POTENTIAL BY-PRODUCTS FROM MICROBIAL TRANSFORMATION OF D-LIMONENE

ELIZABETH R. BOWEN

U.S. Citrus and Subtropical Products Laboratory[1]
Winter Haven, Florida

Abstract. **Two common citrus molds, Penicillium italicum and P. digitatum, were used to transform d-limonene, the major component of citrus oils, to compounds with potentially greater commercial value. Fermentations were carried out in shake flask cultures, and gas-liquid chromatography and infrared spectrometry were used for the identification and quantitation of conversion products. Major transformation products were p-mentha-2,8-dien-1-ol, p-mentha-1,8-dien-4-ol, carvone, cis- and trans-carveol, perillyl alcohol, and p-menth-8-ene-1,2-diol. P. italicum converted more d-limonene than P. digitatum did, and additional carbon sources inhibited the conversions by both molds. This technique has potential for increasing the use of d-limonene.**

As part of an effort to increase the use and value of citrus by-products, a study was undertaken to investigate the possible use of microorganisms to transform d-limonene into compounds with potentially greater commercial value. d-Limonene is the major component of citrus oils, comprising 90-95% of orange and grapefruit oils (11,17), the principal citrus oils produced in Florida. During the 1972-73 Florida processing season, 30 million pounds of citrus oils were recovered; total potential recovery was 92 million pounds (10). The ready availability and high optical purity of d-limonene make it an excellent starting material for bioconversions; the diverse properties of closely related monoterpenes, important in the flavor and perfume industries, suggested the possibility of using microorganisms to effect the desired transformations (4).

Limited work has been done on the microbiological transformation of d-limonene. Murdock investigated the bacterial contamination of citrus oils during processing and found microorganisms capable of producing α-terpineol by the hydrolysis of d-limonene (15,16). Dhavalikar and co-workers in India isolated a soil pseudomonad capable of using limonene as the sole source of carbon; fermentation of limonene by this bacterium resulted in a large number of neutral and acidic products (5,6). Dhere and Dhavalikar used enrichment culture to isolate an Enterobacterium capable of transforming limonene into dihydroperillic acid and perillic acid (7). Mukherjee and colleagues isolated a *Cladosporium Sp.* capable of producing *trans-p*-menth-8-ene-1,2-diol from limonene (14). Mattison found a Penicillium culture on rotting orange rind that converted limonene to a metabolite tentatively identified as α-terpineol (13).

The organisms used in this study, *Penicillium italicum* and *P. digitatum*, are responsible for the blue- and green-mold rots, two of the most common postharvest diseases of citrus fruits (18). These molds were selected for their natural ability to invade citrus peel, which is high in oil content. Thus, these organisms might be capable of metabolizing d-limonene. A study of the transformation of d-limonene by these molds is reported; major transformation products were identified and the percent conversion was determined for each.

Materials and Methods

Organisms

P. italicum and *P. digitatum* were found on decaying oranges stored at 5°C. Pure cultures were isolated by enrichment culture technique on Czapek-Dox broth (8) containing 1% limonene as the sole carbon source. Cultures were transferred several times in this medium so that organisms best able to grow on limonene would be selected. Growth was heavy only when small amounts of ethanol were added as an additional carbon source and carrier solvent for the limonene.

Chemicals

d-Limonene (P&F) was supplied by Glidden-Durkee, Organic Chemicals Division, Jacksonville, Florida. Specifications were: d-limonene by vapor pressure chromatography (VPC) — 99% minimum; octanol — 0.1%; optical rotation (25°C) +100° minimum. Authentic samples of transformation products were from several commercial and

[1]One of the laboratories of the Southern Region, U. S. Department of Agriculture, Agricultural Research Service.

The author thanks Mr. C. W. Wilson, III, Dr. Steven Nagy and Dr. Joseph H. Bruemmer for their invaluable discussions and suggestions throughout the course of this study.

Mention of a brand name is for identification only and does not imply endorsement by the U. S. Department of Agriculture.

in-house sources; all were purified by gas-liquid chromatography (GLC) and their identities confirmed by infrared spectrospectrometry (IR).

