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MINI REVIEW

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Biotransformation of limonene by bacteria, fungi, yeasts, and plants

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Abstract The past 5 years have seen significant progress in the field of limonene biotransformation, especially with regard to the regiospecificity of microbial biocatalysts. Whereas earlier only regiospecific biocatalysts for the 1,2 position (limonene-1,2-diol) and the 8-position (α-terpineol) were available, recent reports describe microbial biocatalysts specifically hydroxylating the 3-position (isopiperitenol), 6-position (carveol and carvone), and 7-position (perillyl alcohol, perillylaaldehyde, and perillic acid). The present review also includes the considerable progress made in the characterization of plant P-450 limonene hydroxylases and the cloning of the encoding genes.

Introduction

The two enantiomers of limonene (Fig. 1) are the most-abundant monocyclic monoterpenes in nature; L-limonene is mainly found in a variety of trees and herbs such as *Mentha* spp., while D-limonene is the major component of peel oil from oranges and lemons, and the essential oil of caraway. Natural functions are thought to include the prevention of dehydration, and the inhibition of microbial, especially fungal, growth. The biosynthesis of limonene and other monoterpenes in plants was recently reviewed by Wise and Croteau (1999).

The era between 1945 and 1960 marked the beginning of the orange juice industry in Florida; the percentage of oranges that was processed to concentrated juice increased from less than 1% to 80% (Murdock and Allen 1960). The consequent availability of vast amounts (50,000 tonnes per year; Braddock and Cadwallader 1995) of low-cost (U.S. $ 1–2/kg; Mazzaro 2000) enantiopure D-limonene from the peel oil has interested chemists and biologists alike. This is because a range of important flavor and medicinal compounds has the same carbon skeleton, suggesting great market potential. Most notable derivatives in this respect are oxygenated compounds, such as α-terpineol, perillyl alcohol, carveol, carvone, and menthol (structures included in Table 1 and Fig. 2). Bulk prices for menthol and both enantiomers of carvone are in the range of U.S. $ 30–60/kg; prices for (–) and (+) perillyl alcohol are at least one order of magnitude higher. Also, the use of D-limonene for the synthesis of larger terpenes has been considered (Mehtra 1990).

The regiospecific introduction of carbonyl or hydroxy groups by chemical means has proved difficult due to the similar electronic properties of the allylic methylene groups (carbon 3 and 6) and the allylic methyl groups (carbons 7 and 10). As a consequence, classical chemical oxidation procedures give rise to mixtures of products. A representative example is the allylic oxidation of limonene using selenium dioxide, yielding limonene-4-ol, *trans*-carveol, *cis*-carveol, perillyl alcohol, and limonene-10-ol (Jensen and Sharpless 1975; Sakuda 1969). There is one notable exception to the general failure to develop regiospecific chemical oxidation processes: D-limonene can be converted into (–) carvone using the nitrosyl chloride method (involving an allylic rearrangement). This process was discovered as early as 1885 (for references see Royals and Horne 1951) and is, as far as the authors know, currently the only existing chemical process carried out on a reasonable scale using D-limonene as a raw material.

For obvious reasons (enzyme regiospecificity and enantiospecificity), biocatalytic conversion of D-limonene was considered as early as the 1960s (Dhavalikar and Bhattacharyya 1966; Dhavalikar et al. 1966), and numerous D-limonene-transforming microbial and plant cells have been described since. Previous reviews dedicated to

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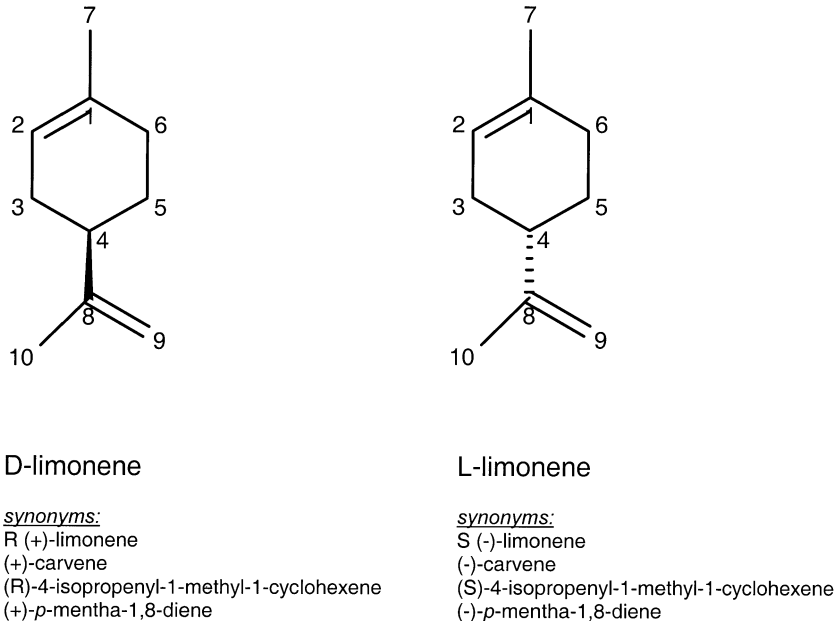


Fig. 1 Chemical structures, carbon atom numbering, and nomenclature of the two enantiomers of limonene

limonene biotransformation include Braddock and Cadwallader (1995) and Gabrielyan et al. (1992). Valuable reviews on bioconversions of terpenoid compounds including limonene were written by Kak (1992), Kieslich et al. (1986), Mikami (1988), Trudgill (1990), and Van der Werf et al. (1997).

