Identification of Fungal Limonene-3-Hydroxylase for Biotechnological Menthol Production

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ABSTRACT More than 30,000 tons of menthol are produced every year as a flavor and fragrance compound or as a medical component. So far, only extraction from plant material and chemical synthesis are possible. An alternative approach for menthol production could be a biotechnological-chemical process with ideally only two conversion steps, starting from (+)-limonene, which is a side product of the citrus processing industry. The first step requires a limonene-3-hydroxylase (L3H) activity that specifically catalyzes hydroxylation of limonene at carbon atom 3. Several protein engineering strategies have already attempted to create limonene-3-hydroxylases from bacterial cytochrome P450 monooxygenases (CYPs, or P450s), which can be efficiently expressed in bacterial hosts. However, their regiospecificity is rather low compared to that of the highly selective L3H enzymes from the biosynthetic pathway for menthol in *Mentha* species. The only naturally occurring limonene-3-hydroxylase activity identified in microorganisms so far was reported for a strain of the black yeast-like fungus *Hormonema* sp. in South Africa. We have discovered additional fungi that can catalyze the intended reaction and identified potential CYP-encoding genes within the genome sequence of one of the strains. Using heterologous gene expression and biotransformation experiments in yeasts, we were able to identify limonene-3-hydroxylases from *Aureobasidium pullulans* and *Hormonema carpetanum*. Further characterization of the *A. pullulans* enzyme demonstrated its high stereospecificity and regioselectivity, its potential for limonene-based menthol production, and its additional ability to convert α - and β -pinene to verbenol and pinocarveol, respectively.

IMPORTANCE (–)-Menthol is an important flavor and fragrance compound and furthermore has medicinal uses. To realize a two-step synthesis starting from renewable (+)-limonene, a regioselective limonene-3-hydroxylase enzyme is necessary. We identified enzymes from two different fungi which catalyze this hydroxylation reaction and represent an important module for the development of a biotechnological process for (–)-menthol production from renewable (+)-limonene.

KEYWORDS menthol, fungal enzyme, terpenes, limonene, oxidation, hydroxylation, CYP, P450, biotechnology, fungal enzymes

The monocyclic monoterpene alcohol (–)-menthol has an annual production volume of more than 30,000 tons (1). It is used as a flavor and fragrance compound, as a cosmetics additive, and as an ingredient of pharmaceutical products, e.g., for the treatment of colds or burns (2). Menthol can be either extracted from the essential oil of *Mentha* plants or produced by various chemical processes. The technical synthesis of (–)-menthol is currently carried out using different processes at Symrise/Lanxess (from *m*-cresol), Takasago (from myrcene), and BASF (from citral) (2, 3). However, no

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FIG 1 Biotechnological-chemical processes for (-)-menthol production (top; green and red) versus the native pathway in peppermint plants (*Mentha × piperita*) (bottom; brown) (adapted from reference 9). L5H, limonene-5-hydroxylase; L3H, limonene-3-hydroxylase; IPD, (-)-*trans*-isopiperitenol dehydrogenase; IPR, (-)-isopiperitenone reductase; IPI, (+)-*cis*-isopulegone isomerase; PR, (+)-pulegone reductase; MR, (-)-menthone reductase.

biotechnological process for (-)-menthol production has been established so far. Since the product yields of *Mentha* plantations are strongly dependent on changing climatic factors and chemical synthesis is partly based on limited fossil resources, biotechnological production processes based on renewable raw materials are an attractive alternative. A sustainable menthol synthesis route could be a biotechnological-chemical two-step conversion (4), starting from the renewable raw material limonene. The conversion of limonene to the intermediate requires specific hydroxylase enzymes. In the case of (-)-limonene, a limonene-5-hydroxylase (L5H) would be necessary to form *trans-p*-1,8-menthadien-5-ol, whereas (+)-limonene has to be hydroxylated at carbon atom 3 by a limonene-3-hydroxylase (L3H) to produce *trans*-isopiperitenol (Fig. 1). *trans*-Isopiperitenol, and presumably also *trans-p*-1,8-menthadien-5-ol, can then be further transformed into (-)-menthol by chemical hydrogenation (4, 5). Many protein engineering strategies have already attempted to create limonene-3-hydroxylases with high regioselectivity and stereospecificity from bacterial cytochrome P450 monooxygenase (CYPs, or P450s) (6–8). However, while these enzymes can be efficiently expressed in bacterial hosts,

their product selectivity is limited [75% *trans*-isopiperitenol from (+)-limonene at best, with formation of several side products] (7).

In the peppermint plant, *Mentha × piperita*, (–)-menthol is produced from (–)-limonene via a six-step metabolic pathway (9) (Fig. 1). The first enzyme of this pathway is a selective limonene-3-hydroxylase which is present in two slightly different forms encoded by CYP71D13 (PM17) and CYP71D15 (PM2) (10). As with other enzymes of the menthol pathway (2), several studies have been conducted to express PM17 heterologously in microbial hosts (11, 12), but they resulted in rather low productivities.

Besides their occurrence in *Mentha* plants (2, 10, 13), the only other limonene hydroxylase activity with clear selectivity for carbon atom 3 was described for a strain of the black yeast-like fungus *Hormonema* sp. (strain UOFS Y-0067) (4). The genus *Hormonema* belongs to the class Dothideomycetes, which in turn forms a subgroup of Ascomycota. Many Dothideomycetes live as endophytes or epiphytes in or on plants or degrade dead plant material as saprobionts (14). The cells of *Hormonema* sp. UOFS Y-0067 were also found to hydroxylate α - and β -pinene. Earlier, van Dyk and colleagues (4) had suggested the potential use of the fungus for (–)-menthol production from (+)-limonene, but the results were not reproducible and the concentrations of the product obtained varied greatly. In addition, the responsible enzyme remained unknown. Therefore, they considered it unlikely “that it will ever be possible to base a commercial biotransformation process on an organism like this *Hormonema* sp.” (4).

In order to identify stereo- and regioselective limonene-3-hydroxylases in *Hormonema* or other fungi, we tested different related fungi for the respective conversion. Candidate CYP enzymes were identified in the fungal genomes, and by gene comparison and heterologous expression of 20 different CYP sequences in yeasts, we identified a limonene-3-hydroxylase from *Aureobasidium pullulans* and also from *Hormonema carpetanum*. Subsequently, the enzyme from *A. pullulans* was further characterized in detail regarding substrate and product selectivity. In addition, a gene encoding a CYP reductase was identified in the genome of *Aureobasidium pullulans*. Such reductases are required as electron donors for the function of many CYPs.

RESULTS

Hydroxylation capability of different Dothideomycetes. In order to investigate if the limonene-3-hydroxylase activity of *Hormonema* sp. UOFS Y-0067 (4) is a common ability among members of the class Dothideomycetes, several fungi, including *Hormonema carpetanum*, *Aureobasidium pullulans*, *Neofusicoccum parvum*, *Ochroconis constricta*, and *Parastagonospora nodorum*, were tested for (+)-limonene hydroxylation. *Hormonema carpetanum* and *Aureobasidium pullulans* were found to convert limonene into *trans*-isopiperitenol (Fig. 2A). Further analyses confirmed that *A. pullulans* produces *trans*-isopiperitenol as the main product in a limonene biotransformation reaction (Fig. 2B to D), and this strain was used to search for the respective hydroxylase enzyme.