Fermentations

Fermentations were carried out at 21°C in cotton-stoppered 500-ml Erlenmeyer flasks, each containing 100 ml total volume. Agitation was provided by a rotary shaker with 3/4 in. orbit, operated continuously at 100 rpm in a dark room. Czapek-Dox broth, pH 6.8, was autoclaved at 121°C for 15 min; all fermentations were run in this medium, both with and without 3% sucrose. Limonene was first dissolved in ethanol, then added to the cooled media. A 10-ml aliquot of a heavy three-day culture of each organism grown as described above was used for the innoculations. One group of flasks received 0.5% (v/v) limonene + 0.25% ethanol, and were incubated for 3 and 9 days. Another group received 0.5% limonene + 0.25% ethanol initially, and like amounts at 3, 5, and 7 days (2.0% total limonene) and was incubated for a total of 9 days. Control flasks containing no organisms were run under identical conditions for each group so that the degree of autoxidation of limonene could be determined.

Analytical

Fermentation broths (100 ml volume) were saturated with sodium chloride and extracted 4 times with 25 ml methylene chloride (100 ml total). Extracts were then concentrated on a rotary evaporator at room temperature (25°C) and 25 in. Hg presure. Final amounts of solvent were removed under a stream of nitrogen, and samples were stored at 5°C until analysis. Chromatography was performed on a Hewlett-Packard Model 5750 Gas Chromatograph equipped with a flame ionization detector. An 8 ft x 1/4 in. O.D. glass column packed with 10% SP-1000 (Supelco, Inc., Bellefonte, Pennsylvania) on Gas-Chrom Q 100/120 mesh was used, with a 10:1 splitter for collection of the various compounds. The carrier gas was helium at a flow rate of 60 ml/min. Temperature was held at 80°C for 8 min, raised to 200°C at 6°/min, than held at 200°C for 8 min. IR spectra were obtained on a Perkin-Elmer Model 137 Sodium Chloride Spectrophotometer. Limonene transformation products were identified by comparison of their IR spectra and retention time data with those of authentic samples. The percent conversion for each compound was determined by the internal normalization method (9) after calculation of the areas under the peaks of the GLC curve.

Results and Discussion

The compounds shown in Figure 1 were identified as the major products of limonene fermentation by both *P. italicum* and *P. digitatum*.

Relative retention times of these compounds are shown in the chromatogram in Figure 2.

Conversion was greatest in the group of flasks receiving 0.5% limonene and incubated for 9 days (Table 1). Maximum conversion after 3 days with 0.5% limonene was only 10%; in these, the same transformation products were found, except in much smaller amounts. Less than 5% conversion was found at the higher limonene level (2%) after 9 days. Limonene has shown antimicrobial action on certain molds (19), and might have been inhibitory at the higher substrate level.

P. italicum converted much more limonene than *P. digitatum* did, as indicated by the figures

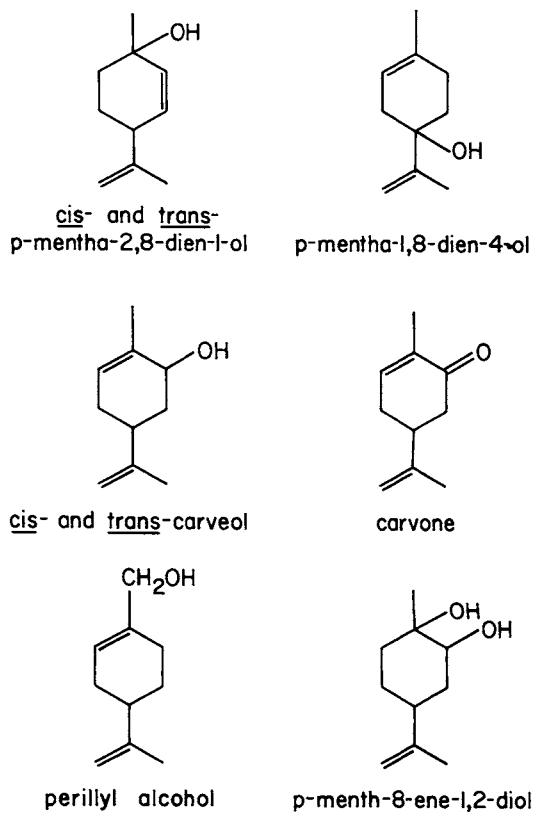

Fig. 1. Major products of limonene fermentation by P. italicum and P. digitatum.