Here, we review the biocatalytical literature on limonene, divided into four sections according to the natural role and source of the enzymes involved. Patents are only cited if results are not (yet) published in journals or books. Other documents cover the physicochemical and toxicological properties (Vonburg 1995; www.floridachemical.com), chemical conversions (Ravindranath 1983), fungicidal properties (Murdock and Allen 1960), autooxidation (Royals and Horne 1955), and anticarcinogenic properties (Crowell 1999).

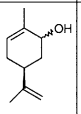
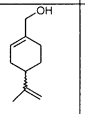
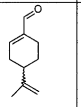
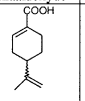
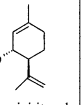
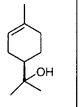
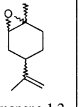
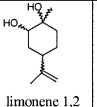
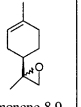
Metabolites formed by limonene-degrading bacterial strains

A common strategy to obtain enzymes for the regio- and/or enantiospecific oxygenation of hydrocarbons is the evaluation of biodegradative pathways in bacterial isolates obtained by selective enrichment; if the target compound is a pathway intermediate in one of the isolates, the use of blocked mutants (gene for subsequent enzyme inactivated) or genetic constructs (heterologous expression) may allow for the biocatalytic production of this derivative.

The application of this strategy for D-limonene has met with only limited success thus far, in part due to a one-sided experimental approach. In most studies on the elucidation of D-limonene degradation pathways, suggested routes were primarily based on (1) the structure of accumulated compounds and (2) simultaneous induction experiments (comparison of oxidation rates of potential intermediates by limonene-induced cells). In the absence of supportive data from blocked mutants or genetic constructs, this 'black box' approach may easily lead to erroneous conclusions, due to the following uncertainties. (1) If a certain compound is found to accumulate, it may either be a dead-end product resulting from an incomplete regiospecificity of the enzyme (often an oxygenase) involved or a (transiently) accumulating intermediate in the pathway, due to a relatively low expression level or a high K_m of the next enzyme in the pathway. (2) The accumulation of multiple compounds (e.g., hydroxy limonene isomers) may be due to either the presence of multiple oxygenases with different regiospecificities or a single enzyme displaying an incomplete regiospecificity. (3) Consumption of a putative intermediate by D-limonene grown cells ("simultaneous induction") is no evidence that it is a true intermediate. Its degradation pathway may be co-induced with the D-limonene degradation pathway.

The first biodegradation study was performed at the Indian National Chemical Laboratory in Poona in the 1960s (Dhavalikar and Bhattacharyya 1966; Dhavalikar et al. 1966). The authors isolated a *Pseudomonas* strain able to grow with D-limonene as the sole carbon and energy source. Compounds accumulating during growth on D-limonene were extracted from the culture medium, and fractionated using different solvents and pH values. Skilful analytical work (mainly thin layer chromatography and nuclear magnetic resonance) showed the accumulation of dihydrocarvone, carvone, carveol, limonene-1,2-*cis*-diol, 1-hydroxy-2-oxo-limonene, limonene-1,2-*trans*-diol, limonene-6,9-diol, perillic acid, β-isopropenyl pimelic acid, 2-hydroxy-8-p-menthen-7-oic acid, and 6,9-dihydroxy perillic acid in unquantified amounts. From these results and simultaneous induction experiments, the

Table 1 Selection of limonene derivatives resulting from microbial biotransformations

