Debittering of Orange Juice by Bacteria Which Degrade Limonin

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A method is described for debittering orange juice by bacteria entrapped in a dialysis sac. A soil bacterium (*Acinetobacter* sp.) was isolated by enrichment culture. It was capable of growth and of using limonin as a sole carbon source. Two nonbitter metabolites were isolated from the reaction mixture and identified as deoxylimonin and deoxylimonic acid. A pathway is proposed for debittering limonin by the bacteria: limonin → deoxylimonin → deoxylimonic acid. The bacteria were immobilized in a dialysis sac and used for debittering early season juice. It was possible to substantially reduce the bitterness of the juice by transfering the same dialysis sac from batch to batch of juice. A total of 120 mg (dry weight) of bacteria was required for the conversion of 1 L of bitter early season orange juice to drinkable juice.

Limonin is the principal bitter constituent of orange juice. The bitterness in juices due to limonin gradually develops after extraction from oranges and is referred to as delayed bitterness. The intact fruit barely contains limonin; however, a nonbitter precursor limonoat A-ring lactone is present. This nonbitter precursor originally present in the insoluble fruit sections passes into the juices after its preparation and is converted to limonin under acidic conditions.

Delayed bitterness has become a serious economic problem to the citrus industry and attempts are being made to solve the probelm by processes such as prevention of limonin formation by choice of rootstock, exposure of the fruit to ethylene, treatment of the juice by enzyme, and, recently, absorption of the limonin already formed on cellulose acetate. All of the above are summarized in a recent review by Maier et al. (1977). At present there are no entirely satsifactory method for preventing or removing limonin bitterness.

Hasegawa et al. (1972b, 1973, 1974b) and Brewster et al. (1976) isolated an enzyme from *Arthrobacter globiformis* and from *Pseudomonas sp.* which oxidizes limonate A-ring lactone to a nonbitter product, 17-dehydrolimonate A-ring lactone. this enzyme was used to prevent limonin bitterness. However, the enzyme acts only on the open ring lactone which exists for a short time in the juice before it is converted to limonin and does not attack the closed ring bitter limonin. The pH optimum of this enzyme is 8.5–9.5, and, therefore, its activity in the natural acid environment of the juice is limited.

This paper describes a method for removing limonin bitterness after its formation in the acidic juice, using bacteria.

MATERIALS AND METHODS

Limonin was prepared from grapefruit seeds according to Emerson (1948). A solution of 1 mg/mL acetonitrile was prepared and used for a standard curve and for the activity assay.

Deoxylimonin, deoxylimonic acid, and methyldeoxylimonic acid were prepared according to Barton et al. (1961).

Randomly tritiated limonin was prepared by Nuclear Research Center, Negev, Israel, with a specific activity of 43 mCi/mmol. However, the limonin was not completely pure as judged by TLC, even after its recrystallization. A solution of 1 mg/mL of the recrystallized limonin in acetonitrile was prepared so that 4 μL of this solution contained 10^5 cpm/TLC spot at the R_f of limonin and 2×10^4 cpm at the R_f's of the two products (see Results and Discussion).

Essential oils were obtained from Pardess Citrus Products, Ltd., Israel.

Orange juice was early season juice containing 18 ppm of limonin, adjusted to pH 4.5 or 6 by NaOH.

Enrichment Culture. An enrichment culture was prepared by inoculating orchard soils and sewage from citrus processing plants in a mineral medium containing 50 mg/L limonin and 0.01% yeast extract. The culture flasks were shaken at 30 °C and were transferred weekly to fresh medium. After 1 month several isolates of microorganisms were obtained from these cultures, and the most active isolate in utilizing limonin at acidic pH was selected for further experiments.

Growth of *Acinetobacter* sp. The bacteria were grown in a 2-L Erlenmeyer flask containing 400 mL of salt medium (1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of KCl, 2 g of $(NH_4)_2SO_4$, and 0.01 g of $FeSO_4 \cdot 7H_2O$ per 1 L), 30 mg/L limonin, and 0.15% casamino acids at 32 °C and rotated at 180 rpm. After 24 h the cells were collected by centrifugation and were kept frozen until use.

