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Production of α-Terpineol from *Escherichia coli* Cells Expressing Thermostable Limonene Hydratase

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ABSTRACT

The genes encoding a thermostable limonene hydratase have been located on a cloned fragment in *Escherichia coli* conferring growth on limonene and production of the monoterpenes perillyl alcohol and α-terpineol. Whole cell bioconversion studies at elevated temperature employing both an aqueous phase and neat limonene phase demonstrated significant production of α-terpineol with additional production of carvone.

Index Entries: Limonene; α-terpineol; thermostable; two-phase.

INTRODUCTION

Monoterpenes constitute a diverse group of C10-based plant secondary metabolites produced in part for defense against microbes and insects. Because of their unique organoleptic properties, certain monoterpenes utilized in fragrances and as food ingredients command some of the highest unit values among biotechnological products *(1)*. Microbial conversion of low value monoterpenes to higher value derivatives has been recognized for some time as an attractive opportunity, but has been thwarted by the lack of knowledge of microbial monoterpene pathways leading to a multiplicity of monoterpene metabolites (for review, *see* ref. 2). Because of its low cost and extensive availability as a waste citrus product *(3)*, the monoterpene R-(+)-limonene has been selected as a target for directed microbial bioconversions. To help avoid problems arising from

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the microbial toxicity of this monoterpene, eubacterial thermophiles were targeted for investigation anticipating that their robust enzymes and growth ability in conditions favoring monoterpene volatilization might provide advantages in bioprocessing applications. In previous studies, a *Bacillus stearothermophilus* strain BR388 was isolated, which proved resistant to limonene toxicity, and which demonstrated production of perillyl alcohol and α-terpineol during growth on limonene *(4)*. The former compound has value as a flavorant *(2)*, whereas the latter is extensively utilized in perfume manufacture *(5)*. In order to study and control the pathway metabolites, the entire pathway was cloned into *Escherichia coli* as a 9.6-kb plasmid insert, conferring to the new host growth on limonene as a sole carbon source and production of perillyl alcohol and α-terpineol *(6)*. It was proposed that limonene degradation in both the thermophile and recombinant proceeded by oxidation of the C-1 methyl to perillic acid with further breakdown utilizing the β fatty acid pathway, whereas α-terpineol was formed as a hydratase-catalyzed reversible side product (Fig. 1). In this paper, information is presented on the cloned hydratase and the hydratase-catalyzed formation of α-terpineol and other products is examined in a two-phase bioreactor at elevated temperature.

MATERIALS AND METHODS

Growth of Microorganisms

E. coli recombinants carrying *B. stearothermophilus* inserts were grown in M9 salts medium containing either yeast extract or the selected monoterpene. M9 minimal medium *(7)* contains per liter: Na_2HPO_4, 6g; KH_2PO_4, 3g; NaCl, 0.5g; NH_4Cl, 1g; pH7.4. After autoclaving and cooling, 2 mL of 1*M* $MgSO_4$ and 0.1 mL of 1*M* $CaCl_2$ were added. Growth was carried out using 20 mL culture volumes in 40 mL serum bottles closed with Teflon-coated butyl stoppers and aluminum caps.

Limonene Hydratase Assay

Recombinant *E. coli* were grown in 50 mL LB medium containing 50 μg/mL ampicillin at 37°C overnight. Cells were centrifuged and resuspended in 5 mL of 50 m*M* sodium phosphate buffer, pH 7.0, and disrupted by 30-s bursts of sonication with cooling on ice for 3–5 min. The crude enzyme extract resulting from centrifugation at 35,000*g* for 30 min was assayed in a procedure adapted from Nagasawa et al. *(8)* using a 2 mL reaction mixture containing 1 m*M* 3-cyanopyridine in 50 m*M* phosphate buffer, pH 7.0. The reaction mixture was incubated at 55°C for 20 min and stopped by addition of 0.2 mL 1*M* HCl. Nicotinamide product was determined using HPLC analysis (Waters HP1050 with 3.9 mm × 15 cm NOVA C-18 column (Waters, Inc.) Peaks eluted using 60% acetonitrile in 5 m*M*

