sophisticated bio- and down-streaming processes can improve the productivity of a bioprocess considerably (Schrader 2007; Hua et al. 2007).

Perillene [3-(4-methyl-3-pentenyl)-furan], a trace constituent of many plant essential oils with a fresh citrus note, was formed by *β*-myrcene supplemented cells of the basidiomycete *Pleurotus ostreatus*. Although the perillene concentration in submerged cultured pellets remained below 1 mg l^{-1}, a study elucidating the metabolic pathway was successful (Fig. 1; Krings et al. 2008a, b). The formation of α-(*Z*)-acaridiol was supposed to be the metabolic bottle neck. This study reports that supplementation of α-(*Z*)-acaridiol with integrated in situ product recovery resulted in high bioconversion rates and quantitative separation of perillene.

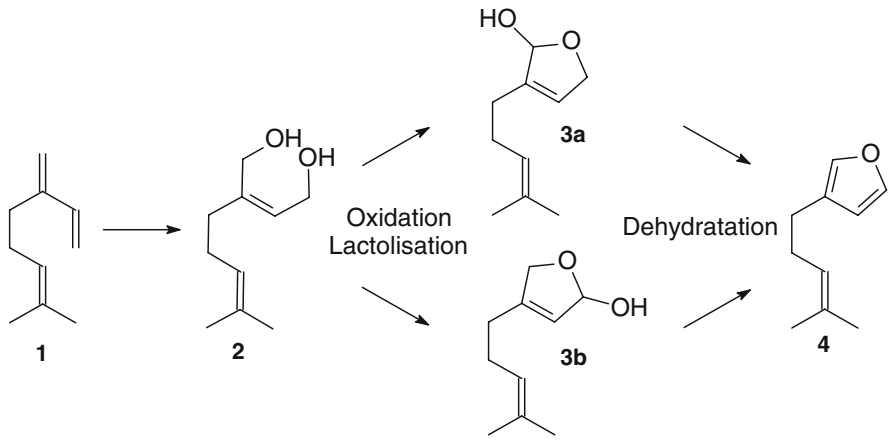

Fig. 1 The perillene **[4]** pathway: β-myrcene **[1]**, α-(*Z*)-acaridiol **[2]**, α,α-acarilactol **[3a]**, α, β-acarilactol **[3b]**

Materials and methods

Fungus: *Pleurotus ostreatus* (DSMZ 1020) was obtained from the culture collection of DSMZ, Braunschweig, Germany. It was grown on glucose/asparagine/yeast extract medium (Onken and Berger 1999). The flasks were sealed with cellulose plugs and autoclaved for 20 min at 121°C.

Chemicals: β-Myrcene (>90%, Fluka, Germany) and azeotropic pentane/diethyl ether (1:1.12 v/v) were distilled before use. α-(*Z*)-Acaridiol was obtained by reduction of α,α-acariolide using diisobutylaluminium hydride (Krings et al. 2008a).

Bioconversion

Bioconversion was performed using 1.8 l medium in a 2 l bioreactor (ISF-100, Infors, Bottmingen, Switzerland) with paddle stirrer (250 rpm), a pH electrode operated at 24°C and aerated at 0.1 vvm with sterile air. The reactor was inoculated with 200 ml homogenised pellets (settled volume after 10 min). The waste air outlet of the bioreactor was equipped with an adsorber (glass column, 12 × 1 cm i.d.) packed with 10 g precleaned (3 times with 30 ml methanol, diethyl ether and n-pentane) and heated (100°C overnight) Lewatit 1064MD (0.32–1.3 mm particle size, Lanxess, Leverkusen, Germany) to trap the stripped volatiles.

As with the previous shake-flask cultures (Krings et al. 2008a) bioconversion was started after 3 days fungal growth by adding 5.2 mmol β-myrcene during the first 2 days and was increased to 26 mmol on the following days to account for the fast evaporation of β-myrcene. α-(*Z*)-Acaridiol was added daily at 171 µmol (29 mg, adapted to the conversion rate found with shake-flask cultivation).