Identification of fungal limonene-3-hydroxylase enzymes. Since preliminary experiments with specific enzyme inhibitors had shown that the responsible protein is most probably a member of the cytochrome P450 monooxygenase family (data not shown), we focused on such enzymes. According to the NCBI database, the genome sequence of *A. pullulans* CBS 100280 (EXF-150) (15) contains 51 genes encoding putative CYP proteins. These annotated CYPs from *A. pullulans* were assigned to different families based on their protein sequences and named accordingly by David Nelson (16) (Fig. 3). The individual proteins were clustered by means of multiple sequence alignment (Fig. 3).

Sixteen of the 51 putative *A. pullulans* CYPs were chosen for expression in *Saccharomyces cerevisiae*, so that candidates from the different subclusters of the phylogenetic tree were included (Fig. 3, blue dots).

In addition to the potential CYP-encoding sequences, a gene encoding a putative CYP reductase (CPR) enzyme was identified in the genome of *A. pullulans* (CYP reductase.Ap), by comparing protein sequences of known CYP reductases from other fungi with the genome of *A. pullulans*.

FIG 2 *trans*-Isopiperitenol production with different Dothideomycetes. (A) *trans*-Isopiperitenol production over time of different Dothideomycetes. Culture samples of different fungi were taken over a period of 5 h (once per hour) after addition of 1 g liter⁻¹ (+)-limonene. Samples were extracted with ethyl acetate, and the *trans*-isopiperitenol amount was determined by GC-MS analysis. The negative control was medium without cells. The data points and error bars represent the mean values and standard deviations for three biological replicates. (B) GC-MS analysis of biotransformation of (+)-limonene by *A. pullulans*. The chromatogram of ethyl acetate extract from (a) *A. pullulans* culture after addition of 1 g liter⁻¹ limonene (0 h) is compared to chromatograms of (b) *A. pullulans* culture 24 h after addition of limonene and (c) isopiperitenol reference substance [2:1 mixture of (+)-*trans*-*cis*-isopiperitenol]. *trans*-Isopiperitenol (peak 2; RT, 14 min) was identified as the main product of (+)-limonene (peak 1) biotransformation by comparison of retention time and (C) mass spectrum to those of (D) the isopiperitenol reference substance.

In an initial investigation, *S. cerevisiae* strains harboring the different CYP genes together with the putative CPR were tested in a (+)-limonene biotransformation experiment. One strain led to a clearly increased *trans*-isopiperitenol concentration compared to the background product level of the strain containing the empty vectors (Fig. 4; also, see Fig. S1 in the supplemental material). Therefore, we proposed that the

FIG 3 Clustering of cytochrome P450 monooxygenases of *A. pullulans* CBS 100280 (EXF-150) and of *H. carpelanum* CBS 115712 (only those with the highest identity to L3H.Ap were selected). The proteins annotated as cytochrome P450 monooxygenases were analyzed for their amino acid sequence identity and subdivided into protein families. CYP candidate genes of *A. pullulans* chosen for expression in *S. cerevisiae* are marked with blue dots. Limonene-3-hydroxylase enzymes from *A. pullulans* (L3H.Ap = CYP65FA1) and *H. carpelanum* (L3H.Hc = CYP65EZ1) as well as other tested CYP65 candidates from *A. pullulans* are marked in red, green, and yellow, respectively. For accession numbers, see Table 4. Clustering was performed with the software Geneious using default settings (ClustalW alignment; Geneious tree builder; resampling method, bootstrap; number of replicates, 1,000). Branch labels show percent consensus support.

corresponding protein of *A. pullulans*, CYP65FA1, is a limonene-3-hydroxylase, referred to here as L3H.Ap. In addition, it was found that *trans*-isopiperitenol was produced by *S. cerevisiae* expressing L3H.Ap in the absence of the putative CYP reductase, indicating that L3H.Ap can perform hydroxylations if the electrons are provided by the baker's yeast CPR.

In order to identify further limonene-3-hydroxylase enzymes in the genomes of *A. pullulans* and *H. carpelanum*, the second fungus for which L3H activity was detected in the previous experiments, additional putative CYP genes from both fungi were selected based on amino acid sequence identity to L3H.Ap. Therefore, CYP65FA2, CYP65EX2, and CYP65EW2 from *A. pullulans* and CYP65EZ1 from *H. carpelanum* were chosen for further tests (Fig. 3, yellow and green fonts). The CYP65EZ1 gene sequence from *H. carpelanum* (CYP65EZ1.Hc) was identified by analysis of partial genome data for *H. carpelanum* CBS 115712. The levels of amino acid sequence identity between L3H.Ap and CYP65FA2.Ap, CYP65EX2.Ap, CYP65EW2.Ap, and CYP65EZ1.Hc are about 53%, 33%, 41%, and 52%, respectively. Whereas no limonene conversion could be observed in the biotransformation experiments with CYP65FA2.Ap, CYP65EX2.Ap, and CYP65EW2.Ap

FIG 4 *trans*-Isopiperitenol concentrations achieved in a (+)-limonene biotransformation experiment with different *S. cerevisiae* CEN-PK2-1C strains. Concentrated cell suspensions were used. Empty vectors were yeast strains harboring empty pPK245 (and pPK448) vectors; CYP reductase.Ap refers to cells expressing a putative CYP reductase gene from *A. pullulans* CBS 100280 (EXF-150) from the pPK448 vector and containing empty pPK245. CYP532A30.Ap to CYP65EZ1.Hc indicate cells expressing different putative CYPs from *A. pullulans* CBS 100280 (EXF-150, Ap) or *H. carpetanum* CBS 115712 (Hc) from the pPK245 vector together with putative CYP reductase from *A. pullulans* from pPK448 plasmid (left) or without reductase.Ap (right). *trans*-Isopiperitenol concentrations were calculated from peak areas, which were determined by GC-MS analysis, and normalized to the peak area of the internal standard (3-carene) and the number of cells corresponding to 150 OD units. The data points and error bars represent the mean values and standard deviations for three biological replicates ($n = 3$).

(data not shown), *trans*-isopiperitenol could be detected in biotransformation samples with the yeast strain expressing CYP65EZ1 from *H. carpetanum* (Fig. 4; Fig. S1), referred to here as L3H.Hc. However, the concentrations achieved were less than a third of the value for strains harboring the L3H.Ap gene. Therefore, the L3H enzyme of *A. pullulans* was chosen for further characterization.

