Näf, F.; Decorzant, R.; Willhalm, B.; Velluz, A.; Winter, M. *Tetrahedron Lett.* **1977**, 1413.
Parliment, T. H. *J. Agric. Food Chem.* **1972**, *20*, 1043.
Rapp, A.; Knisper, W. *Vitis* **1979**, *18*, 229.
Rapp, A.; Knipser, W.; Engel, L. *Vitis* **1980**, *19*, 226.
Schultz, T. H.; Flath, R. A.; Mon, T. R.; Eggling, S. B.; Teranishi, R. *J. Agric. Food Chem.* **1977**, *25*, 446.
Takaoka, D.; Hiroi, M. *Phytochemistry* **1976**, *15*, 330.
Whitfield, F. B.; Stanley, G.; Murray, K. E. *Tetrahedron Lett.* **1973**, 95.
Whitfield, F. B.; Sugowdz, G.; Casimir, D. J. *J. Chem. Ind. (London)* **1977**, *12*, 502.
Williams, P. J.; Strauss, C. R.; Wilson, B. *Phytochemistry* **1980a**, *19*, 1137.
Williams, P. J.; Strauss, C. R.; Wilson, B. *J. Agric. Food Chem.* **1980b**, *28*, 766.
Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. *J. Chromatogr.* **1982a**, *235*, 471.
Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. *Phytochemistry* **1982b**, *21*, 2013.
Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. *J. Agric. Food Chem.* **1982c**, *30*, 1219.
Winter, M.; Furrer, A.; Willhalm, B.; Thommen, W. *Helv. Chim. Acta* **1976**, *59*, 1613.
Winter, M.; Klöti, R. *Helv. Chim. Acta* **1972**, *55*, 1916.
Winter, M.; Näf, F.; Furrer, A.; Pickenhagen, W.; Giersch, W.; Meister, A.; Willhalm, B.; Thommen, W.; Ohloff, G. *Helv. Chim. Acta* **1979a**, *62*, 135.
Winter, M.; Schulte-Elte, K. H.; Velluz, A.; Limacher, J.; Pickenhagen, W.; Ohloff, G. *Helv. Chim. Acta* **1979b**, *62*, 131.

Received for review January 24, 1983. Accepted April 15, 1983.

Metabolism of Limonoids by *Arthrobacter globiformis* II: Basis for a Practical Means of Reducing the Limonin Content of Orange Juice by Immobilized Cells

Shin Hasegawa,* Vickie A. Pelton, and Raymond D. Bennett

Arthrobacter globiformis II, a newly isolated bacterium from soil, metabolized bitter limonin of citrus juice to a nonbitter metabolite, limonol, when the juice was treated on a column packed with immobilized cells. On the basis of this information, a biological process that uses immobilized cells of this bacterium in acrylamide gel was developed for reduction of the limonin bitterness of citrus juice sera. The activity and operational stability of the process were demonstrated.

Bitterness due to limonin in certain citrus juices is one of the major problems of the citrus industry worldwide and has significant economic impact.

Substantial progress has been made in studies of the biochemistry of the bacterial degradation of limonoids during the past 10 years. The occurrence of two metabolic pathways of limonoids in bacteria has been firmly established: one via deoxylimonoids such as deoxylimonin (Hasegawa et al., 1972a) and the other via 17-dehydrolimonoids such as 17-dehydrolimonate A-ring lactone (Hasegawa et al., 1972b). Enzymes involved in the pathways have been isolated and characterized (Hasegawa et al., 1972b, 1974b,c; Hasegawa, 1976).

A continuing study of limonoid-metabolizing microorganisms, metabolic pathways of limonoids, and enzymes involved in the pathways resulted in the finding of a new metabolic pathway of limonoids in bacteria. This paper shows that immobilized cells of a newly isolated bacterium, *Arthrobacter globiformis* II, metabolized bitter limonin of citrus juice to nonbitter limonol.

Recently, biological processes using immobilized bacterial cells have been developed for reduction of the limoin bitterness of citrus juices. The process using *Acinetobacter* cells entrapped in a dialysis sac reduced the limonin content of navel orange juice by converting it to nonbitter deoxylimonin and deoxylimonic acid (Vaks and Lifshitz, 1981). The process using immobilized cells of *Arthrobacter globiformis* in acrylamide gel converted limonin to nonbitter 17-dehydrolimonoate A-ring lactone (Hasegawa et al., 1982). Both processes require multienzyme catalytic systems. In the newly found pathway, the conversion of limonin to limonol is apparently catalyzed by the action of a single enzyme. This paper also shows the results of studies on the reduction of the limonin content of navel orange juice serum using *A. globiformis* II cells immobilized in acrylamide gel.

