

Regio- and Stereoselective Fungal Oxyfunctionalisation of Limonenes

Rüdiger Kaspera^a, Ulrich Krings^a, Michael Pescheck^b, Dieter Sell^b, Jens Schrader^b, and Ralf G. Berger^{a,*}

^a Zentrum für Angewandte Chemie der Universität Hannover, Institut für Lebensmittelchemie, Wunstorfer Str. 14, D-30453 Hannover, Germany.
Fax: +49-511-762-4547. E-mail: rg.berger@lci.uni-hannover.de

^b Karl-Winnacker-Institut der DECHEMA e. V., Theodor-Heuss-Allee 25, D-60486 Frankfurt am Main, Germany

* Author for correspondence and reprint requests

Z. Naturforsch. **60c**, 459–466 (2005); received November 23/December 23, 2004

Selective transformations of limonene by asco- and basidiomycetes were investigated. On the shake flask scale, *Penicillium citrinum* hydrated *R*-(+)-limonene to α -terpineol [83% regioselectivity (rs), more than 80 mg l⁻¹ product yield], and *Gongronella butleri* catalysed the terminal oxidation to yield perillyl alcohol (60% rs, 16 mg l⁻¹). On the laboratory bioreactor scale, *Penicillium digitatum* produced a peak concentration of 506 mg α -terpineol l⁻¹ in the fed-batch mode, equivalent to a theoretical yield of 67%, and no volatile by-products were found. *Fusarium proliferatum* transformed *R*-(+)-limonene enantiospecifically to *cis*-(+)-carveol (98.6% ee, more than 35 mg l⁻¹ product yield) and *S*-(-)-limonene predominantly to *trans*-(-)-carveol (96.3% ee). *Pleurotus sapidus* selectively dehydrogenised the accumulating *trans*-(-)-carveol to the corresponding enantiopure *R*-(-)-carvone. The results show that a careful selection of strain and bioprocess parameters may improve both the yield and the optical purity of a desired product.

Key words: Limonene, Oxyfunctionalisation, Stereoselectivity

Introduction

Bioactive monoterpenoids derived from both limonene enantiomers are among the most frequently investigated products of terpene biotransformations. Especially some of the mono-oxidised derivatives, such as carvone, perillaldehyde, α -terpineol or linalool oxide are value-added products that are used as potent flavours, fragrances, pharmaceuticals, fungistatics, and enzyme inhibitors (Duetz *et al.*, 2003; Grassmann *et al.*, 2002; Schrader and Berger, 2001; Sime *et al.*, 2000). The little odour active precursor limonene, the main constituent of *Citrus* essential oils, occurs in large quantities as a waste stream of *Citrus* oil processing representing a cheap starting material (Duetz *et al.*, 2003; Ohloff, 1994).

Regio- and enantioselectivity of a volatile molecule are often responsible for characteristic properties in flavour perception. The different perception of the carvone enantiomers [(*S*)-(+)-caraway, (*R*)-(-)-mint] is one of the best known examples of stereoselective odour recognition. The stereoselective introduction of the hydroxy functionality is, therefore, one of the main objectives of flavour biotechnology. Previous investigations on limo-

nene transformations have demonstrated at least five different degradation pathways (van der Werf *et al.*, 1999b) which often co-exist without selectivity. For instance, the bacterial transformation of limonene by *Pseudomonas aeruginosa* reached a maximum concentration of 0.63 g l⁻¹ carvone and 0.24 g l⁻¹ α -terpineol in 200 ml shake flasks (Acosta *et al.*, 1996). The basidiomycete *Pleurotus sapidus*, when supplied with (*R*)- or (*S*)-limonene via the gas phase, yielded more than 0.1 g l⁻¹ carveol and carvone (Onken and Berger, 1999) in all possible enantiomer forms. Only a strain of *Rhodococcus opacus* hydroxylated (*R*)-limonene stereospecifically to yield (+)-*trans*-carveol with 94 to 97% ee and with no by-products (Duetz *et al.*, 2001a). This enantioselectivity is crucial for the generation of enantiopure carvone, accessible through the subsequent oxidation, for example, by *R. erythropolis* (van der Werf and Boot, 2000). Apart from the often found lack of regio- and stereoselectivity, another drawback of cell-based transformations is the enzymatic attack of the primary products of oxidation. For instance, the formation of perillyl alcohol was always followed by the oxidation to perillaldehyde and perillic acid followed by β -oxidation (Duetz *et al.*, 2001b;

Chang and Oriel, 1994). As both disadvantages likewise occur with chemosynthesis, there is a strong demand for improved biotransformations for the asymmetric synthesis of terpenoids.