Further characterization of limonene-3-hydroxylase activity of L3H.Ap. In order to obtain larger amounts of protein to further characterize L3H.Ap, we tried to express the protein and several variants in *Escherichia coli*. After all attempts to express the protein in a functional form with *E. coli* failed, we tested expression in the eukaryotic host *Pichia pastoris* (17). For this purpose, two different vector systems were applied. Whereas one of the vectors used is an episomal plasmid with constitutive expression (pBSYA1Z), the pPICZA vector is integrated into the genome of *P. pastoris*, and expression of the CYP is induced by methanol addition. As negative controls, the cells were transformed with the respective empty expression vector. Furthermore, PM17 from *Mentha × piperita*, codon optimized for *P. pastoris* (12), was used as a reference. Since it has been observed that the L3H.Ap enzyme is functional in *S. cerevisiae* with the native yeast CPR, the putative CYP reductase of *A. pullulans* was not coexpressed in *P. pastoris*.

The experiments showed that with both vector systems, larger amounts of *trans*-isopiperitenol were produced from (+)-limonene with L3H.Ap than with PM17 from *Mentha × piperita* (Fig. 5A; Fig. S2). The maximum *trans*-isopiperitenol concentration obtained was around 300 µM (45 mg liter⁻¹) in the concentrated yeast cell suspension with constitutive gene expression from the episomal vector.

Apart from *trans*-isopiperitenol, *p*-1,8-menthadien-4-ol could be detected in the limonene biotransformation reactions with L3H.Ap (Fig. 5B to D; Fig. S2). However, the peak area of produced *trans*-isopiperitenol is around 10 times larger than the *p*-1,8-menthadien-4-ol peak area. Small *p*-1,8-menthadien-4-ol peaks could also be identified in the limonene biotransformation experiments with *A. pullulans* cultures (Fig. 2; retention time [RT], 13.4 min) and *S. cerevisiae* cells harboring the fungal L3H enzyme (Fig.

FIG 5 Hydroxylation of limonene with *P. pastoris* X-33 strains expressing L3H enzymes. (A) *trans*-isopiperitenol peak areas achieved in (+)-limonene biotransformation experiments with different *P. pastoris* X-33 strains (concentrated cell suspensions). pBSYA1Z, episomal, constitutive gene expression; pPICZA, genome integrated, methanol-induced gene expression; empty, *P. pastoris* with empty expression vector; L3H.Ap, with CYP65FA1 of *A. pullulans*; PM17, with limonene-3-hydroxylase of *Mentha × piperita*. (B) Product peak areas achieved in biotransformation experiments with (+)- or (–)-limonene with *P. pastoris* X-33 strains (concentrated cell suspensions) harboring empty pBSYA1Z plasmid or expressing limonene-3-hydroxylase enzymes from *A. pullulans* (L3H.Ap) or *Mentha × piperita* (PM17) from pBSYA1Z backbone. While with (–)-limonene several different products were formed with all strains (see Fig. S3), only peak areas of the main (by-)products are shown for comparison. For chromatograms, see panel C; also see Fig. S2 and S3. For panels A and B, peak areas were determined by GC-MS analysis and normalized to the peak area of the internal standard (3-carene) and the number of cells corresponding to (A) 150 or (B) 225 OD units. The data points and error bars represent the means and standard deviations for six (A) or three (B) biological replicates. (C) GC-MS analysis of (+)-limonene biotransformation experiment shown in panel B. The chromatogram of the negative control with the empty pBSYA1Z expression plasmid (blue) is compared to those of strains harboring PM17 from *Mentha × piperita* (brown) and L3H.Ap (red) and an isopiperitenol reference substance [2:1 mixture of (+)-*trans*-/*cis*-isopiperitenol] (black). *trans*-isopiperitenol (peak 2; RT, 10.3 min) was identified as the main product (>90%) of (+)-limonene biotransformation. As a side product (<10%), *p*-1,8-menthadien-4-ol (peak 3; RT, 9.8 min) was formed by cells expressing L3H.Ap. (D and E) Substrates and main products of bioconversion reactions catalyzed by L3H.Ap. 1, (+)-limonene; 2, (+)-*trans*-isopiperitenol; 3, *p*-1,8-menthadien-4-ol; 4, (–)-limonene; 5, (–)-*trans*-isopiperitenol; 6, carveol (for the MS spectrum, see Fig. S4).

S1; RT, 9.8 min). Further oxidation of the formed *trans*-isopiperitenol or *p*-1,8-menthadien-4-ol was not observed (Fig. S2).

When the limonene enantiomers were compared, higher product peaks were obtained with (+)-limonene than with (–)-limonene in a biotransformation experiment with the

FIG 6 Product peak areas and *trans*-isopiperitenol concentrations achieved in (+)-limonene biotransformation experiments with *P. pastoris* X-33 strains expressing limonene-3-hydroxylase L3H.Ap under different conditions. Concentrated cell suspensions were used. Peak areas were determined by GC-MS analysis and normalized to the peak area of the internal standard (3-carene) and the number of cells corresponding to 225 OD units. *trans*-Isopiperitenol concentrations were calculated from normalized peak areas. The data points and error bars represent the mean values and standard deviations of three biological replicates. (A) Investigation of the influence of buffer pH and glucose feeding on product formation. Empty, *P. pastoris* strain with empty pBSYA1Z expression vector; L3H.Ap, strain expressing CYP65FA1 of *A. pullulans* from the pBSYA1Z backbone. For biotransformation, 100 mM potassium phosphate buffer at pH 6.0, 7.4, or 8.0 was used. "+Gluc" indicates that the cell suspension contained 2% glucose. When glucose was added, the pH of the biotransformation suspension decreased to less than 5 during the experiment. (B) Investigation of the influence of reaction vessel volume on product formation at pH 8.0. For the experiment, a *P. pastoris* strain expressing L3H.Ap from pBSYA1Z backbone was used. Biotransformation was carried out in reaction vessels with a total volume of 100 ml or 2 ml with cell suspension volumes of 7.5 ml or 750 μl, respectively. For bars labeled "100 ml vessel + DMSO" and "100 ml vessel + Lim," samples were taken at different time points (2, 5, 20, 28, and 51 h after DMSO or limonene addition). For bars labeled "100 ml vessel + Lim_closed" and "2 ml vial + Lim," reaction vessels remained closed during the entire experiment, and samples were taken only at 28 h.

L3H.Ap-expressing strain (Fig. 5B and E; Fig. S2 and S3). PM17 showed an inverse preference (Fig. 5B; Fig. S2 and S3).

Unexpectedly, when (–)-limonene was used as the substrate, several different products were formed (Fig. S3). However, this could be observed with all strains harboring the empty vector, L3H.Ap, or PM17 from *Mentha × piperita*, to a similar extent. Only the production of *trans*-isopiperitenol was clearly increased compared to the negative control when the fungal or plant L3H was present (Fig. 5B).

