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Cloning and Expression of the Limonene Hydroxylase of *Bacillus stearothermophilus* BR388 and Utilization in Two-Phase Limonene Conversions

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Abstract

A 3.6-kb fragment of *Bacillus stearothermophilus* BR388 chromosomal DNA that confers growth on limonene to *Escherichia coli* has been sequenced, revealing a single open reading frame encoding a single subunit limonene hydroxylase containing 444 amino acid residues. This enzyme proved capable of limonene hydroxylation to a mixture of carveol and perillyl alcohol as well as dehydrogenation of these products to carvone and perillyl aldehyde. Oxygen, FAD, and NADH were found to stimulate the hydroxylation reaction in cell extracts, and NAD^+ stimulated the dehydrogenase reaction. In two-phase bioconversions using viable *E. coli* cells overexpressing the limonene hydroxylase, perillyl alcohol and carvone were the principal products observed.

Index Entries: Limonene; carveol; perillyl alcohol; two-phase; *Bacillus stearothermophilus*.

Introduction

Microbial bioconversions of poorly water-soluble substrates can often be enhanced by the presence of a second organic solvent phase containing the substrate, thereby maintaining a high concentration of substrate in the aqueous phase while allowing product removal to the organic phase to avoid product inhibition and provide convenient recovery (for a recent review, *see* ref. *1*). Because of its ready availability and low cost from waste citrus skins *(2)*, we have been investigating the utility of the monoterpene limonene as a neat organic phase for the production of higher value specialty monoterpenes. In an earlier publication, we described the isolation of a

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Bacillus thermophile capable of limonene utilization as a sole carbon source, exhibiting production of perillyl alcohol, α-terpineol, and other metabolites of interest *(3)*. It was subsequently found that growth on limonene could be conferred to *Escherichia coli* by introduction and expression of a 3.6-kb chromosomal DNA insert from BR388 *(4)*. In this article, we demonstrate that growth on limonene by the *E. coli* recombinant is conferred by a single gene encoding a novel limonene hydroxylase that oxidizes limonene at two allylic sites, producing carveol and perillyl alcohol together with subsequent conversion to carvone and perillyl alcohol. We describe initial trials utilizing an *E. coli* recombinant overexpressing this enzyme to produce oxidized monoterpenes in a two-phase biotransformation.

Materials and Methods

Chemicals and Reagents

R-(+)-limonene, R-(+)-perillyl alcohol, and (R)-(+) perillic acid were purchased from Aldrich, Milwaukee, WI. (R)-(+) perillyl aldehyde was obtained from Nippon Terpene, Japan, Tokyo. $H_2{}^{18}O$ was purchased from Isotec, Miamisburg, OH. NAD, NADH, NADP, NADPH, FAD, isopropyl-β-D-thiogalactopyranoside (IPTG), antibiotics, lysozyme, Tris buffer, EDTA, and bicine were purchased from Sigma, St. Louis, MO. Agarose and all restriction enzymes were purchased from Boehringer-Mannheim, Indianapolis, IN. The Erase-a-Base system was purchased from Promega, Madison, WI.

Bacterial Strains and Plasmids

E. coli DH5α (F⁻ ϕF80d *lacZ*ΔM15Δ[*lacZYA-argF*] U169 *endA1 hsdR*17[rκ mκ] *deoR recA*1 *supE*44λ *thi*-1 *gyrA*96 *relA*1) from Gibco-BRL and *E. coli* BL21 (DE3) (F⁻ dem ompT hsdS [rB- mB-] gal λ[DE3]) from Novagene were used in this study. Plasmids pBluescript [SK+] from Stratagene (La Jolla, CA) pET-30a from Novagen (Madison, WI) and pKK233-2 from Amersham Pharmacia (Piscataway, NJ) were utilized.

Growth of Microorganisms

E. coli was grown at 37°C on Luria-Bertani (LB) broth (1% bactotryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.2). Antibiotics were used at the following concentrations: ampicillin (100 μg/mL) and kanamycin (20 μg/mL).