The paper summarises results from an extended screening of 50 asco- and basidiomycete species in an attempt to explore their catalytic potential in limonene transformation. The transformation of (*R*)-(+)-limonene to α -terpineol was selected to demonstrate the up-scaleability, and the transformation of limonene isomers to carveol isomers to prove enantiospecificity.

Materials and Methods

Strains

Amariella mellea (CBS 100.12), *Aspergillus niger* (DSM 737), *Botrytis cinerea* (DSM 1179), *Chaetomium cochloides* (DSM 1909), *Chaetomium globosum* (DSM 1962), *Fusarium proliferatum* (DSM 762), *Grifola frondosa* (CBS 480.63), *Gongronella butleri* (DSM 2917), *Penicillium digitatum* (DSM 62840), *Penicillium citrinum* (DSM 1179), *Pleurotus euosmus* (DSM 5331), *Pleurotus ostreatus* (DSM 5339), *Pleurotus sajor-caju* (DSM 1020), *Pleurotus sapidus* (DSM 8266) were obtained from the culture collections of DSMZ, Braunschweig, Germany and Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. The strains were maintained on yeast agar slants.

Cultivation

Standard medium was prepared as described by Onken and Berger (1999). For the screening and shake flask experiments, cultures (200 ml volume) were inoculated with 10 ml of 5 d old pre-cultures which were grown on the same medium and homogenised with an Ultra-Turrax (Janke & Kunkel, Germany) prior to inoculation. Cultivation was performed on an orbital shaker (Multitron, Infors, Bottmingen, Switzerland) at 150 rpm and 24 °C. The cultivation medium of *Penicillium digitatum* was composed of 10 g l⁻¹ glucose, 20 g l⁻¹ malt extract, 10 g l⁻¹ peptone and 5 g l⁻¹ yeast extract. The pH was adjusted to 6.5 by HCl and the medium was autoclaved for 20 min at 121 °C. The inoculum was prepared by washing a 10 d old cultivation plate with 5 ml sterile medium and then transferring the medium with spores to a 1000 ml Erlenmeyer flask containing 400 ml medium. The flask was incubated at 27 °C for 5 d on an orbital shaker (Certomat-R, B. Braun, Melsungen) at

120 rpm. The bioreactor used (KLF, Bioengineering, Switzerland) had a working volume of 2.7 l and a total volume of 3.7 l. It was equipped with a disc stirrer, a dissolved oxygen probe and a pH electrode. Cultivation was started by adding 200 ml inoculum to 2.5 l medium which was autoclaved *in situ* prior to use. The initial agitation speed was 400 rpm, the aeration rate 0.05 vvm (volume air per volume reactor and minute), the temperature 27 °C; the initial pH after inoculation was 6.3 and was not controlled during fermentation. A metal shell excluded light from the bioreactor. The final biomass concentration was determined by measuring dry cell weight after harvesting and homogenising the total bioreactor content; 4 ml samples were filtered through dried, pre-weighed filter paper and then dried at 105 °C to constant weight.

Conversion experiments

Conversion experiments in shake flask cultures were started after 3 d of inoculation of the experimental culture by adding 100 μ l (3.4 mM) of (+)-limonene (Fluka, > 90%) directly into the flasks. Samples were taken at least every day. Cultivation of pure medium and of autoclaved mycelium with addition of limonene were performed to estimate non-catalysed chemical transformation. When the glucose concentration in the bioreactor dropped below 1 g l⁻¹ (after 70–73 h) the biotransformation was started by raising the speed of agitation to 500 rpm and adding 0.84 ml limonene. Subsequently, 0.84 ml of the precursor was added every 24 h. During fed-batch fermentation portions of glucose were additionally supplemented to increase the actual concentration from 0 to 2 g l⁻¹ every 24 h (immobilised enzyme glucose analyser, YSI 2700 Select, Yellow Springs, OH, USA).

GC analysis of terpenoids in whole cell extracts

Samples of cultures were centrifuged at 2,000 \times g. To 2 ml of supernatant 0.2 g of sodium chloride were added and the liquid was extracted with 4 ml *n*-pentane/diethyl ether mixture (azeotropic 1:1.12). After centrifugation the organic phase was dried with anhydrous sodium sulfate and injected into a Fisons GC 8360 gas chromatograph equipped with a cool on-column injector, CW 20 M column (30 m \times 0.32 mm i.d. \times 0.4 μ m), and a flame ionisation detector using a temperature program starting at 100 °C (3 min) to 220 °C with a

rate of $5\text{ }^{\circ}\text{C min}^{-1}$. Quantification was done by electronic integration using internal standard calibration ($50\text{ }\mu\text{g camphor per extract}$).