Reaction parameters were optimized to increase *trans*-isopiperitenol yields. Increasing the pH from 6.0 to 7.4 and 8.0 (Fig. 6A) increased the *trans*-isopiperitenol yields as well as its relative abundance over its side product; increasing the oxygen supply in the reaction vessel (from about 1.5 to 12 volumes air in relation to the reaction volume) (Fig. 6B) increased the *trans*-isopiperitenol yields. The addition of glucose

TABLE 1 Overview of tested substrates and detected products of hydroxylation reactions with *P. pastoris* X-33 expressing L3H of *A. pullulans* CBS 100280 (EXF-150)^a

Substrate	Products detected after biotransformation with L3H.Ap in <i>P. pastoris</i>
(+)-Limonene (1)	(+)- <i>trans</i> -Isopiperitenol (2), <i>p</i> -1,8-menthadien-4-ol (3)
(-)-Limonene (4)	(-)- <i>trans</i> -Isopiperitenol (5), <i>p</i> -1,8-menthadien-4-ol (3), carveol (6)
Isopiperitenol [(+)- <i>trans</i> / <i>cis</i> mixture 2:1]	None
α -Pinene (7)	Verbenol (8), verbenone (9), further unknown products
β -Pinene (10)	Pinocarveol (11), further unknown products
γ -Terpinene	None
α -Phellandrene	None
3-Carene (12)	<i>p</i> -1,5-Menthadien-8-ol (13), further unknown products
Toluene	None
1-Phenylethanol	None
2-Phenylethylalcohol	None
DMSO	None

^aFor easier identification, the substances have been numbered in the relevant figures (Fig. 2, 5, and 7 and Fig. S1 to S7). These numbers are given in parentheses after the substance names.

to the biotransformation buffer to improve cofactor regeneration was not beneficial. With these modified parameters, a maximum concentration of around 1.1 mM (ca. 165 mg per liter of concentrated yeast cell suspension) *trans*-isopiperitenol could be obtained after 51 h of biotransformation, with a constant *trans*-isopiperitenol increase over time.

L3H.Ap can hydroxylate α -pinene, β -pinene, and 3-carene. To further assess the substrate selectivity of L3H.Ap *in vivo*, other substrates were tested in biotransformation experiments (Table 1). While no products could be detected with γ -terpinene, α -phellandrene, toluene, 1-phenylethanol, and 2-phenylethylalcohol, we observed hydroxylation of 3-carene, α -pinene, and β -pinene (Table 1; Fig. 7; Fig. S4 to S7). The oxygenated monoterpene products were not, or only to a lesser extent, detected in experiments with the empty vector control.

DISCUSSION

In this work, we describe two fungal CYP enzymes which can oxidize (+)-limonene at carbon atom 3, resulting in *trans*-isopiperitenol. Based on the protein sequences, both enzymes, L3H.Ap and L3H.Hc, belong to the cytochrome P450 monooxygenase family of CYP65, which includes other fungal terpene hydroxylation enzymes described before (18).

In contrast to L3H.Ap and L3H.Hc, no limonene hydroxylation activity was detected for CYP65FA2.Ap, CYP65EX2.Ap, and CYP65EW2.Ap or for all other tested CYP candidate genes. This could mean either that these enzymes are unable to catalyze the desired reaction or that they are not functionally expressed in the yeast cells under the conditions used here. In addition, CYP enzymes are known to require suitable reductase enzymes for their functionality, because these reductases provide the electrons necessary for the CYP reaction (19). Due to this dependency, it is also possible that no CYP activity could be measured because the CYP reductase provided in the yeast strains was not suitable to enable the function of the tested CYP candidates. Indeed, it is well known that the redox partner can have a significant impact on overall CYP activity (20). Furthermore, it has been reported that the type of coexpressed CYP reductase can even influence the product spectrum (21).

Although the physiological function of the limonene-3-hydroxylases in *A. pullulans*, *H. carpetanum*, and other fungi is unclear, one can assume a role in modification/detoxification or utilization of monoterpenes produced by plants, which are colonized by such fungi (22). One would generally expect enzymes with high specific activity to carry out these functions *in vivo*.

The newly discovered limonene-3-hydroxylase from *A. pullulans* shows high stereospecificity and regioselectivity of the catalyzed reaction. Only small amounts (<10%) of the side product *p*-1,8-menthadien-4-ol were produced from (+)-limonene by *P. pastoris* cells

FIG 7 Hydroxylation of monoterpene substrates with *P. pastoris* X-33 expressing L3H.Ap. (A) Relative quantitative composition of products formed in biotransformation experiments of α -pinene, β -pinene, or 3-carene with *P. pastoris* X-33 cells (concentrated cell suspension) expressing L3H.Ap from the pBSYA1Z backbone. If possible, products were identified via comparison of retention times and mass spectra with those of chemically synthesized reference substances, or by comparison of mass spectra to those of the NIST mass spectral library (v14) (for MS spectra, see Fig. S4). For chromatograms, see Fig. S5 to S7. The data points and error bars represent the mean values and standard deviations for three biological replicates ($n=3$). (B) Substrates and main products of bioconversion reactions catalyzed by L3H.Ap.

expressing L3H.Ap. Although some P450cam variants with selectivity preference for production of *trans*-isopiperitenol from (–)-limonene were described (6), the selectivity was lower than that of the fungal enzyme when (+)-limonene was used as the substrate (7). So far, the only highly specific limonene-3-hydroxylase enzymes are known to occur in *Mentha* plants (2, 10, 13). In peppermint, for example, PM17 catalyzes the key hydroxylation during the (–)-menthol biosynthesis pathway. In the literature, PM17 is reported to convert (–)-limonene and (+)-limonene exclusively into the respective *trans*-isopiperitenols (23). In experiments with microsomal preparations from the epidermal oil glands (trichomes) of *Mentha × piperita*, the (–)-isomer was favored by a factor of 2 over the (+)-enantiomer (24). Our results with *P. pastoris* cells indicate that the fungal L3H.Ap enzyme shows a reversed preference, which would be beneficial for the intended process using (+)-limonene from the citrus industry as starting material.

The titers of *trans*-isopiperitenol produced with *P. pastoris* cells are still low, reaching only around 165 mg liter^{–1} of product in the concentrated cell suspension after around 51 h. Although a similar approach using *P. pastoris* and the PM17 hydroxylase from peppermint demonstrated production of about 35 mg liter^{–1} from (–)-limonene after additional strain modification (12), the same study reported isopiperitenol concentrations of about 400 mg liter^{–1} in whole-cell biotransformations with *S. cerevisiae* strains expressing PM17 and (–)-limonene as the substrate (values were estimated from the graphs and converted into product concentrations in the biotransformation solution according to the given information). In our biotransformation approaches with L3H.Ap-expressing *S. cerevisiae* strains and (+)-limonene, only 5.6 mg liter^{–1} *trans*-isopiperitenol could be detected. A direct comparison between the studies and the enzymes is not possible at this stage, as, e.g., strains, type of CYP reductase, cultivation conditions, cell densities, reaction vessels, and substrates were not identical. However, in our setup, the *P. pastoris* strain expressing the fungal CYP enzyme L3H.Ap produced larger amounts of (+)-*trans*-isopiperitenol than the corresponding PM17-expressing strain.

Since we did not investigate the protein concentrations in the cells, no conclusions about the differences of catalytic properties and substrate selectivity between the fungal enzymes and the peppermint limonene-3-hydroxylase are possible so far. In addition, as mentioned above, low CYP activity can also be caused by the absence of a suitable reductase enzyme. In order to be able to reliably compare the fungal enzymes with the plant protein, this interaction would also have to be optimized in further experiments, for example, by using a plant CPR in combination with PM17.