For experiments testing growth on limonene as a sole carbon source, cells of EC435, whose construction is described in Fig. 2, were inoculated in M9 salts solution *(5)* containing kanamycin and 0.15% limonene at 37°C with shaking. Samples were taken at 6-h intervals and colony-forming units were determined on LB agar plates containing kanamycin. *E. coli* BL21 (DE3) carrying pET-30a vector with no insert was used as a negative growth control.

Nucleic Acid Extraction and Subcloning

Plasmid DNA was prepared by an alkaline lysis method *(5)* or a Qiagen (Santa Clarita, CA) DNA preparation kit. DNA fragments for subcloning

utilized the Qiagen DNA extraction kit or electroelution of bands following electrophoresis on 6% agarose gels. For subcloning of the limonene hydroxylase gene, an *Nco*I site was created using polymerase chain reaction at the initiation site of the open reading frame (ORF) of the 3.6-kb *Hind*III fragment of *Bacillus stearothermophilus* thermophile BR388 that had been previously cloned into the overexpression vector pKK223-2 and designated EC418 *(4)*. The 2.2-kb *Nco*I-*Hind*III fragment was ligated with pET-30a vector DNA digested with *Nco*I and *Hind*III. The ligation mixture was transformed into *E. coli* BL21 (DE3) and designated EC435.

DNA Sequencing

Plasmids with progressive unidirectional deletions were constructed by exonuclease III reaction of the DNA insert of EC418 using the Promega Erase-a-Base system. Double-stranded plasmid DNA prepared on large scale by the alkaline lysis method *(5)* were sequenced by the Michigan State University sequencing facility using the nucleotide chain termination method with Sequenase 3.0, from United States Biochemical, Cleveland, OH. Internal primers utilizing determined sequence were constructed using the GCG program (Genetics Computer Group, Madison, WI), allowing sequence determination of both insert strands. Sequences were also analyzed with the GCG program.

Analytical Methods

Chemical standards, substrates, and reaction products were analyzed with gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography. The GC-MS system was a mass spectrometer HP5970 coupled with a gas chromatograph HP5890 (Hewlett Packard, Palo Alto, CA). The mass selective detector was an MSD HP5970 (Hewlett Packard). A 0.25 mm id × 15 m DB-wax fused silica capillary column was used for monoterpene separations. Conditions were as follows: 1-mL injection; He carrier gas; injection port and detector port at 240°C; column temperature programmed from 40 to 240°C at 7°C/min with a 2-min initial hold time.

Limonene Degradation with Resuspended Whole Cells

EC435 cells from 250 mL of culture grown in LB medium with kanamycin and 0.2% limonene, induced by IPTG at OD_{600} of 0.8, and incubated for another 2 h, were harvested and resuspended in 10 mL of M9 salt solution supplied with 0.4% glycerol and kanamycin. The biphasic reaction mixture containing 5 mL of the resuspended cells and 2 mL of limonene, and 13 mL of M9 salt solution containing 0.4% glycerol and kanamycin were incubated in a 100-mL serum bottle with Teflon-coated stoppers at 45°C using a New Brunswick (Edison, NJ) G76 water bath with shaking at 200 rpm. After centrifugation of reaction mixture samples at 14,000*g* for 10 min, the organic phase was analyzed directly using GC-MS.