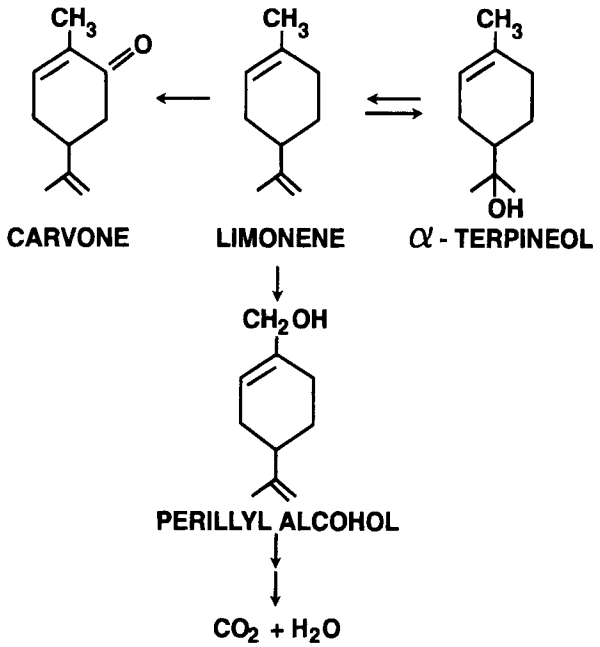


Fig. 1. Proposed pathway for limonene degradation and principal metabolic products of *E. coli* recombinants containing cloned DNA fragments from *B. stearothermophilus* BR388.

sodium phosphate buffer, pH 7.7, were determined at 230 nm. Peaks were identified and quantified using known standards. One unit of activity is defined as the amount of enzyme that catalyzes formation of 1 μ*M*/min nicotinamide under the specified conditions.

GC/MS Product Analysis

Aqueous culture supernants were acidified to pH 2.0 with HCl, extracted three times with ether, evaporated, and analyzed using GC/MS equipment (Hewlett Packard model 5890) and procedures described previously *(4)*. Samples of the limonene phase from bioreactor studies were injected directly.

Subcloning of *E. coli* EC409A Insert

The limonene pathway encoded in the 9.6 kb insert of EC409A was subcloned utilizing HindIII digestion, separation of the 8.2, 3.8, and 0.6 kb fragments by agarose gel electrophoresis, fragment recovery using electroelution, ligation into the HindIII site of pBluescript (SK+), and transformation into *E. coli* DH5α using standard procedures *(7)*. Similar procedures were employed to obtain subcloned fragments utilizing other restriction sites.

Two-Phase Bioreactor Studies

To examine formation of α-terpineol in a whole-cell two phase bioreactor, 5 mL of neat limonene and 50 mL of EC423 cell suspensions (10^9 cells/mL) in M9 salts were shaken in a gyrotory water bath at 250 rpm in 125 mL screw cap bottles at varied temperatures. Samples of aqueous and limonene phases were taken for GC/MS analysis after various periods of incubation of the cell suspension.