Apart from producing *trans*-isopiperitenol, it was found that L3H.Ap can also hydroxylate α - and β -pinene as well as 3-carene. The reactions delivered verbenol, verbenone, pinocarveol, and *p*-1,5-menthadien-8-ol as main products. In the case of α -pinene, the oxidation of verbenol to verbenone might be either caused by L3H activity or by unspecific dehydrogenase activity present in yeast. The production of verbenol and verbenone from α -pinene in the same organism has already been described for bacteria as well as fungi and plant cells (6, 7, 25–28).

The activity of L3H.Ap on substrates other than limonene is in accordance with the conversion of α - and β -pinene by *Hormonema* sp. (4). However, while van Dyk and colleagues (4) could also detect verbenol and verbenone as products of the α -pinene biotransformation experiment, β -pinene was converted to pinocamphone and 3-hydroxy-pinocamphone with *Hormonema* sp. (4). In addition to their experiments, oxidation of α - and β -pinene to several products, including verbenol, verbenone, and pinocarveol, has been also reported for other fungi (4, 28, 29). Moreover, Chiu and colleagues recently described a CYP enzyme from mountain pine beetle that can hydroxylate α - and β -pinene as well as 3-carene (30). However, for this enzyme of the CYP6 family, no activity for limonene isomers was detected. Furthermore, bacterial enzyme variants are known that can use carene, α -pinene, or β -pinene as a substrate; these are, however, converted into a variety of products (7).

In this work, we describe the identification of two fungal limonene-3-hydroxylases that can hydroxylate (+)-limonene with high stereospecificity and regioselectivity to *trans*-isopiperitenol. In addition, further characterization of the L3H enzyme of *A. pullulans* showed that it can also convert (–)-limonene, 3-carene, α -pinene, and β -pinene. By the use of yeasts expressing the CYP enzymes, novel catalysts for the conversion of (+)-limonene into (+)-*trans*-isopiperitenol could be provided that could become part of a (–)-menthol production process. Further investigations of their catalytic properties as well as optimization of cellular expression will be necessary to assess the full potential of the fungal CYP enzymes for biotechnological terpene conversions.

MATERIALS AND METHODS

Chemicals and media. All chemicals were purchased from Merck KGaA (Germany) (previously Sigma-Aldrich [Germany]), Merck Millipore (Germany), or Carl-Roth (Germany) with different purities: (+)-limonene ($\geq 99\%$), (–)-limonene ($\geq 99\%$), α -pinene ($\geq 98\%$), (–)- β -pinene ($\geq 99\%$), γ -terpinene ($\geq 98.5\%$), α -phellandrene ($\geq 95\%$), 3-carene ($\geq 98.5\%$), 1-phenylethanol ($\geq 98\%$), 2-phenylethylalcohol ($\geq 99\%$), (–)-carveol (97%), (–)-*trans*-pinocarveol ($\geq 96\%$), (5)-*cis*-verbenol ($\geq 95\%$), (1S)-(–)-verbenone ($\geq 99\%$).

In addition, a 2:1 mixture of (+)-*trans*- and *cis*-isopiperitenol was obtained from Symrise (Germany).

Fungi, except *S. cerevisiae* and *P. pastoris*, were grown in yeast extract/malt extract (YM) medium (3 g liter^{–1} yeast extract, 20 g liter^{–1} malt extract, 10 g liter^{–1} peptone, 10 g liter^{–1} glucose) (31).

S. cerevisiae strains were cultivated in yeast extract peptone (YEP) medium (10 g liter^{–1} yeast extract, 20 g liter^{–1} peptone) or synthetic complete (SC) medium (6.7 g liter^{–1} yeast nitrogen base without amino acids and with ammonium sulfate and amino acid supplements [32]). SC medium pH was adjusted to 6.3 with potassium hydroxide. If needed for selection of plasmids, medium without uracil and L-tryptophan was used. Glucose (20 g liter^{–1}) was added as the carbon source.

P. pastoris strains were cultured in yeast extract-peptone-dextrose medium (YPD), YPD with sorbitol (YPDS), buffered dextrose complex medium (BMDY), or buffered methanol complex medium (BMMY) medium according to the EasySelect *Pichia* expression kit manual (Invitrogen) and the work of Weis et al. (33).

E. coli was cultured in lysogeny broth (LB) medium (10 g liter^{–1} tryptone, 5 g liter^{–1} yeast extract and 5 g liter^{–1} NaCl) or Terrific Broth (TB) medium (24 g liter^{–1} yeast extract, 12 g liter^{–1} tryptone, 5 g liter^{–1} glycerol, and 89 mM potassium phosphate buffer).

Antibiotics were added at the following concentrations, if required: ampicillin (Amp), 100 μ g ml^{–1}; zeocin, 25 to 50 μ g ml^{–1} (*E. coli*) or 100 to 500 μ g ml^{–1} (*P. pastoris*).

For solid media, 17 to 20 g liter^{–1} agar was added.