	product	substrate (D-or L-)	configuration hydroxy group	other products	act.(U/g dry wt)	organism	main references (in chronological order)
1	 carveol	D	*cis*	2,5,9, others	n.d.	*Pseudomonas* sp. PL	Dhavalikar and Bhattacharyya 1966
		D	*trans* + *cis*	2, others	n.d.	*Penicillium digitatum*	Bowen 1975
		D	*cis*	3,7,9, others	n.d.	*Aspergillus cellulosae M-77*	Noma et al. 1992
		D	*trans* + *cis*	2	< 0.1	*Pleurotus sapidus*	Onken and Berger 1999
		D	n.d.	2,3,4	n.d.	*Escherichia. coli* (3.6 kb from BR388)	Cheong and Oriel 2000
		D	*trans*	none	15	*Rhodococcus opacus* PWD4	Duetz et al. 2001a
		D	*trans*	2	n.d.	*Rhodococcus erythropolis* PWD8	Duetz et al. 2001a
2	 carvone	D	n.a.	1,5,9, others	n.d.	*Pseudomonas* sp. PL	Dhavalikar and Bhattacharyya 1966
		D	n.a.	1, others	n.d.	*Penicillium digitatum*	Bowen 1975
		D	n.a.	1	n.d.	*Pleurotus sapidus*	Onken and Berger 1999
		D	n.a.	1,3,4	n.d.	*Escherichia. coli* (3.6 kb from BR388)	Cheong and Oriel 2000
		D	n.a.	1	n.d.	*Rhodococcus erythropolis* PWD8	Duetz *et al.* 2001a
3	 perillyl alcohol	D	n.a.	1,7,9, others	n.d.	*Aspergillus cellulosae* M-77	Noma et al. 1992
		D	n.a.	4,7	n.d.	*Bacillus stearothermophilus* BR388	Chang and Oriel 1994
		D	n.a.	4,7	n.d.	*Escherichia. coli* (9.6 kb from BR388)	Chang et al. 1995
		D	n.a.	1,2,4	0.1	*Escherichia. coli* (3.6 kb from BR388)	Cheong and Oriel 2000
		D	n.a.	4,5	13	*Mycobacterium* sp. HXN-1500	Duetz et al. 2001b
		L	n.a.	4,5	10	*Mycobacterium* sp. HXN-1500	Duetz et al. 2001b
		D	n.a.	limonene 6,8 diol	n.d.	*P. putida* MTCC 1072	Chatterjee and Bhattacharyya 2001
4	 perillaaldehyde	D	n.a.	3,7	n.d.	*Bacillus stearothermophilus* BR388	Chang and Oriel 1994
		D	n.a.	3,7	n.d.	*Escherichia. coli* (9.6 kb from BR388)	Chang et al. 1995
		D	n.a.	1,2,3	n.d.	*Escherichia. coli* (3.6 kb from BR388)	Cheong and Oriel 2000
		D, L	n.a.	3,5	n.d.	*Mycobacterium* sp. HXN-1500	Duetz et al. 2001b
5	 perillic acid	D	n.a.	1,2,9,others	n.d.	*Pseudomonas* sp. PL	Dhavalikar and Bhattacharyya 1966
		D	n.a.	isopropenyl pimelate	n.d.	*Pseudomonas incognita*	Rama Devi and Bhattacharyya 1977
		D	n.a.	7, other	n.d.	*Pseudomonas gladioli*	Cadwallader et al. 1989
		D	n.a.	none	10	*Pseudomonas putida* GS1	Speelmans et al. 1998
		D	n.a.	none	n.d	*Escherichia coli (cym* genes from *Pseudomonas putida* F1)	Mars et al. 2001
		D, L	n.a.	3, 4	n.d.	*Mycobacterium* sp. HXN-1500	Duetz et al. 2001b
6	 iso pipiritenol	D	*trans*	none	1-2 (see text)	*Hormonema* sp. UOFS Y-0067	Van Dyk et al. 1998
7	 α-terpineol	D	n.a.	none	n.d.	*Cladosporium sp.* T12	Kraidman et al., 1969
		D	n.a.	none	n.d.	*Penicillium digitatum* DSM 62840	Abraham et al. 1986a
		D	n.a.	9	n.d.	*Armillareira mellae*	Draczynska 1987
		D	n.a.	5, other	n.d.	*Pseudomonas gladioli*	Cadwallader et al. 1989
		D	n.a.	1,3,9, others	n.d.	*Aspergillus cellulosae M-77*	Noma et al. 1992
		D	n.a.	3,4	n.d.	*Bacillus stearothermophilus* BR388	Chang and Oriel 1994
		D	n.a	3,4	n.d.	*Escherichia. coli* (9.6 kb from BR388)	Chang et al. 1995
		D	n.a.	none	n.d.	*Penicilium digitatum* NRRL 1202	Tan and Day 1998
8	 limonene-1,2 epoxide	D,L	n.d.	(product is the first intermediate, but does not accumulate)	n.d.	*Diplodia gossypina* ATCC 10936	Abraham et al. 1986b
		D,L	n.d.		n.d.	*Corynespora cassiicola* DSM62474/5	Abraham et al. 1986b
		D	n.d.		n.d.	*Rhodococcus erythropolis* DCL14	Van der Werf and De Bont 1998
9	 limonene 1,2 diol	D	*trans* + *cis*	1,2, 5, others	n.d.	*Pseudomonas* sp. PL	Dhavalikar and Bhattacharyya 1966
		D	*trans* + *cis*	none	n.d.	*Cladosporium* sp. T7	Mukherjee et al. 1973
		D, L	*trans*	none	n.d.	*Diplodia gossypina* ATCC 10936	Abraham et al. 1986b
		D, L	*trans*	none	n.d.	*Corynespora cassiicola DSM62474/5*	Abraham et al. 1986b
		D	n.d.	7	n.d.	*Armillareira mellae*	Draczynska 1987
		D	*trans*	1,3,7,others	n.d.	*Aspergillus cellulosae* M-77	Noma et al. 1992
10	 limonene-8,9 epoxide	D, L	see text	none	n.d.	*Xanthobacter* sp. C20	Van der Werf et al. 2000