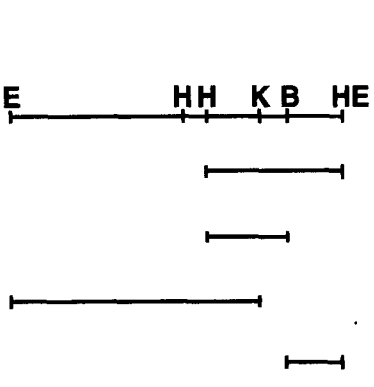
RESULTS

Subcloning of the Limonene Hydratase Gene

Earlier work indicating that both α-terpineol and perillyl derivatives were metabolic products formed during growth of the recombinant *E. coli* EC409A on limonene suggested that both limonene hydratase and methyl oxidase activities were present on the cloned insert *(6)*. In an effort to locate the genes encoding these activities, the transformant was subcloned into 8.2, 3.8, and 0.6 kb HindIII fragments. The transformant EC418 containing the 3.8 kb Hind III insert proved able to grow on limonene (Fig. 2), indicating that genes facilitating limonene catabolism in *E. coli* are retained on this fragment. GC/MS analysis of cell supernatants indicated that perillyl alcohol, α-terpineol, and lesser amounts of carveol were produced, suggesting that both methyl oxidase and hydratase activities were encoded (Table 1). The limonene hydratase gene was further subcloned as a 1.6-kb HindIII-Bg1II fragment as EC419 that expressed hydratase activity, but did not confer growth on limonene nor produce perillyl derivatives. Transformant EC421 carrying the adjacent 2.2-kb Bg1II-Hind III fragment, demonstrated production of perillyl alcohol and growth on limonene, indicating the presence of the gene(s) encoding limonene methyl oxidation. Surprisingly, limonene hydratase activity was also found with transformant EC423, indicating the presence of two distinct limonene hydratase genes in EC409A.

Preliminary Characterization of the Limonene Hydratase

The limonene hydratase in crude extracts demonstrated broad substrate specificity, in that in addition to hydration of limonene, the nitrile group of cyanopyridine could also be hydrated. Since the latter substrate is more soluble and less volatile than limonene, hydration of cyanopyridine to nicotinamide was utilized for standard assay using a published HPLC procedure *(8)*. It was found that the hydratase enzyme expressed from EC419 was thermally unstable, demonstrating no enzymatic activity above 40°C whereas the hydratase expressed by EC423 exhibited an optimum temperature near 55°C which is close to the optimum growth temperature of the thermophile parent (data not shown). During preliminary



Designation	Major products	Limonene growth
EC 409A (9.6)	T, PA	+
EC 418 (3.8)	T, PA	+
EC 421 (2.2)	PA	+
EC 423 (7.3)	T, C	-
EC 419 (1.6)	T	-

Fig. 2. Subcloning of the BR388 limonene degradation pathway. Product designations: T, α-terpineol; PA, perillyl alcohol. Enzyme designations: E, EcoRI; H, HindIII; K, KpnI; B, BglII. (+) Denotes ability to grow on limonene as sole carbon and energy source. Parentheses designate insert size.

Table 1
Monoterpene Products Produced by Recombinant EC418 During Growth on Limonene

	Metabolites and concentration (mg/l*)		
Growth stage	α-terpineol	Carveol	Perillyl alcohol
Early log phase (6 h)	0.7	2.6	0.7
Late log phase (24 h)	1.1	1.7	2.5
Stationary phase (48 h)	29	0.9	72

attempts at purification, the EC423 enzyme could be pelleted by prolonged ultracentrifugation, suggesting that the enzyme may be membrane bound as previously reported for the limonene hydratase of *Pseudomonas gladioli (9)*.

Whole Cell Bioreactor Studies

An attempt was made to utilize whole cells of *E. coli* EC423 in a two-phase bioreactor at elevated temperature as shown in Fig. 3. Neat limonene was utilized as the organic phase to maintain a saturated level of substrate and to facilitate product removal from the aqueous phase. Both actions serve to prevent dehydration of the α-terpineol product in this reversible reaction. As seen in Table 2, significant accumulation of α-terpineol was

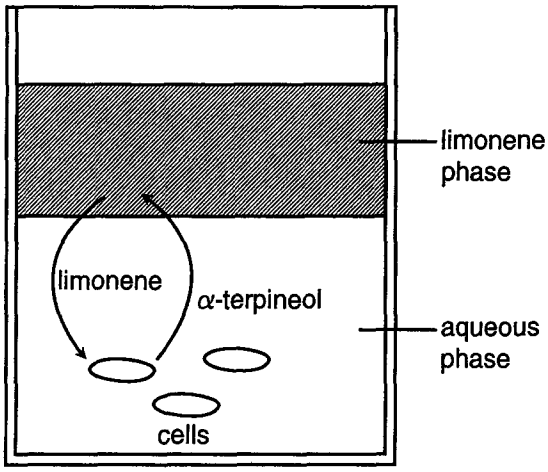


Fig. 3. Cartoon of two-phase whole-cell bioreactor utilizing neat limonene.