Limonene Hydroxylase Assay

Two milliliters of EC435 grown in LB medium containing kanamycin was harvested, washed with 2 mL of M9 salts, and inoculated into 250 mL of M9 minimum salt solution supplied with 0.4% glycerol, 0.05% glucose, 0.2% limonene, and kanamycin. The cells were grown at 37°C to reach OD_{600} of 0.8, supplied with IPTG at a final concentration of 1 m*M*, and allowed to grow for another 2 h. The cells were harvested, washed, resuspended in 10 mL of M9 salt solution, and sonicated using a Cole-Palmer (Vernon Hills, IL) 4710 ultrasonic homogenizer at 4°C for 7 min using 1-min time intervals and cooling on ice. The disrupted cells were centrifuged at 17,000*g* for 30 min. The supernatant was supplied with phenylmethylsulfonyl fluoride (PMSF) (3 m*M*) and used as a crude enzyme extract. The reaction mixture, which contained 500 μL of 0.2% limonene in 100 m*M* Tris-HCl buffer (pH 8.0), 1 m*M* FAD, 1 m*M* NADH, 0.3 m*M* PMSF, 100 μL of water, and 400 μL of crude enzyme solution, was incubated at 45°C for 1 h. Ethyl ether (0.5 mL) was added to the reaction mixture and mixed thoroughly. After standing to allow phase separation, the ether phase was reserved and combined with the ether phase from a second extraction. The combined ether phase was concentrated to 50 μL using nitrogen gas and was analyzed for oxidized monoterpenes using GC-MS with reference to monoterpene standards.

Alcohol Dehydrogenase Assay

For the assay of alcohol dehydrogenase activity, a reaction mixture containing 500 μL of 0.1% perillyl or other alcohol in 100 m*M* bicine-NaOH buffer (pH 9.0), 10 μL of 100 m*M* NAD^+, 400 μL of crude enzyme solution, and 100 μL of water was incubated at 40°C for 30 min. The amount of NADH produced was measured spectrophotometrically at 340 nm. Controls included samples with no cell extract, and extract from *E. coli* BL21(DE3) containing plasmid vector pET-30a with no insert.

Limonene Hydroxylation In Vivo Using $H_2{}^{18}O$

EC435 culture (250 mL) was prepared as described above, harvested, washed with 10 mL of M9 salt solution, separated into two equal samples, and centrifuged. One sample was resuspended in 2 mL of M9 salt solution containing 1.9 mL of distilled water and 0.1 mL of M9 salt solution (10X concentrated), and the other was resuspended similarly, but using 1.9 mL of $H_2{}^{18}O$ (89% isomeric purity). Each reaction mixture was amended with 100 μL of limonene, 80 mg of glycerol, and kanamycin and incubated at 45°C for 72 h using a New Brunswick G76 water bath with shaking at 200 rpm. After centrifugation of the reaction mixture at 9600*g* for 10 min, the limonene phase was analyzed using GC-MS for alteration in mass number of the perillyl alcohol produced. *E. coli* BL21(DE3) containing plasmid vector pET-30a and a reaction mixture without cells was used as a negative control.

Limonene Hydroxylation In Vitro Under Anoxic Conditions

Crude enzyme solution was prepared as described above except that cells were resuspended in 100 m*M* Tris-HCl buffer (pH 8.0) before sonication. Three 10-mL samples of reaction mixture containing 7.6 μL of crude enzyme extract (3.1 mg protein/mL), 1 m*M* each of NADH and FAD, 100 μL of limonene, and 100 m*M* Tris-HCl buffer (pH 8.0) were prepared. Nitrogen gas was bubbled through the reaction mixture for various times prior to enzyme assay. A control without cells was used to determine that limonene loss was negligible under the conditions used, and *E. coli* BL21(DE3) containing plasmid vector pET-30a was used as a negative control.

Determination of Protein Concentration

Protein concentration in enzyme extracts was determined using the Coomassie blue protein assay *(6)*. The assay was determined to be linear using bovine serum albumin as the standard.