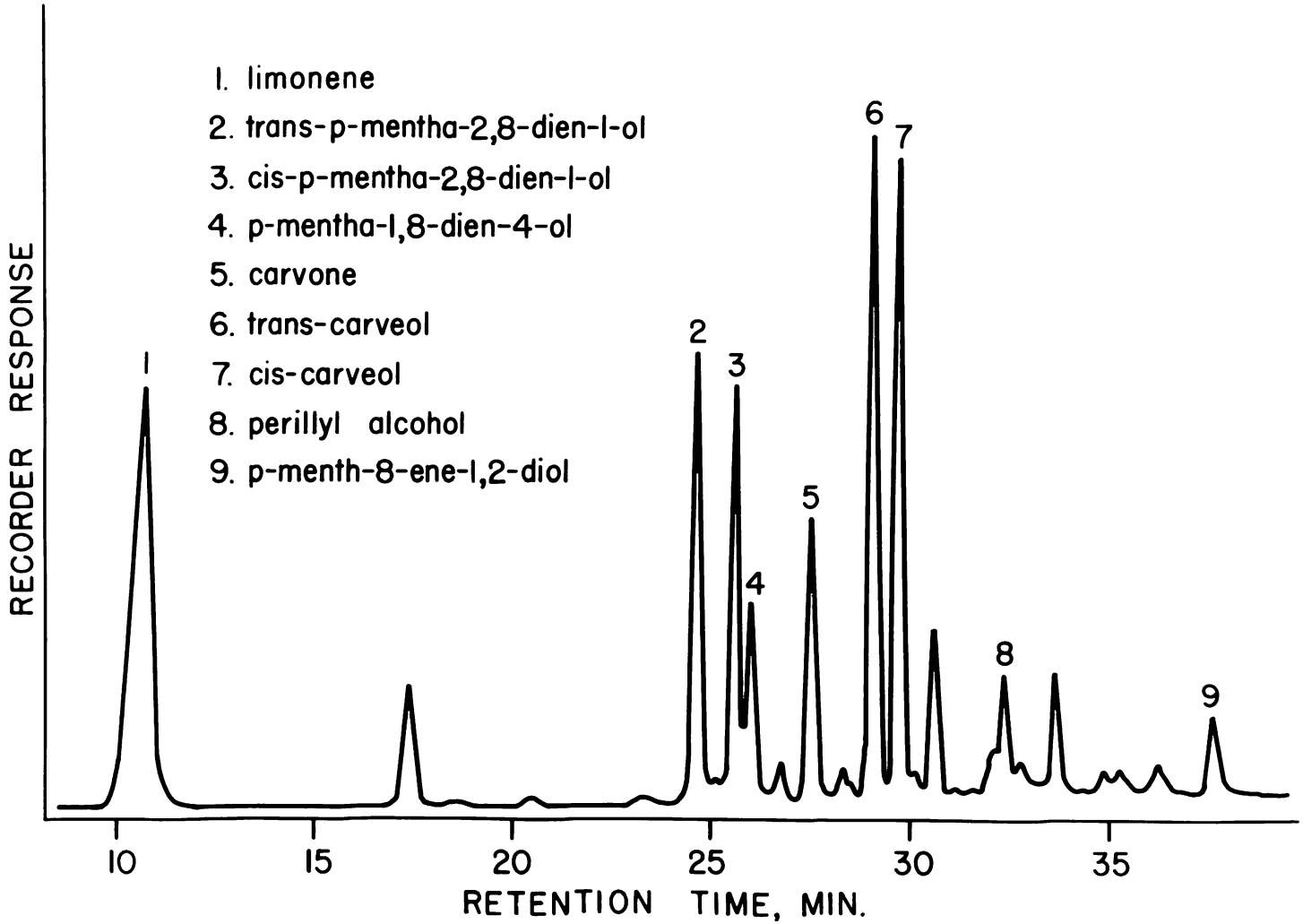

Fig. 2. Chromatogram of limonene conversion products from P. italicum. 0.5%Limonene (no sucrose) incubated for 9 days.

in Table 1. Growth of both molds was heavier with sucrose in the media, but limonene conversion was much less. The addition of sucrose seemed to affect *P. digitatum* much more than *P. italicum*, reducing total conversion by the former from 46% to 16%, and by the latter from 80% to 66%. In preliminary experiments, Orange Serum Broth (Difco Laboratories, Detroit, Michigan), a nutritionally complete medium was used and limonene conversion by these molds was minimal. The additional nutrients in this medium appear to have reduced limonene transformation by providing the organisms with more readily available carbon and energy sources. Small amounts of ethanol were added to all fermentation flasks after preliminary work had shown that growth, as well as limonene conversion, was much better when the organisms were grown on limonene with ethanol than without.