TABLE 2 Fungal and bacterial strains and plasmids used in this study^a

Strain or plasmid	Description	Resource and/or reference
Strains		
<i>Hormonema carpetanum</i> CBS 115712	Wild type	CBS-KNAW, Utrecht, Netherlands
<i>Aureobasidium pullulans</i> CBS 100280 (EXE-150)	Wild type	CBS-KNAW, Utrecht, Netherlands (15)
<i>Neofusicoccum parvum</i>	Wild type	Strain collection, DECHEMA Research Institute, Frankfurt am Main, Germany
<i>Ochroconis constricta</i>	Wild type	Strain collection, DECHEMA Research Institute, Frankfurt am Main, Germany
<i>Parastagonospora nodorum</i>	Wild type	Strain collection, DECHEMA Research Institute, Frankfurt am Main, Germany
<i>S. cerevisiae</i> CEN-PK2-1C	<i>MATa his3D1 leu2-3_112 ura3-52 trp1-289 MAL2-8c SUC2</i>	39
<i>P. pastoris</i> X-33	Wild type	Invitrogen Corporation (Carlsbad, CA, USA)
<i>E. coli</i> DH5 α	<i>fluA2 Δ(largF-lacZ)U169 phaA glnV44 φ.80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	40, 41
Plasmids		
pPK448	Expression plasmid for <i>S. cerevisiae</i> , TRP1	39
pPK245	Expression plasmid for <i>S. cerevisiae</i> , URA3	39
pPK448_CYP reductase.Ap	Expression plasmid with cDNA from putative CYP reductase enzyme from <i>A. pullulans</i>	This work
pPK245_CYP532A30.Ap	Expression plasmid with CYP532A30 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP50915A1P.Ap	Expression plasmid with CYP50915A1P cDNA from <i>A. pullulans</i>	This work
pPK245_CYP65FA1.Ap	Expression plasmid with CYP65FA1 (L3H.Ap) cDNA from <i>A. pullulans</i>	This work
pPK245_CYP53A56.Ap	Expression plasmid with CYP53A56 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP577A26.Ap	Expression plasmid with CYP577A26 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP559K2.Ap	Expression plasmid with CYP559K2 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP609M1.Ap	Expression plasmid with CYP609M1 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP504A38.Ap	Expression plasmid with CYP504A38 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP540B38.Ap	Expression plasmid with CYP540B38 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP5134F1.Ap	Expression plasmid with CYP5134F1 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP52AA9.Ap	Expression plasmid with CYP52AA9 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP617U1.Ap	Expression plasmid with CYP617U1 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP505A51.Ap	Expression plasmid with CYP505A51 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP5104B11.Ap	Expression plasmid with CYP5104B11 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP530F1.Ap	Expression plasmid with CYP530F1 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP545A14.Ap	Expression plasmid with CYP545A14 cDNA from <i>A. pullulans</i>	This work
pPK245_CYP65EZ1.Hc	Expression plasmid with CYP65EZ1 (L3H.Hc) cDNA from <i>H. carpetanum</i>	This work
pBSYA1Z	Expression plasmid for <i>P. pastoris</i> , episomal, GAP promoter, Zeo ^r gene	Blisy e.U. (Hofstätten an der Raab, Austria)
pBSYA1Z_CYP65FA1.Ap	Expression plasmid with CYP65FA1 (L3H.Ap) cDNA from <i>A. pullulans</i>	This work
pBSYA1Z_CYP65EW2.Ap	Expression plasmid with CYP65EW2 cDNA from <i>A. pullulans</i>	This work
pBSYA1Z_CYP65EX2.Ap	Expression plasmid with CYP65EX2 cDNA from <i>A. pullulans</i>	This work
pBSYA1Z_CYP65FA2.Ap	Expression plasmid with CYP65FA2 cDNA from <i>A. pullulans</i>	This work
pBSYA1Z_PM17	Expression plasmid with CYP71D13 (PM17) cDNA from <i>Mentha × piperita</i> , codon optimized for <i>P. pastoris</i> and with FLAG tag (12)	This work
pPICZA	Expression plasmid for <i>P. pastoris</i> , genome integrated, AOX1 promoter, Zeo ^r	Invitrogen Corporation (Carlsbad, CA, USA)
pPICZA_CYP65FA1.Ap	Expression plasmid with CYP65FA1 (L3H.Ap) cDNA from <i>A. pullulans</i>	This work
pPICZA_PM17	Expression plasmid with CYP71D13 (PM17) cDNA from <i>Mentha × piperita</i> , codon optimized for <i>P. pastoris</i> and with FLAG tag (12)	This work

^aCBS-KNAW, Centraalbureau voor Schimmelcultures; Zeo^r, zeocin resistance.

Investigation of different fungi for limonene-3-hydroxylase activity. All strains tested for limonene-3-hydroxylase activity are listed in Table 2.

In order to test the different Dothideomycetes for limonene-3-hydroxylase activities (Fig. 2), 100-ml shake flasks with 25 ml YM medium were inoculated from a 48-h-old preculture and incubated for 48 h at 27°C and 150 rpm (orbit, 2.5 cm). For the biotransformation experiment, 1 g liter⁻¹ (+)-limonene was added to the fungal cultures. Samples (700 μ l each) were taken from the reaction mixtures over a period of 5 h (once per hour). Samples were extracted with 1 volume ethyl acetate, and the *trans*-isopiperitenol amount was determined by gas chromatography-mass spectrometry (GC-MS) analysis. As a negative control, YM medium without cells was used.

Clustering of fungal cytochrome P450 monooxygenase sequences. The protein sequences of all 51 putative CYP enzymes from *A. pullulans* were clustered in a phylogenetic tree with the software Geneious (Biomatters Ltd., New Zealand) using default settings (multiple alignment, ClustalW alignment; cost matrix, BLOSUM; gap open cost, 10; gap extend cost, 0.1; for the Geneious tree builder, genetic distance model, Jukes-Cantor; tree build method, neighbor-joining, no outgroup; resampling method, bootstrap; number of replicates, 1,000).

Construction of fungal CYP gene expression vectors and yeast strains. All yeast and *E. coli* strains, plasmids, and oligonucleotides used in this study are listed in Tables 2 and 3.

cDNA sequences of selected CYP genes from *A. pullulans* EXF-150 (CBS 100280) were obtained through comparison between gene and protein sequence, stored in the NCBI database (for accession numbers, see Table 4). For synthesis of template cDNA, *A. pullulans* CBS 100280 cells were inoculated in 25 ml YM medium in a 100-ml shake flask. After incubation for 24 h at 28°C and 130 rpm (orbit, 2.5 cm), 100 mg liter⁻¹ (+)-limonene was added and incubated for a further 24 h. Afterwards, cells were harvested, and total RNA was isolated with an RNeasy plant minikit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed with an iScript Select cDNA synthesis kit (Bio-Rad) using oligo(dT) primers. Afterwards, cDNAs of CYP genes were amplified via PCR. All expression plasmids used in this study were either constructed by BioCat GmbH (Germany) or constructed using Gibson isothermal assembly cloning (34, 35). *E. coli* cells were grown at 37°C. Plasmids were isolated from *E. coli* using the GeneJET plasmid miniprep kit (Thermo Fisher Scientific).

S. cerevisiae and *P. pastoris* strains were transformed with plasmids according to protocols of Gietz and Schiestl (36, 37) or the EasySelect *Pichia* expression kit manual (Invitrogen). Yeast cells were grown at 30°C on SC selective medium or YPD containing zeocin.

Investigation of fungal CYP candidates for limonene-3-hydroxylation in *S. cerevisiae*. For biotransformation experiments with *S. cerevisiae*, a protocol based on publications by Emmerstorfer et al. and by Emmerstorfer-Augustin and Pichler was used (12, 38). *S. cerevisiae* CEN-PK2-1C strains, containing different pPK245 and pPK448 expression vectors (Table 2), were grown on SC medium agar plates for 3 days. For initial testing of different CYP genes (Fig. 4), main cultures were inoculated directly with a pin-head-sized colony of yeast from agar plate in 50 ml SC medium in a 300-ml Erlenmeyer flask and incubated for a total of 72 h at 30°C and 180 rpm (orbit, 2.5 cm). After 48 h, cells were fed with 1% glucose. From stationary-phase cultures, a number of cells equivalent to 150 units of optical density at 600 nm (OD₆₀₀) were harvested (1,190 $\times$ g, 10 min) and resuspended in 500 μ l of 100 mM potassium phosphate buffer (pH 7.4; total volume, 750 μ l). Cell suspensions were then transferred into 2-ml glass vials and 300 mM (+)-limonene stock solution (in dimethyl sulfoxide [DMSO] with 1% Triton X-100) was added to yield a final limonene concentration of 6 mM. Glass vials were sealed loosely with screw caps and sealing tape. Biotransformation was carried out for 24 h at 900 rpm (orbit, 2 to 3 mm) and 30°C in a tabletop thermal shaker. Terpenoids were extracted with 250 μ l methyl *tert*-butyl ether (MTBE) containing 100 μ M 3-carene as the internal standard. After phase separation by centrifugation for 10 min at 16,000 $\times$ g, organic layers were analyzed via GC-MS. CEN-PK2-1C strains containing the respective empty expression vectors were used as negative controls.