Determination of bioconversion products

Substrate, metabolite and product concentrations were determined as described elsewhere (Krings et al. 2008a, b). In brief, aliquots of the culture broth or disrupted fungal pellets were extracted three times with pentane/diethylether (1/1.12 v/v), dried, concentrated, and analysed by means of high resolution gas chromatography (HRGC) and HRGC-mass spectrometry (HRGC-MS). Intracellular metabolites were isolated after fungal cell disruption using a glass bead mill (Dyno-Mill, Bachofen, Basel, Switzerland) as described elsewhere (Taskova et al. 2006). The loaded adsorber column was replaced each day and desorbed using pentane/diethylether (1/1.12 v/v). The desorbate was dried, concentrated, and 1 µl was analysed by HRGC. One µl of each concentrated sample was injected into a CE Instruments Trace GC 2000 equipped with a cool on-column injector, a Zebron ZB-WAX (Phenomenex, USA) fused silica capillary column (30 m × 0.32 mm i.d. × 0.25 µm film thickness), H_2 as the carrier gas (40 cm s^{-1}), and a FID (230°C) using a temperature program from 40°C (3 min) to 250°C at 3°C min^{-1} and hold for 5 min. Quantification was performed according to the standard n-nonadecane.

All experimental data points are the means of at least two independent internal standard controlled GC quantifications with a standard deviation of less than 10%. The air-stripped perillene was trapped continuously on one adsorber column over 24 h; therefore

duplicates of the adsorption/desorption step were not possible. A column breakthrough did not occur, as was monitored using a serially connected second adsorber column. The exhaustive desorption of the target compounds was controlled by a repeated desorption. The concentrated eluate of the second desorption was regularly found free of any volatile metabolites.

HRGC-MS analysis was carried out using the same chromatographic conditions as for GC-FID analysis and helium as the carrier gas (38 cm s^{-1}). Identification of transformation products was achieved by comparison of EI mass spectra with data from reference compounds or literature (Wiley 6 for MassLab. 1996; NIST 02, June 2002 spectral libraries) using a Fisons GC 8000 gas chromatograph and a Fisons MD 800 mass selective detector (interface: 230°C, ion source: 200°C, quadrupole: 100°C, EI ionisation (70 eV), scan range m/z 33–300 amu).

Results

Pleurotus ostreatus was grown under the controllable conditions of a bioreactor. The water-soluble intermediate, α-(*Z*)-acaridiol, or, for comparison, its volatile precursor, *β*-myrcene, were supplemented daily. During the 10 days of bioconversion of both precursors, the initial pH of 6.8 in the culture medium remained almost constant. The glucose concentration decreased continuously from 30 to 22 g l^{-1}, whereas the wet biomass increased from 61 g to 210 g l^{-1}. After supplementation of *β*-myrcene neither the liquid medium nor the adsorber trap contained detectable products. The daily supplied *β*-myrcene was exhausted within the first hours and entirely trapped on the adsorber column. Disruption of the fungal pellets at the end of the cultivation (Taskova et al. 2006) made four Diels Alder products of *β*-myrcene (>5 mg 210 g^{-1} wet mycelium) and traces (<0.2 mg 210 g^{-1} wet mycelium) of 1,2- and 3,10 epoxy-*β*-myrcene amenable to analysis.

Figure 2 shows the course of metabolite concentrations over 10 days, when a daily portion of 29 mg (171 μmol) of α-(*Z*)-acaridiol was added to the culture medium. Every 24 h after the supplementation of α-(*Z*)-acaridiol, the progress of product formation was monitored and balanced. A small reminder (1 to 2%, w/w) of α-(*Z*)-acaridiol remained in the culture liquid,

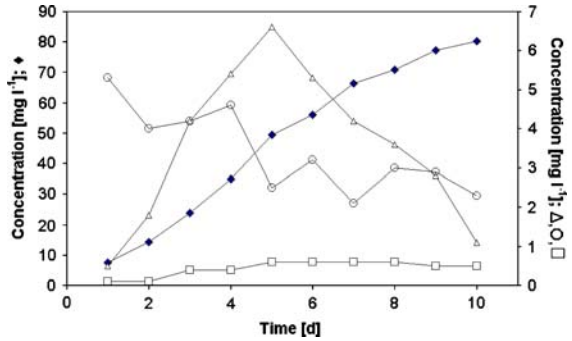

Fig. 2 Actual concentrations of main conversion products after daily supplementation of α-(*Z*)-acaridiol (17.1 μmol) to a liquid culture of *P. ostreatus* grown in a bioreactor (2 l, 250 rpm, 24°C, 0.1 vvm). ◆—Perillene (recovered from the waste air, concentration summed up over time), □—α,α-acarilactol, △—α, *β*-acarilactol, ○—α-(*Z*)-acaridiol