Identification of products

Transformation products were identified by high resolution GC-MS using a Fisons GC 8000 gas chromatograph and a Fisons MD 800 mass selective detector [interface: $230\text{ }^{\circ}\text{C}$; ion source: $200\text{ }^{\circ}\text{C}$; quadrupole: $100\text{ }^{\circ}\text{C}$; EI (70 eV), scan range m/z 33 to 400] under the same chromatographic conditions as for the GC-FID and the spectra obtained were compared with mass spectra from libraries (NIST, Wiley; van Dyk *et al.*, 1998) and retention times of authentic standards [limonene, carvone (*R/S*), perillyl alcohol, perillaldehyde, α -terpineol, limonene epoxide, dihydrocarveol (Fluka, > 99%), carveol as mixture of (*-*)-isomers (Aldrich, > 97%)].

Enantiomeric distribution of carveol isomers was measured using a double oven gas chromatograph (Sichromat 2–8, Siemens) equipped with a PTV (programmable thermal vaporiser), a CW 20 M capillary column in one oven and a life T-switching device to cut onto a chiral β -cyclodextrin (Cyclosil-B., J & W Scientific) column (temperature program: CW 20 M column: $50\text{ }^{\circ}\text{C}-3\text{ min}$, $4\text{ }^{\circ}\text{C min}^{-1}$, $220\text{ }^{\circ}\text{C}-10\text{ min}$; chiral column: $50\text{ }^{\circ}\text{C}-3\text{ min}$, $4\text{ }^{\circ}\text{C min}^{-1}$, $100\text{ }^{\circ}\text{C}-20\text{ min}$, $1\text{ }^{\circ}\text{C min}^{-1}$, $220\text{ }^{\circ}\text{C}$). Identification of enantiomers was performed by authentic standards.

Results

Regioselectivity of limonene transformation

50 Basidiomycete and ascomycete species were cultivated in the presence of (*R*)-(+)-limonene. Hydroxylation, epoxidation, hydration and dehydrogenation reactions were observed. Hydroxylation reactions in positions allylic to the 1,2- or 8,9-double bond of limonene were preferred. Regioselectivity (rs) was calculated as the percentage of oxygen atoms introduced at carbon atoms of the substrate molecule (Fig. 1). The terminal hydroxylation at the C7-position of limonene to perillyl alcohol by *Gongronella butleri* with approximately 60% rs (96 h transformation time) was unusual, particularly because the primary alcohol was not oxidised further to perillaldehyde. *Penicillium citrinum* transformed (*R*)-limonene to α -terpineol with 83% rs. Kinetic investigation showed a maximum product accumulation of 80 mg l^{-1} after 9 d

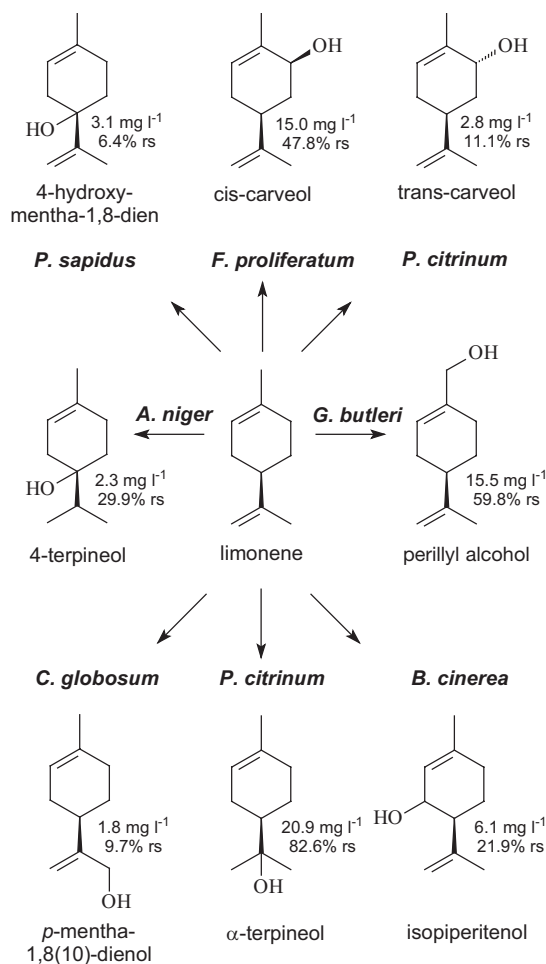


Fig. 1. Regioselectivity of the (*R*)-(+)-limonene hydroxylation in submerged cultures of selected strains [3 mM, transformation time 96 h, orbital shaker at 150 rpm, $24\text{ }^{\circ}\text{C}$, product concentration in mg l^{-1} , regioselectivity (rs) calculated as the percentage portion of hydroxylation products].

of transformation (conversion yield 17.4%). Very similar results were obtained with a strain of *P. digitatum*. A significant chemical hydration of limonene started at pH values below three only.