Table 2
Monoterpene Products Produced by Recombinant EC423 Utilizing Neat Limonene in a Two-Phase Bioreactor

Product	Temperature	Concentration (mg/l) Incubation Time (h) 24	48	72
α - terpineol	40° C	16	117	215
	50° C	19	168	235
	60° C	16	201	209
Carvone	40° C	3.9	20	23
	50° C	8	21	35
	60° C	17	19	28

Concentrations of product determined in the limonene phase are expressed per liter of aqueous cell suspension.

achieved with this simple bioreactor system. Surprisingly, the monoterpene carvone was also found in lesser, but significant amounts. Although minor amounts of carveol were produced during limonene utilization by EC418, production of carvone was not observed with either the parental thermophile or the recombinant EC409A containing the same DNA as part of a larger fragment. For both monoterpene products, optimal production occurred at 50°C, with carvone appearing at earlier times.

DISCUSSION

Cloning of the limonene degradative pathway provides an opportunity for separate examination and utilization of the conversion steps for production of valuable monoterpene metabolites. In this report, we have separated the limonene hydration and methyl oxidation steps, and have obtained additional evidence suggesting that the latter step participates in limonene utilization by the recombinant.

Although formation of carvone has been reported previously for other limonene-degrading bacteria (*2*), formation of this metabolite was not observed during studies of the parental thermophile, and was, therefore, unexpected. From subcloning studies, the gene encoding this ring oxidation activity appears to be distinct separate from that oxidizing the C-1 limonene methyl group. Since carvone is utilized as an important food flavoring (*5*), the enzyme and the encoding gene(s) merit further investigation.

To the authors' knowledge, this is the first attempt to utilize whole cells with thermostable enzymes in a two-phase bioreactor system at elevated temperature. Whereas significant further work is required for optimization and scaleup, the provision of excess monoterpene substrate and removal of reactive product facilitated by the separate phase exhibit significant promise for potential production of the specialty chemicals α-terpineol and carvone using elevated temperature. The introduction of thermostable enzymes catalyzing biotransformation at elevated temperature into a mesophilic bacterial host may also help to eliminate undesired side reactions catalyzed by host enzymes while retaining advantages of whole cell utilization.

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REFERENCES

1. Welsh, F. W., Murray, W. D., and Williams, R. E. (1989), *Crit. Rev. Biotechnol.* **9,** 105–169.
2. Krasnobajew, V. (1984), in *Biotechnology*, Vol. 6a. Lieslich, K. ed., Verlag Chemie, Weinheim.
3. Bowen, E. R. (1975), *Proc. Fla. State Hort. Soc.* **88,** 305–308.
4. Chang, H. C. and Oriel, P. (1994), *J. Food Sci.* **59,** 660–662.
5. Bauer, K., Garbe, D., and Surburg, H. (1990), *Common fragrance and flavor materials*, VCH, Weiheim.
6. Chang, H. C., Gage, D., and Oriel, P. (1995), *J. Food Sci.* **60,** 551, 552.

7. Maniatis, T., Fritsch E. F., and Sambrook, F. (1982), *Molecular Cloning*, Cold Spring Harbor Laboratory, New York.
8. Nagasawa, T., Mathew, C. D., Mauger, J., and Yamada, H. (1988), *Appl. Environ. Microbiol.* **54**, 1766–1769.
9. Cadwallader, K. R. and Braddock, R. J. (1992), in *Food Science and Human Nutrition* Charalambous, G., ed., Elsevier, New York, pp. 571–583.