Figures given for the limonene control indicate the amount of conversion taking place without organisms, due to autoxidation of the limonene. All compounds except *p*-mentha-1,8-dien-4-ol were found in the limonene control and have been previously reported as autoxidation products of limonene (20). Wilson and Shaw (21) reported *p*-mentha-1,8-dien-4-ol as a product of limonene oxidation with selenium dioxide-hydrogen peroxide; an authentic sample for this study was prepared by that method. Preliminary tests indicated it was essential to run this limonene control, otherwise conversion produced by autoxidation would be wrongly attributed to microbial action. Thus, in the figures in Table 1, about 7% of the total conversions attributed to the organisms were actually due to the autoxidation of limonene. In the limonene control, carvone was the major autoxidation product, but the organisms produced more *cis* and *trans* *p*-mentha-2,8-dien-1-ol and *cis*- and *trans*-carveol than carvone.

Most of the compounds produced by *P. italicum* and *P. digitatum* have significant commercial value. Carvone is an important flavor compound, and carveol can be readily oxidized to carvone. Perillyl alcohol is used in flavor compositions and occasionally in perfume (1). *p*-Mentha-2,8-dien-1-ol has been used as an intermediate in the synthesis of (—)-menthol (2,3), and both p-mentha-1,8-dien-4-ol and p-menth-8-ene-1,2-diol have shown promise as attractants for pine tree insects (12).

This work has shown that the citrus molds, *P. italicum* and *P. digitatum*, are capable of producing high yields of commercially important limonene oxidation products. *P. italicum* converted almost twice as much limonene as *P. digitatum* did, and it was essential to use a nutritionally restrictive medium to achieve optimum conversion. Manipulation of the variables of the fermentation — pH, temperature, oxygen absorption rate, media composition, and substrate level — might favor the yields of one or more products. Thus, the microbial transformation of d-limonene shows promise as a process to increase citrus by-product use and value.

Table 1. Limonene conversion products of P. italicum and P. digitatum - 0.5% limonene incubated for 9 days at 21°C with and without sucrose. (GLC area % of recovered material.)

	P. italicum		P. digitatum		
Conversion product	W/o sucrose	W/ sucrose	W/o sucrose	W/ sucrose	Limonene control
trans-p-Mentha-2,8-dien-1-ol	9.23	8.30	6.27	2.19	.66
cis-p-Mentha-2,8-dien-1-ol	8.46	8.42	5.01	2.03	.57
p-Mentha-1,8-dien-4-ol	4.10	3.35	3.01	.93	--
Carvone	6.02	4.69	4.01	1.26	1.29
trans-Carveol	13.46	10.05	7.52	2.57	.79
cis-Carveol	13.07	9.42	7.02	2.47	.28
Perillyl alcohol	3.23	1.79	.65	.28	.17
p-Menth-8-ene-1,2-diol	3.10	3.55	1.88	.88	.29
Other compounds	19.60	16.88	10.87	4.00	3.13
Total conversion	80.27	66.45	46.24	16.61	7.18
Limonene remaining	19.75	33.56	53.75	83.40	92.84