To quantify the produced *trans*-isopiperitenol amounts, stock solutions of isopiperitenol (2:1 mixture of *trans* and *cis*) with different concentrations were added to buffer aliquots of 750 μ l and extracted with MTBE in the same way as the biotransformation samples.

Biotransformation experiments with *Pichia pastoris*. A modified version of the approach described by Emmerstorfer et al. and Weis et al. was used (12, 33, 38). *P. pastoris* X-33 strains containing different pBSYA1Z or pPICZA expression plasmids (Table 2) were grown on YPD or BMDY medium agar plates containing 500 μ g ml⁻¹ zeocin for 2 to 3 days at 30°C. BMDY medium (25 ml) in a 300-ml Erlenmeyer flask with 500 μ g ml⁻¹ zeocin was inoculated directly with a pin-head-sized colony of *P. pastoris* and incubated for around 24 h at 30°C and 200 rpm (orbit, 2.5 cm). Afterwards, 25 ml BMDY or BMMY medium was added to cultures with pBSYA1Z or pPICZA plasmids, respectively. After 24 h, 1% glucose was added to strains with pBSYA1Z vectors. Strains with pPICZA plasmids were fed two or three times with 1% methanol. Portion (150 or 225 OD₆₀₀ units) of the 72- to 76-h-old cultures were harvested (1,190 $\times$ g, 10 min) and resuspended in 375 or 500 μ l of 100 mM potassium phosphate buffer (pH 6.0, 7.4, or 8.0). If glucose was added to the biotransformation reaction mixture, 425 μ l of buffer and 75 μ l of a 20% glucose solution were used. Cell suspensions were then transferred into 2-ml glass vials, and 300 mM (+)- or (-)-limonene stock solution (in DMSO with 1% of Triton X-100) was added to yield a final concentration of 6 mM limonene. Glass vials were sealed loosely with screw caps and sealing tape. Biotransformation was carried out for 24 to 28 h at 900 rpm (orbit, 2 to 3 mm) at 30°C in a tabletop thermal shaker. Terpenoids were extracted with 250 μ l MTBE containing 100 μ M 3-carene as the internal standard. After phase separation by centrifugation for 10 min at 16,000 $\times$ g, organic layers were analyzed via GC-MS.

For experiments in 100-ml reaction vessels, for each biotransformation batch 2,250 OD₆₀₀ units was harvested (1,190 $\times$ g, 10 min) and resuspended in 5 ml of 100 mM potassium phosphate buffer (pH 8.0).

TABLE 3 Oligonucleotides used in this study

Oligonucleotide or PCR primer	Sequence	Purpose
P1	TCGAATAAACACACATAAACAAACGAATTCATGCGCAACTCGACTCTCTC	Cloning of CYP reductase.Ap gene in pPK448 backbone
P2	TAITTAAGTGAAGATTTAAAGTAGGTACCTCATGACACAGACATCTCTTGG	
P3	TACAAAAACACATACATAAACTAGAATTCATGATCATCGGCGAGTTAGTACC	Cloning of CYP532A30 gene in pPK245 backbone
P4	TAITTTTATAAATTATTAATCTTAGGTACCTTATACAGGAACCTCGGAG	
P5	TACAAAAACACATACATAAACTAGAATTCATGTTCTCGGAGCAATCACC	Cloning of CYP65FA1 gene in pPK245 backbone
P6	TAITTTTATAAATTATTAATCTTAGGTACCTCAAGTCGTAGTCTCGAAGGTG	
P7	TACAAAAACACATACATAAACTAGAATTCATGGGTGCTCGAGCTCC	Cloning of CYP53A56 gene in pPK245 backbone
P8	TAITTTTATAAATTATTAATCTTAGGTACCTCAAAATTCCTCTTCATACCAAC	
P9	TACAAAAACACATACATAAACTAGAATTCATGGATTTCTGATCGCTACTTC	Cloning of CYP559K2 gene in pPK245 backbone
P10	TAITTTTATAAATTATTAATCTTAGGTACCTTACACCGTGCCTG	
P11	TACAAAAACACATACATAAACTAGAATTCATGCTGCTCTCTCTCTCAC	Cloning of CYP5134F1 gene in pPK245 backbone
P12	TAITTTTATAAATTATTAATCTTAGGTACCTTACCACTGCTCTCTTCAAC	
P13	TACAAAAACACATACATAAACTAGAATTCATGGCGAGCATCTGTATTC	Cloning of CYP52AA9 gene in pPK245 backbone
P14	TAITTTTATAAATTATTAATCTTAGGTACCTCAGTGAGAGGCGACATCTT	
P15	TACAAAAACACATACATAAACTAGAATTCATGGGTTTGACGGCTG	Cloning of CYP617U1 gene in pPK245 backbone
P16	TAITTTTATAAATTATTAATCTTAGGTACCTTAATCTCTATACATCTCAAAATTAACATAACAC	
P17	TCAATTGAAACAACTATCAAAACACAATGTTCTCGGAGCAATCACC	Cloning of CYP65FA1 gene in pBSYA1Z backbone
P18	TCTGACATCCTCTTGAGCGGCCGCTCAAGTCGTAGTCTCGAAGG	
P19	TCAATTGAAACAACTATCAAAACACAATGGAGCTCGAAGGACTC	Cloning of CYP65EW2 gene in pBSYA1Z backbone
P20	TCTGACATCCTCTTGAGCGGCCGCTCACCGGACCACTCGTC	
P21	TCAATTGAAACAACTATCAAAACACAATGCTGACCTTTGCTTTGTC	Cloning of CYP65EX2 gene in pBSYA1Z backbone
P22	TCTGACATCCTCTTGAGCGGCCGCTCAATCTGTACAGCAAGTAAGTTTG	
P23	TCAATTGAAACAACTATCAAAACACAATGGCACTTCTCGACAG	Cloning of CYP65FA2 gene in pBSYA1Z backbone
P24	TCTGACATCCTCTTGAGCGGCCGCTCACCAACACTACCTCTAGC	
P25	TCAATTGAAACAACTATCAAAACACAATGGAATGCAATCTCTCTGC	Cloning of PM17 gene in pBSYA1Z backbone
P26	TCTGACATCCTCTTGAGCGGCCGCTTATTAATCTGTCGTCATCCTTGT	
P27	AGATCAAAAAACAACTAATTATCGAAGCATGTTCTCGGAGCAATCACCAGG	Cloning of CYP65FA1 gene in pPICZA backbone
P28	CAGATCCTCTCTGAGATGAGTTTTTTGTTTCAGTCGTAGTCTCGAAGGTG	
P29	AGATCAAAAAACAACTAATTATCGAAGCATGGAATGCAATCTCTCTG	
P30	CAGATCCTCTCTGAGATGAGTTTTTTGTTTCCTTATCGTCGTCATCCTGTGATC	Cloning of PM17 gene in pPICZA backbone

TABLE 4 Overview of clustered and partially tested genes of *A. pullulans* and *H. carpetanum*, with database identification numbers