Results and Discussion

Sequence and Initial Characterization of Limonene Hydroxylase

Sequencing of the 3.6-kb insert in EC418 that conferred growth on limonene and production of oxidized limonene metabolites revealed the existence of a single reading frame (Fig. 1). Although purified enzyme is not yet available to unambiguously determine the start codon, a sequence resembling a Shine-Dalgarno sequence in the appropriate position and complementary with the *B. stearothermophilus* 3' terminus of the 16S RNA gene *(7)* suggests the protein start at the position shown. To verify that this ORF encoded the limonene monooxygenase activity, a 2.2-kb sequence spanning the ORF was subcloned into the vector pET-30a utilizing the existing *Hind*III site near the terminus and an *Nco*I site introduced at the N-terminus to create a recombinant designated EC435 (Fig. 2). Growth on limonene as a sole carbon and energy source by EC435 is shown in Fig. 3, in which growth is followed by a sharp decrease in viability after 24 h, which we attribute to formation of toxic limonene metabolites (see below). No growth on limonene or decrease in inoculum viability was observed under the same conditions in the absence of the limonene hydroxylase gene (data not shown). Comparison of the limonene hydroxylase sequence with the Genbank library did not identify sequences with significant sequence similarity. Because oxidoreductases containing a single subunit are usually flavoproteins *(8)*, the enzyme sequence was examined for FAD fingerprints. As shown in Fig. 4, a sequence was identified with similarity to the concensus sequence encoding an FAD-binding region deduced by Eggink et al. *(9)*. Although the limonene hydroxylase encodes 9 rather than 11 amino acids and no aromatic residues, the other key characteristics of the concensus sequence are present.