Estimation of Residual Limonin. One milliliter of the solution containing limonin was extracted with 2 mL of chloroform. The chloroform was evaporated to dryness in vacuo or under N_2, and the residue was dissolved in 0.2 mL of chloroform. The limonin content was estimated by two methods: (a) for limonin solution and juice, by TLC according to Maier and Grant (1970) (0.02 mL); (b) for limonin solution only, by a novel spectrophotometric method (0.18 mL) described under Results and Discussion.

Estimation of labeled limonin and its products was made by running a TLC in benzene–ethanol–water–acetic acid (200:47:15:1, upper phase), scraping the relevant spots from the TLC and transferring them into a scintillation vial for counting.

Activity Assay. A bacterial pellet [usually 0.2 mg (dry weight)/mL] was suspended in 1 mL of 0.1 M phosphate buffer, pH 6, containing 40 μg of limonin. After 1.5 h of incubation at room temperature the reaction was stopped by the addition of 0.5 mL of 5% Cl_3AcOH. The residual limonin was estimated as described previously. Activity was expressed as percent conversion of limonin. Assay with immobilized bacteria was done in a similar manner.

Isolation of Deoxylimonic Acid (II) from Culture Medium. *Acinetobacter* was grown as described but with the addition of 500 mg/L limonin. After 72 h the cells were removed by centrifugation, and the supernatant was acidified with HCl and extracted with chloroform. The chloroform was evaporated to dryness, and the residue was

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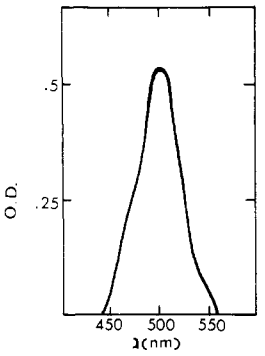

Figure 1. Spectrum of red color development in the spectrophotometric method for the estimation of limonin.

dissolved in a small volume of dichloromethane. Deoxylimonic acid (II) was crystallized from 2-propanol.

Bacterial Immobilization. Acrylamide gels were prepared with bacterial suspensions according to Tosa et al. (1974) and Shimizu et al. (1975) in different concentrations of monomers at 0 and 37 °C.

Agarose Immobilization. A total of 0.2 mg (dry weight) of bacterial cells was suspended in 0.04 mL of 0.1 M phosphate buffer, pH 6. The suspension was transferred to 1 mL of a 5% agarose solution (Sigma) in a 46 °C water bath. After being stirred in a stream of N_2, the mixture was transferred to an ice bath until gelation occurred. The gel was washed and cut into small sections.

Dialysis Sac. A bacterial suspension in 0.04 mL of 0.1 M phosphate buffer, pH 6, was placed into a 4 cm length dialysis sac. (Thomas, $^7/_8$ in. diameter cutoff 12000). The bacteria used for immobilization and other experiments were intact living cells.

RESULTS AND DISCUSSION

Development of a Method for Estimation of Limonin Concentration. Nomura and Saito (1966) determined limonin by a spectrophotometric method using Ehrlich reagent. However, it was found that the method was not sensitive enough. Burnham (1970) used Ehrlich reagent to determine indole. The difference between those methods was the composition of the reagent and the acidic environment required for the reaction. A sensitive method was achieved by modification of Burnham reagent for indole and applying it to limonin. It was found that the optimal concentrations of the reagent components were 0.1 g of 4-(dimethylamino)benzaldehyde, 3 mL of acetic acid, and 2.4 mL of 70% perchloric acid. The red color which fully developed after 30 min (and was stable for more than 1 h) had maximal absorbance at 503 nm (Figure 1). The limonin estimation was performed as follows: To 1 mL of chloroform containing 0–100 μg of limonin was added 1.5 mL of the reagent. After vigorous mixing and a waiting period of 30 min at room temperature, the absorbance of the red upper phase was read in a spectrophotometer at 503 nm. The limonin in the unknown samples was estimated from a standard curve of pure limonin prepared similarly.

So that this sensitive method can be used after bacterial degradation of limonin, deoxylimonic acid—the main product which reacts similarly to limonin—must be removed by converting it to tis chloroform-insoluble sodium salt. So that this could be accomplished, 1 mL of water and 0.04 mL of 2 N NaOH were added to the chloroform containing the unknown limonin, and after short vigorous mixing by a vortex mixer the chloroform layer was transferred to another tube for limonin estimation.