Assigned name	Fungus	Protein (NCBI ^a) or GenBank accession no.	Sequence ID (GI – NCBI)
CYP532A30	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ81481.1	662524092
CYP50915A1P	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ78964.1	662521555a
CYP65FA1 (L3H.Ap)	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ84190.1	662526809
CYP53A56	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ80869.1	662523476
CYP577A26	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ83870.1	662526489
CYP559K2	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ79812.1	662522412
CYP609M1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ82851.1	662529043
CYP504A38	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ83160.1	662525777
CYP540B38	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ83654.1	662526272
CYP5134F1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ87691.1	662530317
CYP52AA9	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88534.1	662531161
CYP617U1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ85574.1	662528196
CYP505A51	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88047.1	662530673
CYP5104B11	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ89245.1	662531873
CYP530F1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ86419.1	662525467
CYP545A14	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ87624.1	662530250
CYP65FA2	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ82374.1	662524989
CYP65EW2	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88046.1	662530672
CYP65EW4	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ86879.1	662529504
CYP65EX2	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ83455.1	662526073
CYP53E8	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ80659.1	662523265
CYP59E7-partial	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ82102.1	662524716
CYP61A6	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88356.1	662530983
CYP62V1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88283.1	662530910
CYP62K3	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ84873.1	662527494
CYP65EW1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ80659.1	662523265
CYP504B35	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88139.1	662530765
CYP504E24	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ83873.1	662526492
CYP505G	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ83999.1	662526618
CYP534D6	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ89839.1	662532467
CYP537J1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ79075.1	662521667
CYP540B39	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ81219.1	662523828
CYP543C11	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ86091.1	662528714
CYP570G6	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ87812.1	662530438
CYP578V1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ87430.1	662530056
CYP584AK1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88250.1	662530877
CYP584AL1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ86261.1	662528885
CYP584AM1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ82069.1	662524682
CYP617G17	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ87191.1	662529816
CYP660H1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ82176.1	662524790
CYP5104G1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ80674.1	662523280
CYP5195G1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ78830.1	662521419
CYP5201C1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ80264.1	662522867
CYP5259A5	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ80252.1	662522855
CYP5326B1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ81109.1	662523718
CYP5053C20	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ81134.1	662523743
CYP5607B1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ78829.1	662521418
CYP6462A2	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ87937.1	662530563
CYP6472A2	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ87621.1	662530247
CYP6921A1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ86214.1	662528838
CYP50407A1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ83496.1	662526114
CYP50914A1	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ86212.1	662528836
CYP reductase.Ap (putative CYP reductase)	<i>A. pullulans</i> CBS 100280 (EXF-150)	KEQ88142.1	662530768
CYP65EZ1 (L3H.Hc)	<i>Hormonema carpetanum</i> CBS 115712	MW239664	
CYP65EY1	<i>Hormonema carpetanum</i> CBS 115712	MW239669	
CYP65EX1	<i>Hormonema carpetanum</i> CBS 115712	MW239665	
CYP5188D1	<i>Hormonema carpetanum</i> CBS 115712	MW239666	
CYP65EW3	<i>Hormonema carpetanum</i> CBS 115712	MW239667	
CYP65EW1	<i>Hormonema carpetanum</i> CBS 115712	MW239668	

^a<https://www.ncbi.nlm.nih.gov>.

The cell suspensions were transferred to 100-ml glass bottles, and limonene stock solution was added to a final concentration of 6 mM limonene. The vessels were tightly closed with screw caps and Parafilm. The glass bottles were incubated for 51 h in a cabinet incubator at 30°C and 250 rpm (orbit, 2.5 cm). Samples of 750 μ l were taken at 2, 5, 20, 28, and 51 h and extracted as described for the other *Pichia* samples. Some batches were kept closed throughout the experiment without sampling to make sure that no *trans*-isopiperitenol evaporated.

P. pastoris strains containing the respective empty expression vectors were used as negative controls in all experiments.

To quantify the produced *trans*-isopiperitenol amounts, stock solutions of isopiperitenol (2:1 mixture of *trans* and *cis*) with different concentrations were added to cell suspension aliquots of a *P. pastoris* strain, containing the empty pBSYA1Z vector, and extracted with MTBE in the same way as the biotransformation samples.

For the bioconversion experiments with substrates other than (+)- and (–)-limonene, the different substances (Table 1) were dissolved in DMSO with 1% of Triton X-100 and added to the biotransformation reaction mixtures to give a final concentration of 6 mM. Only in the case of isopiperitenol (2:1 mixture of *trans* and *cis*), 0.6 μ M was used. Biotransformation samples were extracted as described above, but without the internal standard.

GC-MS analysis. For GC-MS analysis of fungal biotransformation samples, except *S. cerevisiae* and *P. pastoris*, a GC-17A QP5050A system (Shimadzu) with a VB-5 column (30 m by 0.25 mm by 0.25 μ m; ValcoBond; Valco Instruments Co. Inc., and VICI AG) or a GCMS-QP2010 SE system (Shimadzu) containing a DB-5 column (30 m by 0.25 mm by 0.25 μ m; Agilent) was used. Measurements were conducted as follows: helium as carrier gas, split ratio of 10, injection at 250°C with a sample volume of 1 μ l, a column flow of 1.2 ml min^{–1}, and a linear velocity of 39.2 cm s^{–1}. The column temperature was programmed as follows: 60°C for 3 min, with 10°C min^{–1} up to 240°C, followed by 3 min at 240°C.

Samples of biotransformation experiments with *S. cerevisiae* and *P. pastoris* were analyzed by a GCMS-QP2010 SE (Shimadzu) system containing a DB-5 column (30 m by 0.25 mm by 0.25 μ m; Agilent). Measurements were conducted as follows: helium as carrier gas, split ratio 1 or 5, injections at 270°C, column flow of 0.7 ml min^{–1}, and a linear velocity of 30 cm s^{–1}. The column temperature was programmed as follows: 40°C, 9°C min^{–1} up to 175°C, followed by 30°C min^{–1} up to 300°C.

trans-Isopiperitenol, carveol, verbenol, verbenone, and pinocarveol were identified by comparison of retention times and mass spectra with those of chemically synthesized reference substances (see “Chemicals and media”). *p*-1,8-Menthadien-4-ol, *p*-1,5-menthadien-8-ol, and other products were identified by comparison of mass spectra to those of the NIST (National Institute of Standards and Technology) mass spectral library (v14) or the MassLib mass spectral library (Symrise Holzminden; v9.4.0). Identity was assumed if the similarity index was equal to or higher than 90%. Absolute concentrations of *trans*-isopiperitenol were calculated from chromatogram peak areas by comparison to a calibration curve prepared by measuring a dilution series of isopiperitenol reference substance with known concentrations.

Data availability. The accession numbers of the genes and proteins used in this study are listed in Table 4, and the sequences are available via the NCBI database (<https://www.ncbi.nlm.nih.gov>).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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F.M.S., I.S., M.M.W.E., H.S., J.S., and M.B. designed the research. F.M.S., I.S., M.M.W.E., and E.B. performed the experiments. J.P. contributed reagents and analysis tools. F.M.S., I.S., M.M.W.E., J.P., and M.B. analyzed the data. F.M.S. and M.B. wrote the manuscript with input from all authors.

We declare that we have no conflicts of interest regarding the content of this article.

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