n.d = not determined,
n.a. = not applicable

authors concluded that the organism attacks the limonene molecule at several positions, and that a major pathway in this strain probably starts with hydroxylation at the 7-position yielding perillyl alcohol. Subsequent oxidation to perillic acid, hydration of the 6–10 C=C double bond, followed by a pathway analogous to regular β-oxidation, results in complete degradation. Unfortunately, further articles on this particular strain (*P. putida* PL*)* have not been published. However, more recently, a number of similar *Pseudomonas* strains have been studied. A *Pseudomonas incognita* strain (originally isolated with linalool as the sole carbon source) converted D-limonene to perillic acid and β-isopropenyl pimelic acid (Rama Devi and Bhattacharyya 1977). Cadwallader et al. (1989) obtained a D-limonene-degrading *Pseudomonas gladioli* strain by selective enrichment, and observed the transient accumulation of perillic acid (probably as a pathway intermediate), and the formation of α-terpineol and an

unidentified compound as dead-end products. Speelmans et al. (1998) also isolated a strain (*P. putida* GS1) with D-limonene as the sole carbon source by selective enrichment. In the presence of excess D-limonene, (+) perillic acid was formed at a specific activity of around 10 U/g of dry weight. In a follow-up study, Mars et al. (2001) concluded that the first three enzymes from the cymene degradation pathway in *P. putida* GS1 (cymene monooxygenase and two dehydrogenases) are responsible for this bioconversion. The observation that a recombinant *Escherichia coli* strain expressing the genes of the *cym* pathway of *P. putida* F1 also converted limonene to perillic acid (albeit at low rates) strengthened this hypothesis (Mars et al. 2001). In this context it is interesting to note that the pseudomonad used by the pioneering Indian group was also capable of growth with *p*-cymene as the sole carbon source (Dhavalikar and Bhattacharyya 1966). Retrospectively, it may be speculated that the formation of perillic acid they observed was due to enzymes from the *cym* pathway as well. Another D-limonene-degrading pseudomonad (*P. putida* MTCC 1072) was found to accumulate perillyl alcohol and limonene 6,8 diol in a 4:5 ratio during growth on D-limonene (Chatterjee and Bhattacharyya 2001). As the results in this study were only based on the wild-type strain, it remains unclear if the simultaneous formation of these two compounds is due to an incomplete regiospecificity of a single enzyme or to the simultaneous expression of two limonene-oxidizing enzyme systems.

A number of D-limonene-degrading *Bacillus* strains was studied by the group of Patrick Oriel from Michigan. *Bacillus stearothermophilus* BR388 was found to convert D-limonene to perillyl alcohol, α-terpineol, and perillaaldehyde (Chang and Oriel 1994). The same metabolites were found to accumulate during growth of an *E. coli* construct carrying a 9.6-kb chromosomal fragment from this strain, which allowed the construct to grow with D-limonene as the sole carbon source (Chang et al. 1995). In a later study (Cheong and Oriel 2000), a 3.6-kb subfragment was found to encode a limonene hydroxylase that hydroxylated limonene in the 6 or 7 position, giving rise to a mixture of (mainly) perillyl alcohol and carvone (the latter formed from carveol by means of an nonspecific dehydrogenase from the host strain) in a 3:1 ratio. These results suggest strongly that the occurrence of multiple products is due to incomplete enzyme regiospecificity (rather than the simultaneous expression of multiple enzymes with different regiospecificities). In contrast to the wildtype *Bacillus* strain and the construct carrying the 9.6-kb fragment, α-terpineol did not accumulate (Cheong and Oriel 2000). Apparently, the formation of this compound was due to another enzyme encoded on the 9.6-kb fragment but not on the 3.6-kb fragment.

It seems reasonable to assume that the D-limonene-degrading *Pseudomonas* and *Bacillus* strains described above gain most of their carbon and energy from limonene via the route of progressive oxidation of the C7 methyl group (leading to perillic acid), followed by a pathway resembling β-oxidation. Dependent on the strain, the initial hydroxylases seem to display various degrees of regiospecificity, giving rise to a variety of side-products.

A completely different degradation route was found in *Rhodococcus erythropolis* DCL14. This strain appeared to initiate D-limonene degradation by epoxidation of the 1,2 C=C double bond, yielding limonene 1,2 epoxide. Subsequent hydrolysis leads to the corresponding diol that may be oxidized to a hydroxyketone, which is in turn a substrate for a Baeyer-Villiger oxygenase (Van der Werf and De Bont 1998; Van der Werf et al. 1999). The strain was originally selected from a collection of 120 strains isolated with D-limonene as the sole carbon and energy source, on the basis of its low rate of perillyl alcohol oxidation (Van der Werf and De Bont 1998).

Several of the above studies indicate that initial enzymes involved in limonene catabolic pathways are not always completely regiospecific. Possibly this is due to the absence of a sufficiently strong selective pressure; complete regiospecificity of catabolic oxygenases is only essential if the regioisomers cannot be assimilated (and would thus be lost as a source of carbon and energy, after the investment of NADH). However, many strains capable of growth on D-limonene can utilize a wide range of hydroxylated derivatives (Dhavalikar et al. 1966; Van der Werf and De Bont 1998). The wide substrate range may be related to the observation that such oxidized limonene derivatives are generally co-produced by the limonene-producing plants or trees (Wise and Croteau 1999) or generated by autooxidation of limonene (Royals and Horne 1955).