U.S. Department of Agriculture, Agricultural Research Service, Fruit and Vegetable Chemistry Laboratory, Pasadena, California 91106.

MATERIALS AND METHODS

Limonol was synthesized from limonin by the procedure of Melera et al. (1957). Navel oranges were purchased from a local market. The juices were extracted with a Sunkist juicer, and the sera were obtained from the juices by centrifugation at 2500*g* for 10 min. Limonin was added to the sera to obtain convenient working concentrations.

Growth of Cells. The substrate, 500 mL of a mineral salt (Hasegawa et al., 1972b), 0.2% nutrient broth (Difco Laboratory, Detroit, MI), and 0.2% limonate (both A and D rings of limonin open), was placed in a 2.8-L Fernbach flask and incubated with 20 mL of 72-h culture of *Arthrobacter globiformis* II. Incubation was at 25 °C on a shaker. After 48-h incubation, cells were harvested by centrifugation, washed with 0.5 M potassium phosphate buffer at pH 7.0, and frozen until used for immobilization.

Immobilization of Cells in Acrylamide Gel. Cells were immobilized in acrylamide gel by the method of Tosa et al. (1974). The resulting gel was then gently ground with a Polytron and packed in a column.

Metabolism of Limonin with Immobilized Cells. Since limonin is almost insoluble in aqueous solution, 20% navel orange juice serum was used as a carrier solution. A total of 45 mg of limonin was dissolved in 1000 mL of

Table I. R_f Values of Limonin and Its Metabolite[a]

	A	B	C
limonin	0.27	0.38	0.38
limonol	0.23	0.42	0.23
metabolite	0.23	0.42	0.23

[a] Composition of solvent systems: A, toluene-EtOH-H_2O-HOAc (200:47:15:1, upper layer); B, EtOAc-cyclohexane (3:2); C, CH_2Cl_2-MeOH (97:3).

the diluted serum, and the serum was then passed through a column (2 × 15 cm) packed with 3 g of cells immobilized in acrylamide gel at a rate of 100 mL/h at room temperature. This process metabolized 60–70% of the serum limonin.

Isolation of Metabolite. The effluent obtained above was acidified with HCl to pH 2 and extracted twice with CH_2Cl_2. The extracts were combined and evaporated. TLC analyses showed two major components, one of which was limonin. The second compound was neutral and Ehrlich's positive. The extract was therefore fractionated on a silica gel column (2 × 30 cm) with EtOAc–hexane. The column was eluted, stepwise, by increasing concentrations of EtOAc in hexane by the following schedule: at first 500 mL of EtOAc–hexane (2:8) followed by 500 mL of EtOAc–hexane (2.5:7.5), 500 mL of EtOAc–hexane (3:7), and finally 1000 mL of EtOAc–hexane (3.5:6.5). Fractions of 15 mL each were collected. The fractions containing the unknown were combined and evaporated to give 23 mg of a single chromatographically pure compound.

Identification of Metabolite. Silica gel G plates were used for TLC. Plates were developed with three solvent systems (Table I). Chromatograms were revealed by spraying with Ehrlich's reagent and exposing to HCl gas (Dreyer, 1965b). NMR spectra were run at 100 MHz in $CDCl_3$-$(CD_3)_2SO$ (98:2) by using a JEOL JNM-PS-100 spectrometer.

Analysis of Limonoids. Limonin and its metabolites were quantitatively analyzed by the procedure of Maier and Grant (1970). Silica gel plates were developed routinely with toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer) and revealed by the method described previously.

RESULTS AND DISCUSSION

In a continuing survey of microoorganisms, *A. globiformis* II was isolated from soil. Like the earlier isolate, *A. globiformis* (Hasegawa et al., 1972b), the organism grew very well on a limonoate–mineral salt medium and produced 17-dehydrolimonoate (the A ring of 17-dehydrolimonoate A-ring lactone is open) as the majoor metabolite in its growth medium. The major difference in characteristics of limonoid metabolism between the two organisms was that *A. globiformis* II cells immobilized in acrylamide gel converted limonin of citrus juice to an unidentified compound as the major metabolite, whereas *A. globiformis* cells immobilized in acrylamide gel converted limonin to 17 -dehydrolimonoate A-ring lactone (Hasegawa et al., 1982).