Enrichment Culture. The few microorganisms that could utilize limonin as a sole carbon source were isolated by enrichment culture. The one which was most active in utilizing limonin at acidic pH's was found to be a Gram-negative soil bacterium, *Acinetobacter* sp.

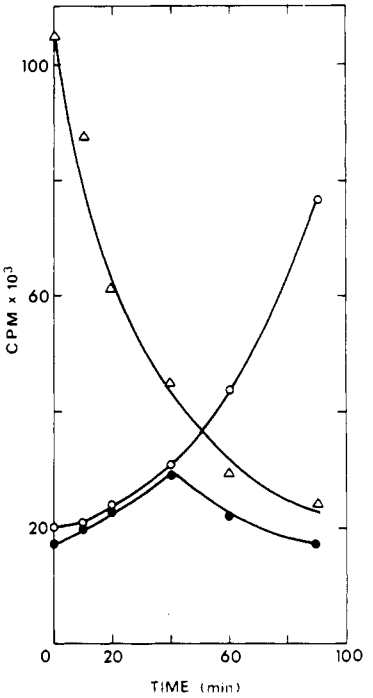

Figure 2. Kinetics of limonin conversion to its products. 1 mL of 1 OD (0.4 mg/mL, dry weight) bacterial suspension in 0.1 M phosphate buffer at pH 6 was incubated with 40 μg of radioactive limonin. Limonin (Δ) and its products, deoxylimonin (●) and deoxylimonic acid (○), were assayed at various intervals after limonin addition, by methods described under Materials and Methods.

Reaction Conditions. The optimal activity of the bacteria was found to be at pH 6 and 32 °C. Adding cations to the buffer reaction or pretreatment of the cells with organic solvents or detergents (Martin and Perlman, 1975) did not raise its activity.

Conversion of Limonin to Nonbitter Metabolites. While limonin disappeared, four other limonoids (stained by Ehrlich reagent), with lower R_f values, could be detected on the TLC plate. The color intensity of two of these, i.e., I and II, was much stronger than that of the other two.

The R_f of the unknown compound which was isolated from the culture media (see Materials and Methods) was the same as II. An NMR of this unknown was run and its signals were compared with those of functional groups of limonoids (Dreyer, 1965).

A shift of H-17 signal to a higher field than in limonin, namely, 5.4 to 5.1 ppm, indicates that II lacked the epoxide group (deoxy). Appearance of a new signal at 1.65 ppm, which did not exist in limonin, proved the existence of a carboxylic group in II. After methylation of II, its R_f value was higher than that of the original compound. In the NMR spectrum of the methylated compound, a new signal appeared whose intensity and position were those of a methoxy group. These findings indicated that II is deoxylimonic acid.

It was not possible to isolate enough material of I from the culture medium for chemical identification. However, as could be inferred from Figure 2, I must be an intermediate in the conversion of limonin to deoxylimonic acid. Logically, this intermediate could be deoxylimonin, since the chemical preparation of deoxylimonic acid from limonin is done through deoxylimonin (Barton et al., 1961). Deoxylimonin was chemically prepared, and the R_f and the

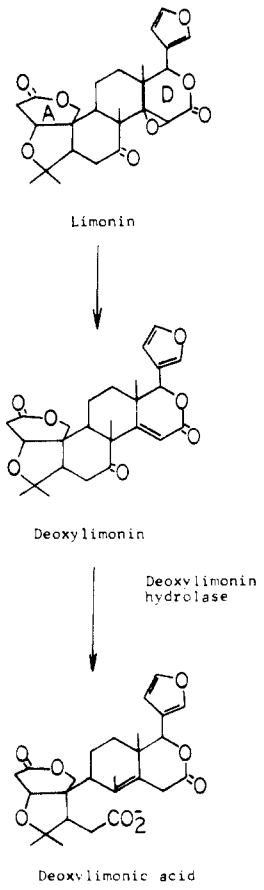

Figure 3. Proposed pathway for debittering limonin.

IR spectra were found to be identical with those of I.

Figure 2 describes an experiment in which a bacterial suspension was incubated with radioactive limonin. It can be seen that while limonin is disappearing, labeled deoxylimonin and deoxylimonic acid are being formed, and after 90 min of incubation practically only deoxylimonic acid remained. The total counts at each time period are equal, indicating that the two limonoids are direct degradation products of limonin.