Co-metabolic conversions by oxygenases involved in the degradation of other compounds

The distinction in this review between bioconversions by microbial enzymes involved in natural limonene biodegradation (previous section) and bioconversions by enzymes involved in the degradation of other compounds (present section) is arbitrary to some extent. For example, the biodegradation route of limonene involving the cymene degradation enzymes (Mars et al. 2001; Speelmans et al. 1998) might be considered primarily a co-metabolic conversion, as a large part of the D-limonene supplied is not mineralized but accumulates as perillic acid.

A clear example of a co-metabolic conversion is the epoxidation of D-limonene in the 8–9 position by cells of *Xanthobacter* sp. C20, isolated and grown on cyclohexane. Apparently, the monooxygenase catalyzing the hydroxylation of cyclohexane (a cytochrome P-450 enzyme) does not hydroxylate the cyclohexene ring of D-limonene, but performs this epoxidation instead, giving rise to (4R,8R)-limonene-8,9-epoxide as the only reaction product. L-Limonene was converted into a (78:22) mixture of (4S,8R)- and (4S,8S)-limonene-8,9-epoxide (Van der Werf et al. 2000).

Screening of 1,800 bacterial strains known to possess catabolic pathways for alkanes, toluene, naphthalene, phenylacetic acid, cinnamic acid, cresol, and other compounds at the ETH Zurich yielded two types of commercially interesting co-metabolic conversions. Firstly, a range of alkane-degrading strains mainly from the genera *Rhodococcus* and *Mycobacterium* were found to hydroxylate D-limonene and L-limonene exclusively in the 7-position, yielding perillyl alcohol (Duetz et al. 2001b). In some strains, the perillyl alcohol was partially or completely oxidized to the corresponding aldehyde and acid, probably by the action of non-specific dehydrogenases. Interestingly, archetypal Gram-negative alkane degraders such as *P. putida* (*oleovorans*) GPo1 were not capable of hydroxylating limonene (Duetz et al. 2001b). The second co-metabolic conversion found was the formation of *trans*-carveol from D-limonene by the action of toluene and naphthalene dioxygenases (Duetz et al. 2000, 2001a). The relatively high specific activity (up to 15 U/g dry weight) and complete regiospecificity (no detectable regioisomers) found for *Rhodococcus opacus* PWD4 are promising characteristics for an industrial application of this biocatalyst. The biocatalytic production of (+) carvone is presently under investigation, since another strain (*R. globerulus* PWD8) was found to convert part of the formed *trans*-carveol to carvone (Duetz et al. 2000, 2001a). The two-step bioconversion of limonene to carvone is attractive since the NADH consumed in the first step (an oxygenase) is regenerated in the second step (a dehydrogenase); only oxygen is stoichiometrically consumed. The thermodynamics of this conversion (e.g., Gibbs free energy changes) are favorable for the full conversion to carvone (Duetz et al. 2001a).

Bioconversions by fungi and yeasts

Although not proven in all instances, all bioconversions of D-limonene by yeasts and fungi seem to be initiated by P-450 monooxygenases. The conversion of limonene to α-terpineol by *Penicillium digitatum* DSM 62840 was first thought to be catalyzed by a hydratase (Abraham et al. 1986a), which would have been attractive for a biocatalytical process since no oxygen or co-factors would have been required. However, the initial step was later found to be epoxidation of the 8,9 double bond. The subsequent conversion to α-terpineol was stated to involve 'reductive cleavage of the epoxide' but no details on the enzymes involved were provided (Kieslich et al. 1986). Interestingly, even racemic mixtures of D- and L-limonene yielded optically active α-terpineol: only D-α-terpineol was found to accumulate, probably because the L-α-terpineol formed is further degraded, possibly via sobrerol (Kieslich et al. 1986). The formation of α-terpineol was first reported in a short symposium abstract by Kraidman et al. (1969). The responsible strain was designated *Cladosporium* sp. T12. No experimental details were given and no follow-up studies on this strain appeared. More recently, the formation of α-terpineol by *P. digitatum* NRRL 1202 was examined in more detail (Tan and Day 1998a, 1998b; Tan et al. 1998). The proposed involvement of a P-450 monooxygenase rather than a hydratase was supported by three experimental findings; both the iron-chelating agent phenanthroline and a reduced oxygen concentration lowered the rate of α-terpineol formation, while cyanide did not have any inhibitory effect (Tan et al. 1998). The specific activity (less than 0.1 U/g dry weight; Tan et al. 1998), however, is too low for the economical biocatalytical production of α-terpineol. Immobilization in alginate beads as described by Tan and Day (1998b) increased the stability, but did not lead to a significant improvement of the specific activity. Recently, Demyttenaere et al. (2001) tested three different *P. digitatum* strains (DSM 62840, ATCC 201167, and an own isolate) that were all found to transform D-limonene mainly (but not exclusively) to α-terpineol.