Bioreactor experiments

As this product concentration was the highest of all shake flask experiments, *P. digitatum* was inoculated into a lab-scale bioreactor to elucidate up-scalability. The fungus required approximately 70 h to form adequate biomass and to completely consume the glucose. By maintaining a minimum

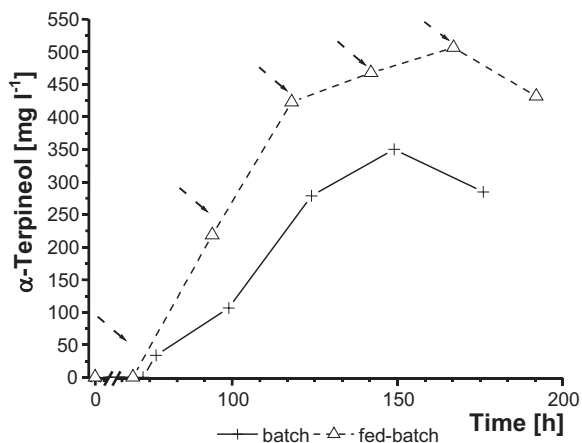


Fig. 2. Comparison of α -terpineol formation by *P. digitatum* in batch and fed-batch mode. In both fermentations the biotransformation was started after total completion of the glucose ($t = 73$ h batch, $t = 70$ h fed-batch) by addition of 0.84 ml limonene, which was repeated every 24 h. In the fed-batch mode glucose was additionally supplemented to increase the actual concentration from 0 to 2 g l⁻¹ every 24 h indicated by arrows.

glucose concentration during the biotransformation period (fed-batch), a maximum product concentration of 506 mg l⁻¹ α -terpineol was achieved after 127 h (Fig. 2). In the batch mode, where no further glucose was added after its completion, 350 mg l⁻¹ α -terpineol were formed in 76 h. The maximum molar conversion of 67% (*i.e.* related to the total amount of limonene added) already occurred after a biotransformation time of 24 h in the fed-batch fermentation and remained nearly constant for further 24 h, compared with 31% maximum molar conversion after 51 h in the batch mode (Fig. 3). The final cell dry weight in the fed-batch was 4.5 g l⁻¹, and 4.2 g l⁻¹ in the batch fermentation. The precursor limonene was not detected in the samples from either bioreactor during fermentation.

Enantiopure carveols

For the generation of enantiopure carveols, limonene has to be regio- and stereospecifically oxidised to carveol. The basidiomycetes and ascomycetes selected showed a different ratio of formation of carveol isomers (Table I). In contrast to ascomycetes, basidiomycetes such as *Pleurotus* species generated all four possible carveol isomers in almost equal quantities. As an exception, *Amarilliella mellea* formed predominantly *trans*-carveol.

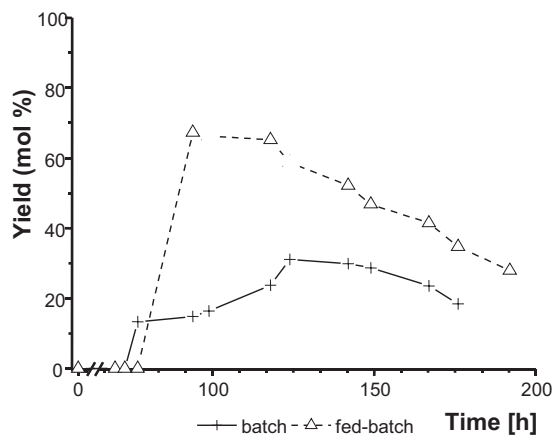


Fig. 3. Comparison of product yield of batch and fed-batch cultivation. The yield refers to the total amount of limonene added.