The metabolite produced by immobilized cells of *A. globiformis* II was a neutral and Ehrilich's-positive compound, which differed from any of the metabolites of the deoxylimonoid pathway established previously (Hasegawa et al., 1972a). Therefore, the metabolite was isolated by column chromatography on a silica gel. The column fraction contained 23 mg of a single pure compound. TLC analysis showed that the isolate had the same mobility as a synthetic sample of limonol with three solvent systems (Table I). Furthermore, its NMR spectrum was identical with that of authentic limonol (Dreyer, 1965a). These data show that the immobilized cells of *A. globiformis* II converted limonin to limonol. Thus, this organism is capable of metabolizing limonoids through at least two metabolic pathways. In this experiment, the same column was used several times to produce the metabolite with similar results in each case.

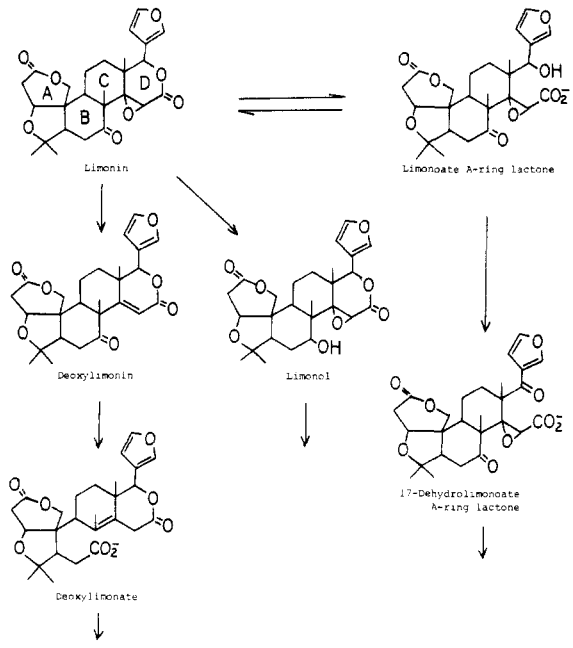
One of the advantages of the biocatalytic system with immobilized bacterial cells over the conventional isolated enzyme system is that the immoblized cell system can be used many times with continuing effectiveness. In this study feasibility of the immobilized cell system of *A. glo-*

Table II. Reduction of Limonin Content of Navel Orange Juice Sera with *A. globiformis* II Cells Immobilized in Acrylamide Gel[a,e]

	column 1,[b] limonin content			column 2,[c] limonin content			column 3,[d] limonin content		
run no.	control, ppm	treated, ppm	reduction, %	control, ppm	treated, ppm	reduction, %	control, ppm	treated, ppm	reduction, %
1	18.9	7.2	62	16.7	3.9	77	20.8	5.6	73
2	20.0	7.8	61	17.5	3.3	81	16.1	5.0	69
3	21.7	8.3	62	15.0	3.9	74	21.7	7.2	72
4	13.7	7.8	43	17.5	8.3	53	23.3	6.1	74
5	17.5	11.1	37	17.5	3.9	78	22.5	7.8	65
6	21.6	9.4	57	20.8	7.2	65	19.2	4.4	77
7	14.2	6.7	53	16.1	4.4	73	22.5	6.7	70
8	16.7	6.1	63	21.7	10.6	51	18.3	3.3	82
9	17.5	7.8	55	23.3	8.9	62	18.3	7.2	61
10	15.0	6.1	59	22.5	7.2	68	22.5	9.4	58
11	17.5	6.7	62	19.2	7.2	63	24.2	10.0	59
12	17.5	10.6	39	22.5	10.0	56	23.0	8.9	61
13	20.8	11.7	44	18.3	6.1	67	22.5	9.4	58
14	16.1	11.7	27	18.3	10.0	45	17.5	7.8	55
15	21.7	16.1	26	22.5	12.8	43	18.3	8.9	51
16	23.3	15.5	33	24.2	13.3	45	19.2	7.8	59
17	22.5	16.1	28	23.0	15.0	35	17.5	8.9	49
18	19.2	17.2	10	22.5	13.3	41	19.2	7.8	59
19	22.5	18.3	19	17.5	12.2	30	19.2	9.4	51
20				18.3	12.8	30	15.8	9.4	41
21				19.2	13.9	28	15.8	7.2	54
22				17.5	15.0	14	17.5	10.6	33
23							19.2	13.9	28

[a] A total of 30 mL of juice serum was passed once through a 1.5-cm diameter column packed with immobilized cells at a rate of 1 mL/min and at room temperature. [b] Column 1: 1.20 g of cells. [c] Column 2: 1.8 g of cells. [d] Column 3: 1.84 g of cells. [e] Serum pH 3.5–3.7.