The other double bond of limonene (1,2 position) was first found to be attacked by *Cladosporium* sp. T7 yielding *trans*-limonene-1,2-diol (1.5 g/l), and small amounts of the cis-diol (0.2 g/l) in a 4-day fermentation (Mukherjee et al. 1973). A later screening of 800 strains yielded three other fungi, *Diplodia gossypin* ATCC 10936, and two *Corynespora cassiicola* strains (DSM 62474 and DSM 62475), with the same capability (Kieslich et al. 1986). The authors suggested that the limonene-1,2-*trans*-diol is formed by hydrolysis of the initially formed epoxide. The specific activity was not reported, but sufficed for the production of 900 g of the (1S, 2S)-*trans*-diol from D-limonene in a 70-l reactor within 96 h (Abraham et al. 1986b; Kieslich et al. 1986). The same screening of 800 strains also yielded a variety of other less regiospecific fungi converting D-limonene to mixtures of multiple oxidation products at relatively low rates (Kieslich et al. 1986). A systematic screening of more than 60 fungal strains by Demyttenaere et al. (2001) also yielded the formation of 1,2 diols from D- and L-limonene by *Corynespora cassiicola* strains as one of the major results.

Trans-isopiperitenol is a hydroxylated product that has never been reported to be formed by bacterial strains, but was the sole biotransformation product of D-limonene of the black yeast *Hormonema* sp. UOFS Y-0067 at a reasonable, but not quantified, activity (Van Dyk et al. 1998; Van Rensburg et al. 1997). The maximal specific activities obtained with this strain (kind gift of Van Dyk) in our own laboratories were 1-2 U/g dry weight (unpublished results).

Another fully regiospecific hydroxylation was found to be carried out by the basidiomycete *Pleurotus sapidus*, which converts D-limonene to *cis*- and *trans*-carveol, albeit at a low rate. The carveol formed was partially oxidized further to carvone (Onken and Berger 1999). The P-450 systems from *Aspergillus cellulosae* M-77 did not display a high degree of regiospecificity and resulted in the formation of a mixture of isopiperitenone, limonene-1,2 *trans* diol, *cis*-carveol, perillyl alcohol, isopiperitenol, and α-terpineol (Noma et al. 1992) A

Penicillium digitatum strain isolated from overripe oranges converted limonene to *cis*- and *trans*-carveol, carvone, limonene-4-ol, and *cis*- and *trans*-mentha-2,8-dien-1-ol as major products (Bowen 1975). Finally, the honey fungus *Armillareira mellae* was reported to transform D-limonene to α-terpineol and limonene-1,2-diol (Draczynska 1987).

Plant enzymes

As described in the previous sections, the bioconversion of limonene by micro-organisms is often catalyzed by catabolic enzymes, with the main purpose of making limonene available as an energy source. Although there are some examples of micro-organisms that carry out such oxidations with rather high specificity (Table 1), many other micro-organisms oxidize limonene with a low specificity, leading to a large number of unwanted side products (Chang and Oriel 1994; Kieslich et al. 1986; Noma et al. 1992).

Also in plants, catabolic oxidizing enzyme activities are present, for example the cytochrome P-450 enzymes that are involved in the detoxification of xenobiotics such as herbicides, and are implicated in herbicide resistance in a number of plant species (Schuler 1996). A number of studies suggest that the oxidation of these exogenous substrates may be carried out by biosynthetic enzymes, but at much lower efficiency than the endogenous substrates (Schuler 1996). Also for limonene there is an example of non-specific plant enzymes that carry out bioconversions. Both enantiomers of limonene were oxidized in the 6-position to racemic *cis*- and *trans*-carveol and carvone by *Solanum aviculare* and *Dioscorea deltoidea* plant cells (Vanek et al. 1999).

The principle difference between specific, biosynthetic, and non-specific catabolic enzymes implies that plant enzymes may be valuable biocatalysts for a range of bioconversion processes. Many plant species have evolved highly specific biosynthetic pathways for the production of a vast number of terpenoids. These terpenoids apparently have evolved to increase the fitness of the species, e.g., by increasing the resistance against micro-organisms or insects, by improving the attraction of pollinators or seed-dispersing organisms (flower fragrance, fruit flavor), or by improving the fragrance or flavor of commercial products (under breeding selective pressure). Several plant species have evolved biosynthetic pathways geared for the production of limonene and an array of different limonene-derived compounds. These include (if applicable both enantiomers of) the alcohols perillyl alcohol, *cis*- and *trans*-carveol, iso-piperitenol, limonen-10-ol, and their further conversion products such as ketones, aldehydes, and acids (e.g., carvone, perilla aldehyde and acid, and iso-piperitenone and all its derivatives in the menthol pathway), expoxides such as limonene oxide and carvone oxide, and any corresponding esters and glycosides (e.g., carvylacetate, menthylacetate) in such diverse plant species as several *Mentha, Citrus, Cymbopogon, Artemisia* spp, caraway and dill seeds, black currant, wild strawberry, celery, perilla, *Stanhopea pulla, Dracocephalum foetidum*, and *Heracleum canescens* (Chapman and Hall 2002; Seigler 1998). The formation of all these limonene-derived compounds must have been catalyzed by an array of plant enzymes, and there is increasing evidence that the enzymes involved in the first modification of the limonene backbone have a high substrate and product specificity (Bouwmeester et al. 1998; Karp et al. 1990). In all these studies, the first step in the bioconversion sequence is carried out by a cytochrome P-450 enzyme that hydroxylates limonene at the allylic C3, C6, or C7 positions (Fig. 2). C10 hydroxylation has not been reported so far, but can be postulated to occur in *D. foetidum* because this species contains limonen-10-ol and the corresponding aldehyde (Chapman and Hall 2002).