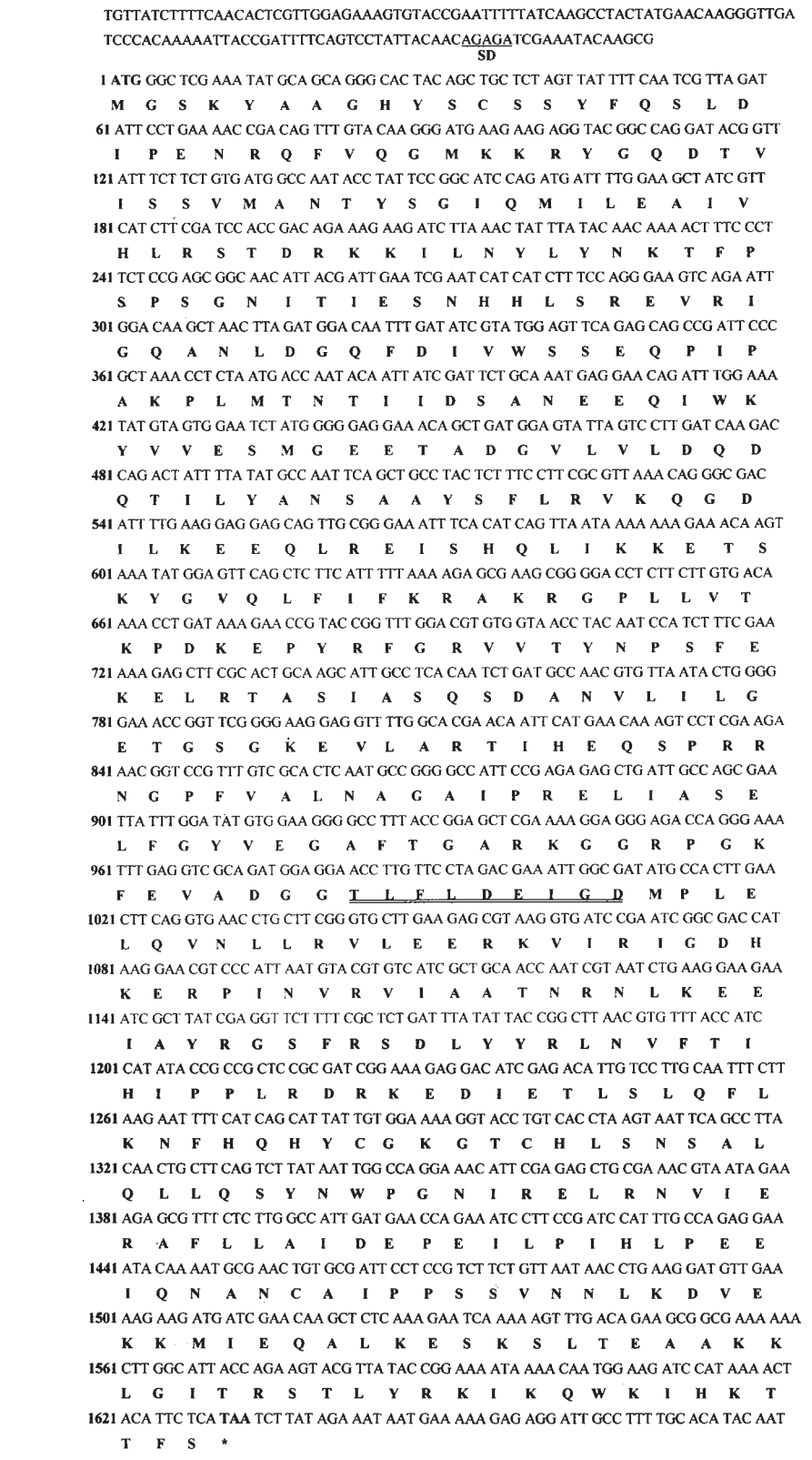


Fig. 1. Nucleotide sequence and deduced amino acid sequence of BR388 limonene hydroxylase.

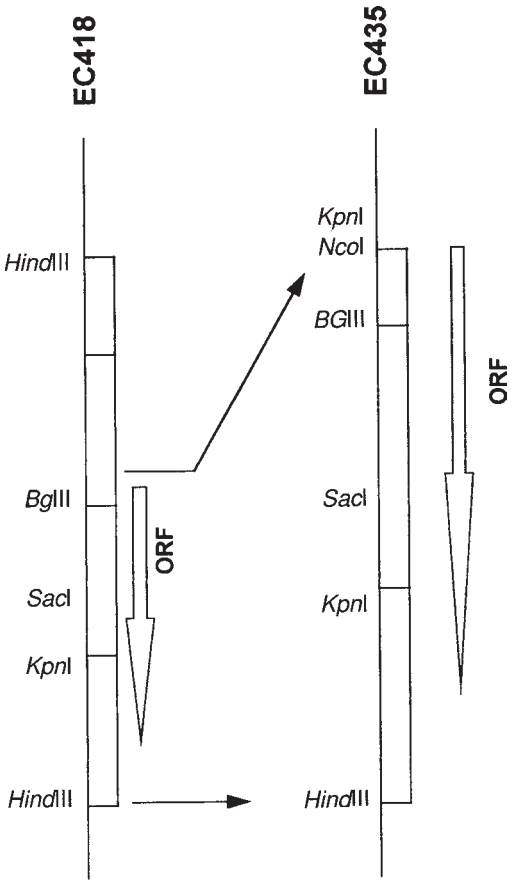


Fig. 2. Construction of EC435.

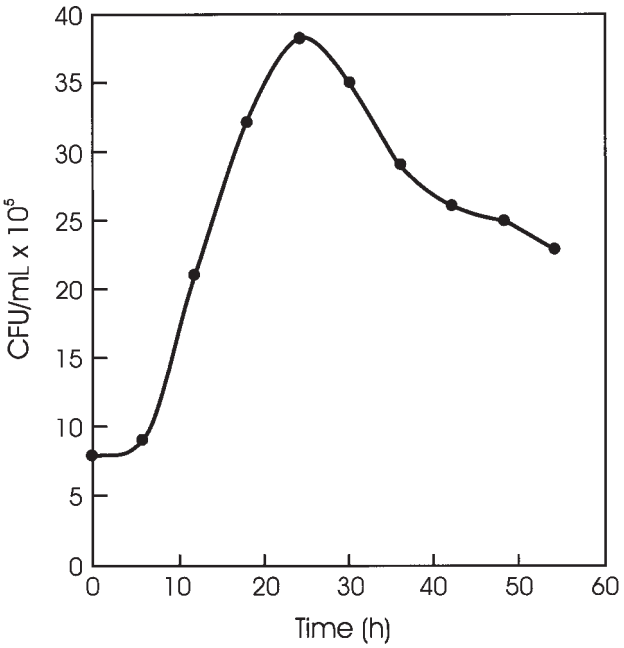


Fig. 3. Growth of EC435 on limonene. CFU, colony-forming units.