Scheme I. Metabolic Pathways of Limonoids



biformis II was investigvated with regard to its activity and operational stability in developing a process for reduction of limonin bitterness of citrus juices. Such an experiment was done in triplicate with navel orange juice sera. The results are shown in Table II. A 30-mL portion of the navel orange juice serum was once passed through a column packed with immobilized cells at a rate of 1 mL/min at room temperature. The column was then washed thoroughly with 0.05 M potassium phosphate butter at pH 7.0 and kept in a 4 °C room until next use. The effluent and the washings were combined and used for limonoid analyses. The control was treated similarly by using a column packed with acrylamide gel without cells. These experiments were repeated once a day until activity was gone.

The limonoid-metabolizing enzyme activity in the immoblized cells very effectively reduced the limonin content of the serum. Column 1 was least effective. This may be because the column had only 1.20 g of cells whereas columns 2 and 3 had 1.80 and 1.84 g of cells, respectively. Column 3 was most effective. For instance, on the 1st run the treatment reduced a 20.8-ppm limonin juice serum to 5.6 ppm and on the 21st run it reduced a 15.8-ppm limonin juice serum to 7.2 ppm. This column was used with continuing effectiveness in 23 consecutive tests during a period of about 40 days. Limonin was almost completely metabolized when the sera containing limonin below 7 ppm were treated with the column. The conversion of limonin to limonol in the system was as effective as the conversion of limonin to 17-dehydrolimonoate A-ring lactone by the immobilized cells of *A. globiformis* (Hasegawa et al., 1982).

A biological process that uses the specific biocatalytic action of immobilized microbial cells represents a new approach to processing citrus juices. Catalysis by fixed cells is quite complex and many basic aspects are yet to be understood. However, this study and previous studies (Hasegawa et al., 1982; Hasegawa and Pelton, 1983) demonstrate technical feasibility of the immobilized cell process as shown by limonin debittering of navel orange juice and juice serum through intact function of limonoid-metabolizing enzymes. Although substantial further work is needed, sufficient progress has been made to show that this type of biological process has potential for practical application in improving the quality of citrus juices.

Scheme I summarizes the metabolic pathways of limonoids in bacteria. Comparative biochemical studies of limonoid metablism in bacteria and *Citrus* have shown that both the 17-dehydrolimonoid and deoxylimonoid pathways are also operative in *Citrus* (Hasegawa et al., 1974a, 1980). The presence of limonol in grapefruit seeds (Bennett and Hasegawa, 1982) suggests that the limonol pathway also might be operative in *Citrus*.

ACKNOWLEDGMENT

We thank Dr. V. P. Maier for his help in the preparation of the manuscript.

LITERATURE CITED

Bennett, R. D.; Hasegawa, S. *Phytochemistry* **1982**, *21*, 2349.
Dreyer, D. L. *Tetrahedron* **1965a**, *21*, 75.
Dreyer, D. L. *J. Org. Chem.* **1965b**, *30*, 749.
Hasegawa, S. *J. Agric. Food Chem.* **1976**, *24*, 24.
Hasegawa, S.; Bennett, R. D.; Maier, V. P. *J. Agric. Food Chem.* **1972a**, *20*, 435.
Hasegawa, S.; Bennett, R. D; Maier, V. P.; King, A. D., Jr. *J. Agric. Food Chem.* **1972b**, *20*, 1031.
Hasegawa, S.; Bennett, R. D.; Verdon, C. P. *Phytochemistry* **1980**, *19*, 1445.
Hasegawa, S.; Maier, V. P.; Bennett, R. D. *Pytochemistry* **1974a**, *13*, 103.
Hasegawa, S.; Maier, V. P.; Border, S. N.; Bennett, R. D. *J. Agric. Food Chem.* **1974b**, *22*, 1093.
Hasegawa, S.; Maier, V. P.; King, A. D., Jr. *J. Agric. Food Chem.* **1974c**, *22*, 523.
Hasegawa, S.; Patel, M. N.; Snyder, R. C. *J. Agric. Food Chem.* **1982**, *30*, 509.
Hasegawa, S.; Pelton, V. A. *J. Agric. Food Chem.* **1983**, *31*, 178.
Maier, V. P.; Grant, S. R. *J. Agric. Food Chem.* **1970**, *18*, 250.
Melera, A.; Schaffner, K.; Arigoni, D.; Jeger, O. *Helv. Chim. Acta* **1957**, *40*, 1420.
Tosa, T.; Sato, T.; Mori, T.; Chibata, I. *Appl. Microbiol.* **1974**, *27*, 886.
Vaks, B.; Lifshitz, A. *J. Agric. Food Chem.* **1981**, *29*, 1258.

Received for review September 13, 1982. Revised manuscript received March 7, 1983. Accepted April 28, 1983. Reference to a product name or company does not imply endorcement of that product or company by the U.S. Department of Agriculture to the exclusion of others that may be suitable.