The biosynthetic P-450s that have been characterized to date were shown to have an extremely high regiospecificity, but low substrate enantioselectivity. For example, the spearmint(−)-limonene-6-hydroxylase and the caraway (+)-limonene-6-hydroxylase with their natural enantiomeric substrate exhibited a high product specificity, producing for over 95% the *trans*-isomer (Bouwmeester et al. 1998; Karp et al. 1990). Both enzymes also accepted the other enantiomer as substrate at about 10–25% of the rate with the natural substrate, and both yielded mainly the *cis*- instead of the *trans*-isomer. Also the peppermint C3 and perilla limonene C7 hydroxylases proved to have a low substrate enantioselectivity (Karp et al. 1990). The three (−)-limonene hydroxylases did have a high substrate specificity. Of a whole range of monoterpene hydrocarbons tested, only the 8,9-dihydro-analogues of limonene [(+)- and (−)-*p*-menth-1-ene] were also accepted as a substrate by the two *Mentha* limonene hydroxylases, but not by the perilla C7 hydroxylase.

The studies described above were carried out using only partially purified enzyme preparations. This makes it difficult to obtain definite proof of substrate and regiospecificity. The recent cDNA cloning of a number of plant cytochrome P-450 terpene hydroxylases, including the *Mentha* limonene C3 and C6 hydroxylases (Haudenschild et al. 2000; Lupien et al. 1999), has opened up new possibilities for the study of these enzymes. The *Mentha* P-450s group in the CYP71 family of terpenoid hydroxylases and show the characteristic conserved P-450 domains, such as the haem-binding region FxxGxRxCxG, and conserved regions in the ER targeting signal, the central region of helix I, and the PERF motif. These conserved regions also form the basis for the PCR cloning strategy, for which degenerate primers designed to anneal to these regions are used (Schuler 1996). Interesting studies by Croteau and coworkers with the heterologously produced *Mentha* C3 and C6 hydroxylases shed light on the reaction mechanism and the effect of the absolute configuration of the substrate on the product specificity. They also showed that the regiospecificity is determined by a single amino acid substitution, F363I, that converts the C6 into a C3 hydroxylase (Schalk and Croteau 2000;