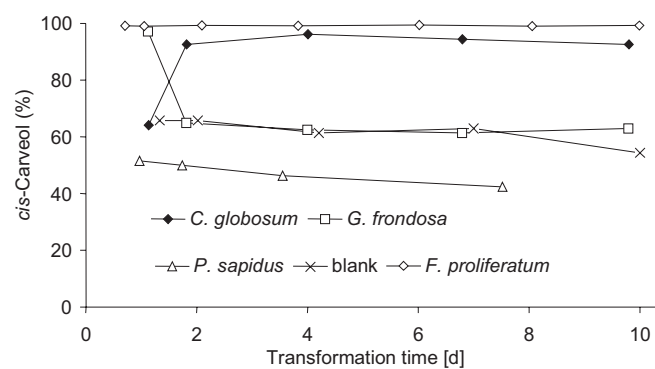
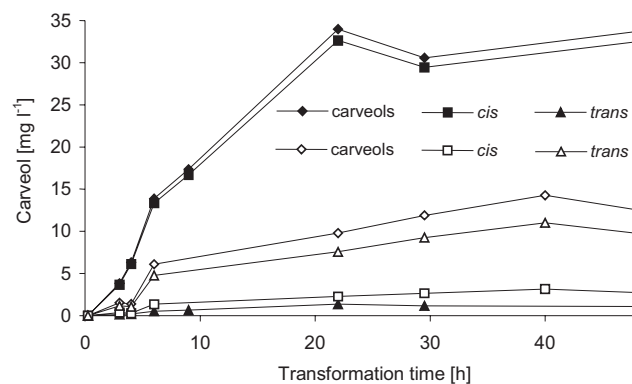
Following the carveol formation over a period of 7 d it was found that *P. sapidus* transformed limonene in a constant ratio (1:1) of *cis/trans*-isomers (Fig. 4). In contrast, ascomycetes such as *Chaetomium* species, generated predominantly *cis*-carveol (Table I, Fig. 4). The catalysis of *Chaetomium globosum* was not very selective at the beginning, but turned into a stereoselective reaction to yield *cis*-carveol (more than 95% diastereomeric excess, *de*) after 2 d. The most selective (*R*)-(+)-limonene transformation was observed with *Fusarium proliferatum* yielding the *cis*-carveol isomer with 98.4% *de* and an enantiospecificity of 99.8% *ee* (enantiomeric excess) for the *cis*-(+)-carveol from the very beginning. In comparison, the basidiomycete *Grifola frondosa* started with the generation of more than 95% *de* of *cis*-carveol, but lost its selectivity after 2 d of cultivation approaching a *cis/trans*-carveol distribution similar to the chemical blank sample (65:35 w/w). The concentrations of carveols in transformation experiments were much higher than those of limonene autooxidation which gave less than 0.5 mg l⁻¹ carveol under conditions (Table I).

The highest concentration of 35 mg l⁻¹ enantiopure *cis*-(+)-carveol accumulated in (*R*)-(+)-limonene supplemented cultures of *Fusarium proliferatum* after 22 h (6.6% molar yield, Fig. 5). The transformation of the (*S*)-(-)-enantiomer yielded a maximum of 12 mg l⁻¹ and a *trans/cis*-ratio of 76.4 to 22.4%. The *cis*-(+)- and *trans*-(-)-diastereomers were of high enantiopurity (*trans* 90.5% *ee* and *cis* 96.3% *ee*, Table I). Preliminary attempts

Table I. Carveol concentration, distribution and enantiomeric excess in submerged cultivation of selected basidio- and ascomycetes after 96 h of transformation (3 mM limonene, orbital shaker at 150 rounds per min, 24 °C).

| Substrate | Carveol [mg l ⁻¹] | % of isomers | | % | |
|------------------------------|----------------------------------|--------------|------------|-------------------------------------|---|
| | | <i>trans</i> | <i>cis</i> | <i>cis</i> -(+)/ <i>cis</i> -(-) | <i>trans</i> -(+)/ <i>trans</i> -(-) |
| (R)-(+)-Limonene | | | | | |
| Basidiomycetes | | | | | |
| <i>Amariella mellea</i> | 4.3 | 76.5 | 23.5 | – | – |
| <i>Grifola frondosa</i> | 3.8 | 47.4 | 52.6 | – | – |
| <i>Pleurotus euosmus</i> | 5.3 | 46.7 | 53.3 | – | – |
| <i>Pleurotus ostreatus</i> | 9.7 | 43.2 | 56.8 | – | – |
| <i>Pleurotus sajor-caju</i> | 5.3 | 51.5 | 48.5 | – | – |
| <i>Pleurotus sapidus</i> | 9.8 | 47.2 | 52.8 | 41.9/58.1 | 48.3/51.7 |
| Ascomycetes | | | | | |
| <i>Chaetomium cochloides</i> | 6.3 | 4.3 | 95.7 | 99.4 | – |
| <i>Chaetomium globosum</i> | 7.3 | 3.3 | 96.7 | 99.5 | – |
| <i>Fusarium proliferatum</i> | 30.6 | 0.8 | 99.2 | 99.8 | – |
| (S)-(-)-Limonene | | | | | |
| <i>Fusarium proliferatum</i> | 13.8 | 76.9 | 22.4 | 90.5 | 96.3 |

–, Not determined.

Fig. 4. Stereoselectivity of the biotransformation of (*R*)-(+)-limonene by different basidiomycetes, *cis*-carveol portion of the generated carveol isomers (limonene dosage 3 mM in 100 ml medium, 24 °C, orbital shaker at 150 rpm).Fig. 5. Time course of carveol generation from (*R*)-(+)- (solid symbols) and (*S*)-(-)-limonene (open symbols) catalysed by *Fusarium proliferatum* (substrate dosage 3 mM).

to isolate active enzymes from one of the species failed; neither a cooled ball mill nor grinding of the frozen pellets in various buffers allowed a successful solubilisation.