Literature Cited

1. Arctander, S. 1969. Perfume and Flavor Chemicals. Vol. I. Steffen Arctander, Publisher. Montclair, New Jersey.
2. Bain, J. P. and W. Y. Gary. 1958. Preparation of unsaturated 3-keto compounds of the p-menthane series. U. S. Patent 2,831,028. April 15.
3. Bain, J. P., A. B. Booth and W. Y. Gary. 1959. Isomerization of terpenic alcohols. U. S. Patent 2,894,040. July 7.
4. Ciegler, A. 1969. Microbiological transformation of terpenes, p. 689-714. In Fermentation Advances. Academic Press, Inc., New York, New York.
5. Dhavalikar, R. S. and P. K. Bhattacharyya. 1966. Microbiological transformations of terpenes. VIII. Fermentation of limonene by a soil pseudomonad. *Indian J. Biochem.* 3:144-157.
6. Dhavalikar, R. S., P. N. Rangachari and P. K. Bhattacharyya. 1966. Microbiological tranformations of terpenes. IX. Pathways of degradation of limonene in a soil pseudomonad. *Indian J. Biochem.* 3:158-164.
7. Dhere, S. G. and R. S. Dhavalikar. 1970. Microbiological transformations of terpenoids: Limonene. *Sci. Cult.* 36:292.
8. Difco Manual. 1974. Difco Laboratories, Inc., Detroit, Michigan. p. 245.
9. Ettre, L. S. 1973. Practical gas chromatography. Perkin-Elmer Corp., Norwalk, Conn. p. 6-17.
10. Kesterson, J. W. and R. J. Braddock. 1975. Total peel oil content of the major Florida citrus cultivars. *J. Food Sci.* 40:931-933.
11. Kirchner, J. G. 1961. Oils in peel, juice sac, and seed. p. 265-301. In W. B. Sinclair (ed.), The Orange. Univ. of California Press, Berkeley, California.
12. Kondo, T. and M. Summimoto. 1974. Limonene oxidation products as insect attractants. *Jap. Kokai.* 74, 124, 239. November 28.
13. Mattison, J. E., L. L. McDowell and R. H. Baum. 1971. Cometabolism of selected monoterpenoids by fungi associated with monoterpenoid-containing plants. *Bact. Proc.* 141.
14. Mukherjee, B. B., G. Kraidman and I. D. Hill. 1973. Synthesis of glycols by microbial transformation of some monocyclic terpenes. *Appl. Microbiol.* 28:447-453.
15. Murdock, D. I., G. L. K. Hunter, W. A. Bueck and J. A. Brent. 1967. Relationship of bacterial contamination in orange oil recovery system to quality of finished product. *Proc. Fla. State Hort. Soc.* 80:267-280.
16. Murdock, D. I. and G. L. K. Hunter. 1970. Bacteriological contamination of some citrus oils during processing. *J. Food Sci.* 35:652-655.
17. Sinclair, W. B. 1972. Grapefruit by-products. p. 502-551. In The Grapefruit. University of California Press, Berkeley, California.
18. Smoot, J. J., L. G. Houck and H. B. Johnson, 1971. Market diseases of citrus and other subtropical fruits. *U. S. Dept. of Agri. Handbook No.* 398. p. 9-12.
19. Subba, M. S., T. C. Soumithri and R. Suranarayana. 1967. Antimicrobial action of citrus oils. *J. Food Sci.* 32:225-227.
20. Verghese, J. 1968. The chemistry of limonene and its derivatives. II. Oxidation of limonene. *Perf. Essent. Oil Rep.* 59:876-886.
21. Wilson, C. W. and P. E. Shaw. 1973. (+)-Limonene oxidation with selenium dioxide-hydrogen peroxide. *J. Org. Chem.* 38:1684-1687.

ANTHRACNOSE, A SERIOUS DECAY OF DEGREENED 'ROBINSON' TANGERINES

G. ELDON BROWN

Florida Department of Citrus
IFAS Agricultural Research and Education Center
Lake Alfred

Abstract. **Anthracnose, caused by the fungus** Colletotrichum gloeosporioides, **is normally a minor decay of oranges and grapefruit. Decay only occurs when infection develops through a severe peel injury or in senescent, overly mature fruit. 'Robinson' tangerines, on the other hand, are extremely susceptible to anthracnose, especially after ethylene degreening. Appressoria, dormant structures of the fungus which are present on the surface of citrus fruits, form infection hyphae during degreening. These penetrate the cuticle and the intact peel. The hyphae continue to develop in degreened 'Robinson' tangerines and eventually result in decay. Ethylene concns of 50 ppm, as opposed to 5, and high densities of appressoria (200 or greater/mm² of fruit surface) increased the incidence of anthracnose. Preventing infection hyphae formation by removing appressoria with washing before, rather than after, degreening reduced anthracnose. Degreening time should be minimal with a low ethylene concn to prevent extensive losses from anthracnose in 'Robinson' tangerines.**

Anthracnose, a postharvest decay of citrus fruits, is caused by the fungus, *Colletotrichum gloeosporioides* Penz. Latent infections are established in the grove where water dispersed spores germinate on immature fruit forming appressoria which can produce infection hyphae that penetrate into the peel (1).

Anthracnose is of minor importance on oranges, grapefruit, tangelos, 'Dancy' tangerines, and 'Temples' where decay only occasionally develops in rather severe injuries to the peel. Degreened 'Robinson' tangerines, however, are very susceptible to anthracnose, particularly when degreening exceeds 36 hr (7). Anthracnose can be reduced substantially by washing fruit before, rather than after, degreening (4,8,9). This is often a more effective control than applying a postharvest fungicide (4,8,9).

Florida Agricultural Experiment Stations Journal Series No. 7072. Cooperative research of the Florida Department of Citrus and the University of Florida, IFAS, Agricultural Research and Education Center, Lake Alfred.