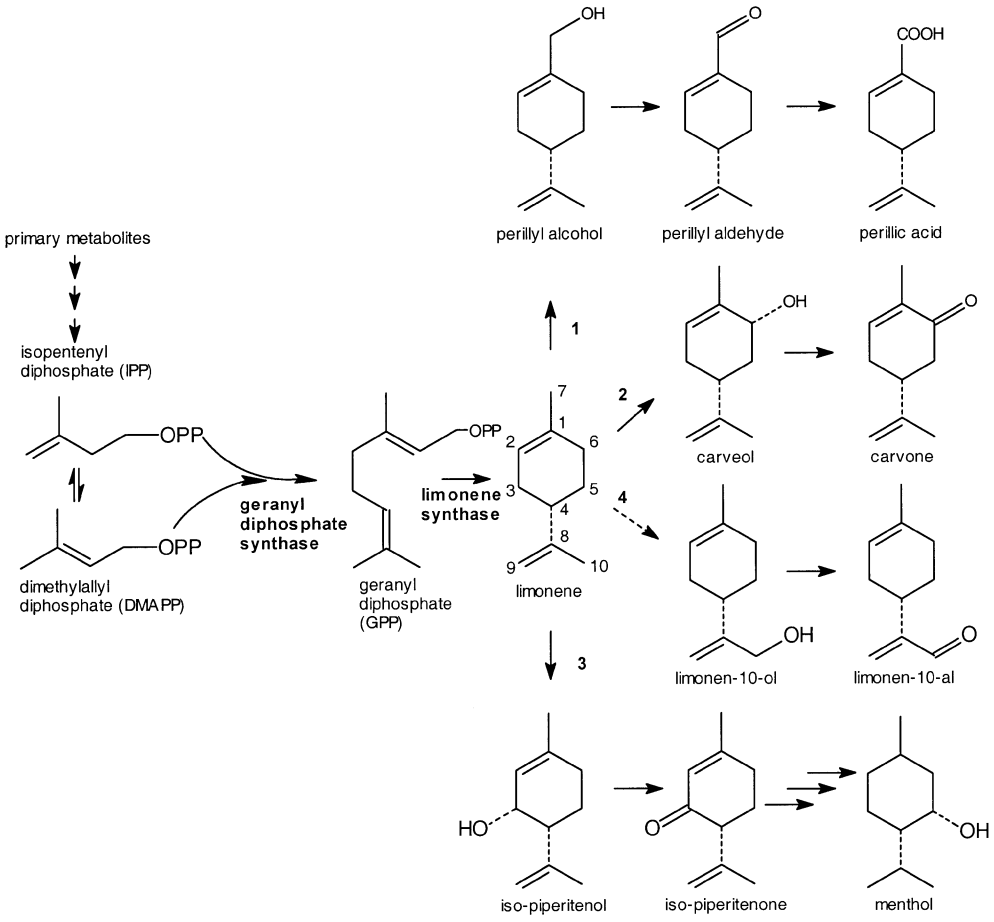


Fig. 2 Biosynthetic pathways in plants starting from limonene, showing the first cytochrome P-450-catalyzed steps and a selection of further modified conversion products. Although these pathways are always stereospecific and mostly the stereochemistry is known, this has been deliberately omitted to indicate that the pathways may occur for both enantiomers. *Solid arrows* indicate proven activities, *broken arrows* indicate putative enzymes. Numbers in *bold* indicate: 1, the perilla (−)-limonene-7-hydroxylase; 2, the caraway (+)- and spearmint (−)-limonene-6-hydroxylase; 3, the peppermint (−)-limonene-3-hydroxylase; 4, the putative limonene-10-hydroxylase. Further conversions are carried out by alcohol and aldehyde dehydrogenases, and reductases

Wüst and Croteau 2002; Wüst et al. 2001). These studies suggest that in the future we may also be able to engineer cloned plant P-450s to change the substrate or product specificity.

The recent advances in the cloning of plant P-450 terpene hydroxylase cDNAs and the high substrate and regiospecificity of these plant enzymes makes them suitable candidates for biocatalysts (De Kraker et al. 2003). In plants this possibility has already been demonstrated (Lücker et al. in preparation). They managed to introduce a catalytically active lemon (+)-limonene synthase and a mint (−)-limonene-3-hydroxylase into tobacco using *Agrobacterium tumefaciens*-mediated transformation. The transgenic tobacco plants produced ample amounts of (+)-*trans*-isopiperitenol. Also, the expression of these cDNAs in micro-organisms is feasible, as yeast and *E. coli* are used to heterologously express and characterize many plant enzymes, including cytochrome P-450s (Haudenschild et al. 2000). The challenge will be to express functional enzymes with high efficiency in micro-organisms suitable for fermentation. If this can be achieved, the high catalytic efficiency and superb regiospecificity of plant enzymes could be combined with the suitability of micro-organisms for industrial production processes, such as fermentation.

Outlook

The past 5 years have seen significant progress in the field of limonene biotransformation, especially with regard to the regiospecificity of microbial biocatalysts. Whereas previously only regiospecific biocatalysts for the 1,2 position (D- and L-limonene-1,2-diol) and the 8-position (α-terpineol) were available, recent reports also describe microbial biocatalysts specifically hydroxylating the 3-position (D-isopiperitenol), 6-position (D-carveol and D-carvone), and 7-position (D- and L- perillyl alcohol, D- and L-perillaaldehyde, and D- and L-perillic acid). The largest remaining biochemical challenge is the search for a hydratase specifically attacking the 8,9 double bond, yielding α-terpineol (instead of the existing oxygen-dependent biocatalysts involving epoxidation as the first step).

Whether these regiospecific hydroxylations can be incorporated into industrial processes depends on (1) the success with which the enzymes involved can be expressed at high levels in safe (preferably food-grade) host strains and (2) the willingness of the fine-chemical industry to invest in expertise and technology for large-scale microbial hydroxylations.

The high-level heterologous expression of bacterial oxygenases is now a rather straightforward exercise,

although the expression levels in artificial host strains are seldom improved significantly in comparison with the original (wild-type) host strain (Duetz et al. 2001c). This may be due to different membrane properties of the artificial host, instability of the electron transfer component, the requirement for a reactivating component, or a shorter half-life (Duetz et al. 2001c). The same problems are encountered – but generally more severely – for eukaryotic (P-450) oxygenases. Other factors such as unfavorable codon usage may be tackled by an adapted codon usage and the co-expression of genes encoding rare codon tRNAs. One of the ultimate challenges for the future is the high-level heterologous expression of the P-450 oxygenases from plants responsible for the natural synthesis of the different enantiomers and stereoisomers of isopiperitenol, piperitenol, carveol, perillyl alcohol, and limonene-10-ol in hosts that allow large-scale production in bioreactors.

In the light of (1) the availability of various oxygenases with suitable regiospecificities, (2) fast developments in the application of oxygenases, and (3) the large price difference between limonene and various oxygenated derivatives, we expect the first large-scale D-limonene bioprocesses to be used in industry within the next decade.

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