For the subsequent oxidation of enantiopure carveol to the corresponding carveone submerged pellets of *Pleurotus sapidus* were used. Kinetic investigations showed that enantiopure (–)-carveols were transformed into enantiopure carveone (> 99% ee). Carvone and carveol reduction products, such as dihydrocarvone and neo-dihydrocarveol were also formed (data not shown). *Trans*-Carveol was preferentially converted up to 94% yield within 4 d, whereas only 55% *cis*-carveol was oxidised in the same time.

Discussion

(*R*)-(+)-Limonene forms the major volatile constituent in *Citrus* essential oil (up to 97%), while (*S*)-(–)-limonene predominates in conifer resins (up to 90%). The limonenes possess little odour activity and are prone to off-flavour formation and polymerisation after exposure to oxygen. Therefore, they are separated from their natural sources and form a waste stream of 50,000 to 100,000 tons per year (Ziegler, 1998). This turns the compound into one of the most favourable starting materials for chemical and biochemical conversion processes. A recent review described five major microbial transformation pathways (van der Werf *et al.*, 1999b). The fungal species of the present screening showed little formation of epoxide and 1,2-diol, as was shown for a *Rhodococcus* (van der Werf *et al.*, 1999a), but operated the perilla, the carveone, the isopiperitone and the α -terpineol pathways. In contrast to prokaryotic transformer strains, a broad range of mentha-skeleton based alcohols was found indicating a larger enzymatic diversity (Fig. 1).

While some fungi accumulated a considerable number of oxyfunctionalised products, others were distinguished by a pronounced regioselectivity. The regioselective generation of perillyl alcohol by *G. butleri*, for example, was superior to the bacterial transformation by a *Myobacterium* sp. (Duetz *et al.*, 2001b) in showing no subsequent oxidation to perillaldehyde. The best regioselectivity, however, was observed for the hydration of the 8,9-double bond of limonene to α -terpineol catalysed by *Penicillium* species (Fig. 1). Few microbial hydratases have been isolated; the one from *Pseu-*

domonas was described as a transmembrane protein (Cadwallader *et al.*, 1992). Furthermore, cytochrome P450-monooxygenases, as a part of a multienzyme complex, were postulated for the formation of α -terpineol (Tan and Day, 1998).

A scale-up from the shake flask to the bioreactor is supposed to increase process efficiency, because the chemical and physical environment is much better controllable. While scale-up experiments quite often do not meet expectations, α -terpineol formation by *Penicillium digitatum* was increased immediately (Fig. 2). One reason for the approximately 45% higher product concentration in the fed-batch fermentation may be the ample supply of the required reduction equivalents for the enzymes formed by intracellular cofactor regeneration as no significant increase in biomass was observed in the fed-batch mode. Problems are still the loss of the precursor *via* the exhaust air and possibly its total catabolic degradation, as indicated by the failure to recover limonene from the nutrient medium during the process. It is assumed that the true product yield, calculated in relation to the unknown, actually available amount of precursor was much higher than the maximum 67% of the theoretical yield (Fig. 3). With respect to productivity, the optimum biotransformation time of the fed-batch process was 48 h resulting in a product formation rate of 8.8 mg l⁻¹ h⁻¹. This exceeded batch fermentation which resulted in a product formation rate of approximately 5.94 mg l⁻¹ h⁻¹ over the same period of time. In addition to the good yields, one great advantage of a biotransformation with this organism is its high selectivity. No unwanted by-products were detected during the whole fermentation period which significantly facilitates downstream processing.

Most of the aforementioned mono-oxyfunctionalisation reactions are believed to be catalysed by cytochrome P450-monooxygenases, enzymes that occur in eukaryotic cells as membrane-bound proteins requiring a separate NAD(P)H-dependent reductase for the electron transfer (van den Brink *et al.*, 1998). Fungal mechanisms of the stereospecific hydroxylation of limonene are not well understood. For the generation of racemic carveol by basidiomycetes (Table I, Fig. 4) a two-step mechanism is proposed *via* an intermediary allylic carbocation or radical. The transient cation or radical can rearrange along the double bond which inevitably results in the loss of the stereochemical infor-

mation at C4. Non-specific introduction of the oxygen atom then leads to the formation of all four possible stereoisomers of carveol. Upon carveol oxidation an enantiomeric distribution of 67% (*R*)-(-) to 33% (*S*)-(+)-carvone was observed, regardless of the limonene enantiomer fed (Onken and Berger, 1999).