but no α-(*Z*)-acaridiol was trapped on the adsorber column. This indicates both low volatility and a fast bioconversion of this compound. Except α,α- and α,*β*-acariolide, products of the oxidation of lactols **[3a,3b]**, the concentrations of all other conversion products in the liquid medium were below 0.2 mg l^{-1}. The kinetics of formation of the two lactones were different. A slow increase of the concentration of α,α-acariolide was observed until day five which remained at a constant level of about 0.6 mg l^{-1}. In contrast, α,*β*-acariolide increased rapidly and reached a peak concentration of 6.6 mg l^{-1} on the fifth day to decrease then with the same rate.

In contrast to the experiment, in which *β*-myrcene served as the substrate, the daily desorbate of the adsorber column now yielded considerable quantities of perillene beside traces of α,α-acarilactol and both acariolides (<0.2 mg l^{-1}). The target compound was recovered quantitatively from the waste air outlet; no perillene remained in the liquid phase. After 10 days (ten successive supplementations of α-(*Z*)-acaridiol) a calculated total of 80.3 mg l^{-1} perillene was recovered (Fig. 2). The highest rate of perillene formation (78%, mol/mol) was observed on day five and showed an almost even mass balance (96%, mol/mol) (Table 1).

Discussion

The bioconversion of monoterpene hydrocarbons holds much promise for aroma biotechnology, but

Table 1 Bioconversion of α-(*Z*)-acaridiol (171 µmol each day) by *P. ostreatus*

Time (days)	Daily conversion to perillene (%)[a]	Summed-up conversion to perillene (%)	Summed-up conversion to all metabolites (%)[b]
1	59	59	75
2	52	55	84
3	76	62	88
4	87	68	92
5	109	76	96
6	55	73	87
7	80	74	85
8	36	69	80
9	49	67	77
10	25	63	74

2 l Bioreactor with in situ product recovery of volatile products

[a] Molar percentage of perillene generated within 24 h, respectively

[b] Molar percentage of products from α-(*Z*)-acaridiol amenable to GC-analysis

the inevitable losses of substrate and product due to evaporation in open cultivation systems often impede high yields (Kaspera et al. 2006). Fed-batch addition of monoterpene hydrocarbons is a proven procedure to reduce the cytotoxic effects of the conversion substrate (Zorn et al. 2004). Compared to shake flask cultures where α-(*Z*)-acaridiol supplementation did not result in a significant increased perillene concentration due to evaporation the in situ recovery of perillene enabled

(i) a quantitative precursor consumption and perillene separation,
(ii) an increased bioconversion rate of α-(*z*)-acaridiol and
(iii) an about 100fold increase of recovery of almost pure perillene.

Some biotransformations improved by in situ recovery have been published (Rito-Polomares et al. 2001; Miyazawa and Ohsawa 2002; Ezeji et al. 2003; Ribeiro et al. 2004; Hua et al. 2007). For an industrial production of aroma compounds in situ product recovery offers fundamental advantages:

(i) no further downstream processing is required,
(ii) target products are protected against further enzymatic or chemical alterations during the cultivation,
(iii) non-favoured metabolic or chemical equilibria can be shifted towards the desired products.
(iv) polystyrene resin adsorber show high capacity, good kinetic performance, stability and re-usability and are thus particularly useful for the adsorption of aroma compounds (Gehrke et al. 2000).

The in situ recovery of perillene demonstrated that the outstanding metabolic potential of fungal biocatalysts, especially of basidiomycetes, may be translated into industrial processes. Conversion rates of more than 90% and high product yields, as obtained with the process presented in this paper, have been rarely achieved before. For comparison, citric acid, a non-volatile flavour, is produced in fed-batch cultivation of Aspergillus niger on starch hydrolysates or molasse in large stirred or tower fermenters with a conversion rate of more than 90%. This demonstrates that a profitable and large-scale use of fungal biocatalysts is an industrial reality in flavour production (Schrader 2007).

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