FAD-binding concensus sequence	T X X X X I Y I I G D
	V W V V
	A F A A
	L L
Limonene monooxygenase	T L F - - L D E I G D

Fig. 4. Putative FAD-binding sequence of BR388 limonene hydroxylase. Concensus sequence from Eggink et al. *(9)*.

Table 1
Stimulation of Limonene Hydroxylase Activity in Cell Extracts

	Perillyl alcohol formed (n*M*/min × mg protein)	
	EC435	Vector pET-30a (no insert)[a]
FAD (1 m*M*)	0.169	ND
NADH (1 m*M*)	0.158	ND
FAD + NADH (1 m*M* each)	0.960	ND

[a]ND, not detected.

Limonene Hydroxylation Using Crude EC435 Enzyme Extracts

Hydroxylation of limonene with crude enzyme extracts of EC435 in the presence of various cofactors was tested. Slight stimulation of hydroxylation was noted with the addition of NADH and FAD as cofactors, with significant stimulation in the presence of both cofactors, although absolute reaction rates were low (Table 1). In this reaction, perillyl alcohol was observed as the major product, although smaller amounts of carveol were also formed. Optimal hydroxylation rates in the presence of combined FAD and NADH were at 45°C and pH 7.7 (Fig. 5). When reactions were carried out in an anoxic state produced by nitrogen sparging of the reaction mixture, significant rate reductions were observed (Table 2), which together with the NADH stimulation support the designation of the enzyme as a monooxygenase.

Dehydrogenase Activity of Limonene Hydroxylase

In addition to hydroxylation of limonene, the limonene hydroxylase produced by EC435 proved capable of oxidation of perillyl alcohol to perillyl aldehyde utilizing NAD^+ as cofactor. The pH and temperature optimum for this reaction was similar to that of hydroxylation (data not

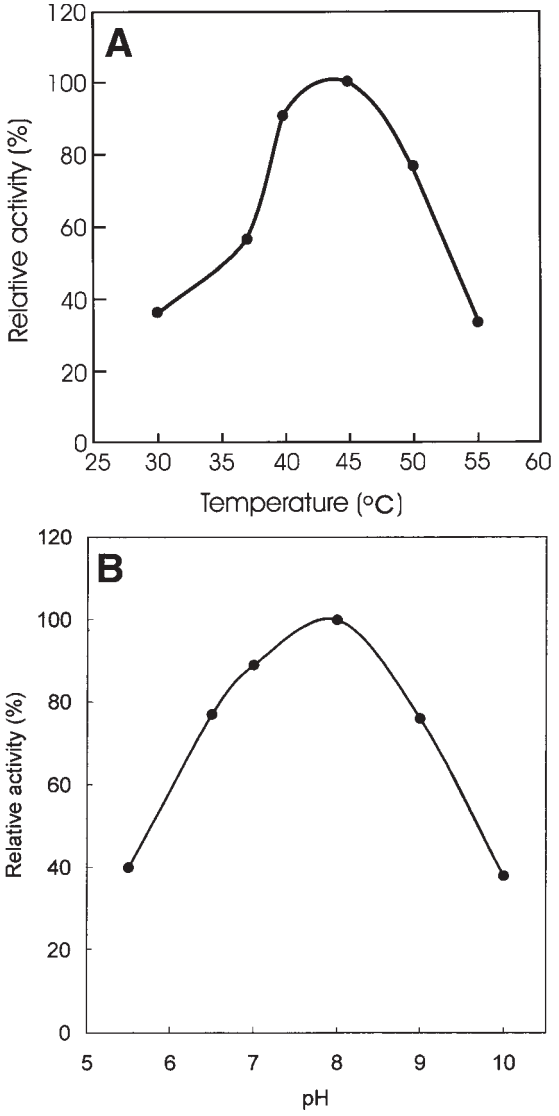


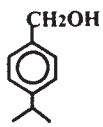
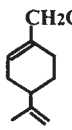
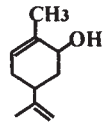
Fig. 5. **(A)** Temperature and **(B)** pH hydroxylation profiles of BR388 limonene hydroxylase.