The stereoselective carveol formation by ascomycetes, such as *F. proliferatum*, gives evidence for an enzymatic mechanism different from the one working in basidiomycetes (Fig. 5). Using (*R*)-(+)-limonene deuterated at C6 and C3 as the substrate and cells of *Mentha* species the abstraction of the C6-deuterium was proved during a hydroxylation by a selective cytochrome P450-monooxygenase (CYP71D18) (Wüst and Croteau, 2002). Kinetic studies showed that the binding energy of the substrate to the heme and a hydrophobic binding motive were responsible for a rigid substrate orientation and, therefore, caused a regio- and stereospecific hydroxylation. During the *cis*-(+)-carveol genesis of *F. proliferatum*, formation of the regioisomer isopiperitenol was observed, a by-product also occurring in *Mentha*.

In contrast to *Pleurotus sapidus*, *Fusarium proliferatum* did not form measurable amounts of carvone, but converted both limonene enantiomers: (*R*)-(+)-limonene to *cis*-(+)-carveol, and (*S*)-(-)-limonene predominantly to *trans*-(-)-carveol. Further oxidation of the enantiopure terpene alcohols yields (*R*)-(-)-carvone [from *trans*-(-)-carveol], and (*S*)-(+)-carvone (Fig. 6). However, transformation of (*R*)-(+)-limonene using a *Rhodococcus opacus* strain resulted in the formation of *trans*-(+)-carveol (Duetz *et al.*, 2001a). Obviously, two hydroxylating enzymatic activities with opposite stereospecificity occur in these microorganisms. During characterisation of limonene degrading enzymes from *R. erythropolis*, both (+)- and (-)-limonene degrading activities were found in the same cell fractions after chromatography. It was suggested that the enzymes involved converted both limonene enantiomers stereospecifically (van der Werf *et al.*, 1999b). In mint plants two separate monooxygenases, with nearly identical amino acid sequence, are responsible for the regioselective hydroxylation of limonene in C3- or C6-position, respectively (Lupien *et al.*, 1999).

In order to oxidise enantiopure carveol to the corresponding carvone, *P. sapidus* was identified

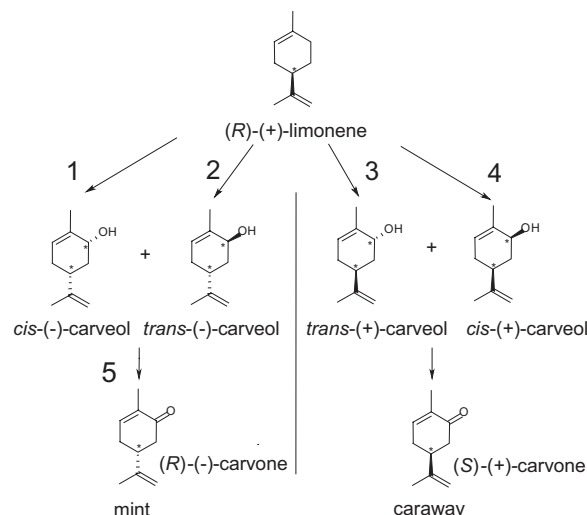


Fig. 6. Generation of enantiopure carvones by enantioselective limonene hydroxylation and subsequent dehydrogenation (all pathways from 1 to 5 by *Pleurotus sapidus*, pathway 3 *Rhodococcus opacus*, pathways 2 and 4 *Fusarium proliferatum*).

as a suitable catalyst for the second step of an integrated bioprocess. The substrate specificity of the carveol dehydrogenase activity [*trans*-(-)-carveol as the predominantly accepted isomer] was similar to the activity observed in *Rhodococcus erythropolis* DCL14 (van der Werf *et al.*, 1999a). This substrate selective oxidation was also used in a bioprocess to obtain enantiopure *cis*-(-)-carveol by racemic separation (Tecelao *et al.*, 2001).

In summary, regio- and stereoselective transformation of the (*R/S*)-limonenes opens access to flavour active terpenoids, such as α -terpineol, perillyl alcohol and both carvone enantiomers with high ee-values. The results indicate that basidiomycetes are the more versatile catalysts, but ascomycetes are more suitable to introduce oxygen atoms stereospecifically than basidiomycetes.

Acknowledgement

We are grateful for the support by the Federal Minister of Education and Research (BMBF 0330062) and by Deutsche Bundesstiftung Umwelt, DBU (AZ 13053/17). The project is part of the joint initiative project "Biologisch aktive Naturstoffe – Chemische Diversität" at the University of Hannover.