Table 2
Limonene Hydroxylase Activity Using Anoxic Cell Extracts

N_2 sparging time (min)	Perillyl alcohol formed (n*M*/min × mg protein)	
	EC435[a]	Vector pET-30a (no insert)[b]
0	0.88 (100)	ND
10	0.47 (52)	ND
30	0.274 (30)	ND

[a]Numbers in parentheses are relative activities (%).
[b]ND, not detected.

Table 3
Relative Rates of Alcohol Dehydrogenase Activity with Varied Substrates

Substrate	 Benzyl alcohol	 Myrtenol	 Cyclohexyl methanol	 4-Isopropyl benzyl alcohol
Activity*	0.049 (3.1)	0.064 (4.0)	0.253 (15.8)	0.674 (42.2)
Substrate	 4-Methyl benzyl alcohol	 3-Cyclohexene 1-methanol	 Perillyl alcohol	 Carveol
Activity*	0.273 (17.1)	0.154 (9.6)	1.596 (100)	1.368 (86)

*, nmol NADH/(mg protein · min). Numbers in parentheses are relative enzyme activities (%).

shown). Table 3 presents the dehydrogenase activity of the hydroxylase with varied alcohol substrates. Although perillyl alcohol gave the highest activity, that with carveol was almost as high, and aromatic alcohols also served as substrates at reduced rates. For either aromatic or cyclohexene substrates, the highest activities were observed for those containing an isopropyl or isopropenyl group at ring position 4.

In Vivo Monoterpene Oxidation

To examine accumulation of extracellular monoterpene metabolites by the EC435 recombinant, cells grown in LB medium and induced with IPTG were employed in two-phase incubations utilizing limonene as a neat organic solvent phase. GC-MS analysis of the limonene phase indicated perillyl alcohol as the principal metabolite, with carvone also evident, together with traces of perillyl aldehyde and carveol (Fig. 6). No detectable limonene oxidation products were observed in the aqueous phase, or when using a recombinant in which a segment of the limonene hydroxylase gene between the vector and insert *Kpn*I sites was deleted (data not shown). Similar experiments with limonene hydroxylase expressed in the recombinant EC418 yielded similar results, although with lower concentrations of perillyl alcohol and carvone (Fig. 6). To provide confirmation regarding molecular oxygen as the oxygen source in the oxidized limonene metabo-

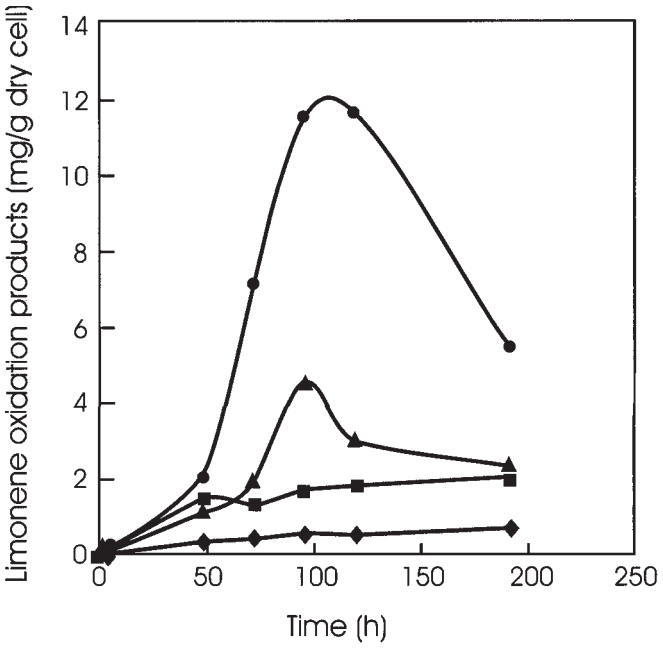


Fig. 6. Perillyl alcohol and carvone formation during two-phase incubations with resuspended cells of EC435 or EC418. (●), perillyl alcohol from EC435; (▲), carvone from EC435; (■), perillyl alcohol from EC418; (◆), carvone from EC418.

lites, a similar two-phase incubation was carried out using ^{18}O-enriched water in the aqueous phase. GC-MS analysis of the perillyl alcohol product did not reveal a shift in product mass number (data not shown), indicating that the oxygen inserted in limonene originated from atmospheric oxygen rather than water, confirming the conclusion obtained from cell extracts.

Conclusion

The limonene hydroxylase described in this study confers growth on limonene by oxidation to perillyl alcohol and perillyl aldehyde, with subsequent catabolism provided by the *E. coli* host *(3)*. Alternative oxidation at the allylic ring carbon 6 provides carveol and carvone as additional but dead-end metabolites. Carvone has biotechnological value as a food flavoring *(10)*, and perillyl alcohol has been demonstrated to possess potent antitumor activity *(11)*. Previous findings of multiple metabolites during microbial limonene degradation have been attributed to the existence of multiple metabolic pathways *(12)*. For BR388, the production of four oxidized metabolites by the limonene hydroxylase (Fig. 7) indicates that multiple metabolite formation in this case results from the unusual enzymatic characteristics of the limonene hydroxylase.

Although biochemical examinations with purified enzyme are needed to elucidate the role of cofactors in the initial limonene oxidation step, it seems likely from the NADH and oxygen requirements, as well as the

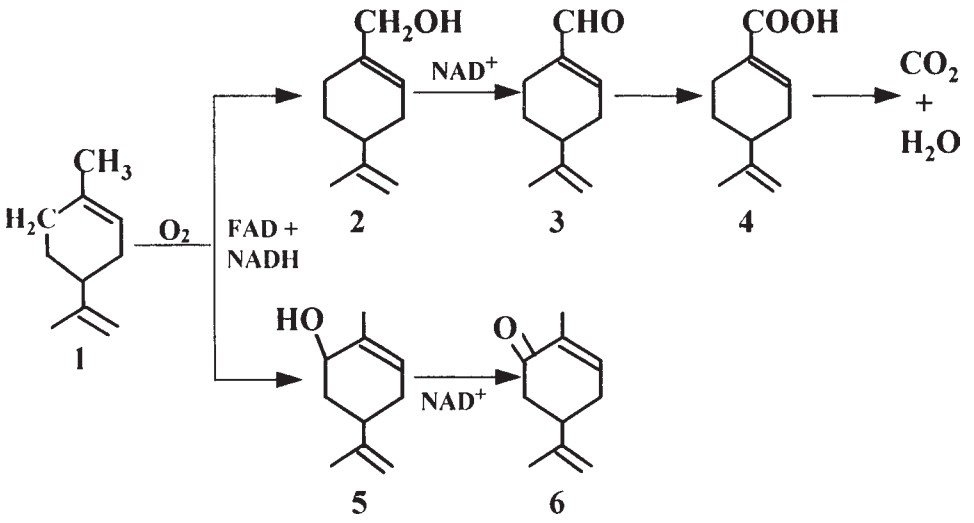


Fig. 7. Limonene metabolism by EC435. 1, limonene; 2, perillyl alcohol; 3, perillyl aldehyde; 4, perillic acid; 5, carveol; 6, carvone.

putative FAD-binding signature, that the enzyme is a monooxygenase. Furthermore, the unique primary sequence and preference for monoterpenoid-type substrates suggests that the enzyme is a member of a new monooxygenase family.

The utilization of recombinant EC435 in a two-phase biotransformation proved successful in providing accumulation of carvone and perillyl alcohol in the organic limonene phase. However, experiments utilizing growth on limonene as a sole carbon source, and the ability of untransformed *E. coli* to grow readily in M9-glycerol broth containing a second limonene phase (data not shown), suggest that at least one of the limonene metabolites is toxic to the cells. Efforts to identify the toxic metabolite and to optimize carvone and perillyl alcohol production using the two-phase system are under way.

Acknowledgment

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