- Acosta M., Mazas N., Mejias E., and Pino J. (1996), Obtención de aromatizantes mediante biotransformación del limoneno. *Alimentaria* **272**, 73–75.
- Cadwallader K. R., Braddock R. J., and Parish M. E. (1992), Isolation of α -terpineol dehydratase from *Pseudomonas gladioli*. *J. Food Sci.* **57**, 241–244.
- Chang H. C. and Oriol P. J. (1994), Bioproduction of perillyl alcohol and related monoterpenes by isolates of *Bacillus stearothermophilus*. *J. Food Sci.* **59**, 660–662.
- Duetz W. A., Fjallman A. H., Ren S., Jourdat C., and Witholt B. (2001a), Biotransformation of D-limonene to (+)-*trans*-carveol by toluene grown *Rhodococcus opacus* PWD4 cells. *Appl. Environ. Microbiol.* **67**, 2829–2832.
- Duetz W. A., Jourdat C., and Witholt B. (2001b), Process for the preparation of perillyl alcohol. European Patent Application, EP 1236802.
- Duetz W. A., Bouwmeester H., van Beilen J. B., and Witholt B. (2003), Biotransformation of limonene by bacteria, fungi, yeast, and plants. *Appl. Microbiol. Biotechnol.* **61**, 269–277.
- Grassmann J., Hippeli S., and Elstner E. F. (2002), Plant defense and its benefits for animals and medicine: Role of phenolics and terpenoids in avoiding oxygen stress. *Plant Physiol. Biochem.* **40**, 471–478.
- Lupien S., Karp F., Wildung M., and Croteau R. (1999), Regiospecific cytochrome P450 limonene hydroxylases from mint (*mentha*) species: cDNA isolation, characterisation, and functional expression of (-)-4*S*-limonene-3-hydroxylase and (-)-4*S*-limonene-6-hydroxylase. *Arch. Biochem. Biophys.* **368**, 181–192.
- Ohloff G. (1994), *Scent and Fragrances*. Springer-Verlag, Berlin.
- Onken J. and Berger R. G. (1999), Effects of *R*-(+)-limonene on submerged cultures of the terpene transforming basidiomycete *Pleurotus sapidus*. *J. Biotechnol.* **69**, 163–168.
- Schrader J. and Berger R. G. (2001), Biotechnological production of terpenoid flavor and fragrance compounds. In: *Biotechnology, Vol. 10: Special Processes* (Rehm H. J., ed.). Wiley-VCH, Weinheim, Chapter 13, pp. 374–422.
- Sime J. T., Cheetham S. P. J., Gradley M. L., and Banister N. E. (2000), Use of sesquiterpenes for inhibiting oxidative enzymes. US Patent Application, US 6,054,490.
- Tan Q. and Day D. F. (1998), Organic co-solvent effects on the bioconversion of (*R*)-(+)-limonene to (*R*)-(+)- α -terpineol. *Process Biochem.* **33**, 29–37.
- Tecelao C. S. R., van Keulen F., and da Fonseca M. M. R. (2001), Development of a reaction system for the selective conversion of *trans*-(-)-carveol to (-)-carvone with whole cells of *Rhodococcus erythropolis* DCL14. *J. Mol. Catal. B: Enzym.* **11**, 719–724.
- van den Brink H. M., van Gorcom R. F. M., van der Hondel C. A. M. J. J., and Punt P. J. (1998), Cytochrome P450 enzyme systems in fungi. *Fungal Genet. Biol.* **23**, 1–17.
- van der Werf M. J. and Boot A. M. (2000), Metabolism of carveol and dihydrocarveol in *Rhodococcus erythropolis* DCL14. *Microbiology* **146**, 1129–1141.
- van der Werf M. J., van der Ven C., Barbirato F., Eppink M. H. M., de Bont J. A. M., and van Berkel W. J. H. (1999a), Stereoselective carveol dehydrogenase from *Rhodococcus erythropolis* DCL14. A novel nicotino-protein belonging to the short chain dehydrogenase/reductase superfamily. *J. Biol. Chem.* **274**, 26296–26304.
- van der Werf M. J., Swarts H. J., and de Bont J. A. M. (1999b), *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. *Appl. Environ. Microbiol.* **65**, 2092–2102.
- van Dyk M. S., van Rendsburg E., Rensburg I. P. B., and Moleleki N. (1998), Biotransformation of monoterpene ketones by yeasts and yeast-like fungi. *J. Mol. Catal. B: Enzym.* **5**, 149–154.
- Wüst M. and Croteau R. B. (2002) Hydroxylation of specifically deuterated limonene enantiomers by cytochrome P450 limonene-6-hydroxylase reveals the mechanism of multiple product formation. *Biochemistry* **41**, 1820–1827.
- Ziegler E. (ed.) (1998), *Flavourings*. Wiley-VCH, Weinheim.