It can be seen that as the reaction progresses limonin is converted to deoxylimonin and the latter to deoxylimonic acid. After approximately 50 min the rate of conversion of deoxylimonin to deoxylimonic acid becomes more pronounced as is evident from the curvature of the line. This is explained by the observation that limonin competitively inhibits the second enzyme which converts deoxylimonin to deoxylimonic acid (Vaks, 1980) and its full capacity is only manifested when the concentration of limonin is reduced.

Deoxylimonin and deoxylimonic acid were found to be nonbitter even at high concentrations as 200 ppm. Limonin, on the other hand, is bitter at 10 ppm (Levi et al., 1974).

Considering all these results it is suggested that the pathway for debittering of limonin is limonin → deoxylimonin → deoxylimonic acid (Figure 3). The same pathway was proposed by Hasegawa et al. (1972a, 1974b) with *Pseudomonas* sp.

The first enzyme in this pathway which converts limonin to deoxylimonin could not be isolated. No activity was found either in the culture medium (cell-free supernatant) or in extracts of cells even when they were disrupted under the most gentle conditions. The same results were found by Hasegawa et al. (1974a) with *Pseudomonas*. The second enzyme in the pathway (as mentioned before) was isolated, partially purified, and characterized (Vaks, 1980). This enzyme was found to be similar to that of deoxylimonin hydrolase which was found in *Pseudomonas* by Hasegawa et al. (1974a).

The failure in isolating the first enzyme prompted us to investigate the possibility of using a whole cell preparation for debittering orange juice.

Table I. Properties of the Immobilized Cells As Compared to Those of Free Cells

preparation	act., % of control	no.[a] of transfers possible	optimal pH	optimal temp, °C
free bacteria (control)	100	3	6-7	30
acrylamide	3			
agarose	75	3	6-7	30
dialysis sac	73	3	6-7	30

[a] Number of possible transfers until activity has fallen to 50% of the initial. Bacterial concentration was 0.2 mg/mL (dry weight).

Table II. Bacterial Activity in Juice and Buffer[a]

medium	% conversion of limonin
buffer	93
natural juice	45
centrifuged juice	50
dialyzed juice	93

[a] The buffer and juice were at pH 6, containing 18 ppm of limonin. Bacterial concentration was 0.4 mg/mL (dry weight).

Table III. Inhibition of Bacterial Activity by Juice Constituents[a]

addition (to dialyzed juice)	% conversion of limonin
none	93
1% citric acid	90
0.2% malic acid	91
8% glucose	90
0.05% essential oils	52

[a] The constituents were added to dialyzed juice at pH 6, containing 18 ppm of limonin. Bacterial concentration was 0.4 mg/mL (dry weight).

Properties of the Cells after Immobilization. The properties of the immobilized cells are summarized in Table I. Immobilization of the bacteria resulted in a residual activity of 3% in acrylamide and about 75% in agarose and in the dialysis sac as compared with the activity of the free bacteria. The slight activity of the cells in acrylamide is probably a result of damage to the bacteria during the immobilization process. The 25% loss in activity in agarose and the dialysis sac is a result of diffusion limitations. Optimal pH, temperature, and result of repeated usage were identical for the immobilized and the free bacterium.

Removal of Limonin from Juice. *Activity of Bacteria in Buffer As Compared to That in Juice.* The activity of *Acinetobacter* in buffer and in juice is shown in Table II. The activity in natural juice, even after centrifugation, was only half of that in buffer. This eliminates the possibility that the bacteria adhere to the juice particles. After dialysis the activity reached that of the buffer which proves that the juice contains a low molecular weight constituent which inhibits the bacterial activity. Various low molecular weight constituents of orange juice (Sinclair, 1961) were assayed for their ability to hinder bacterial activity (Table

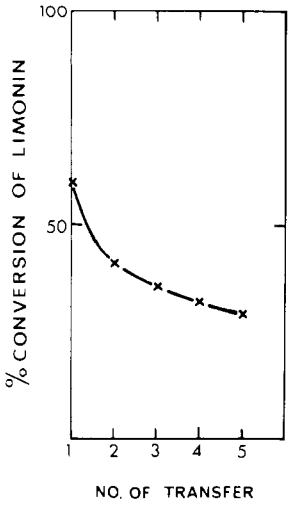

Figure 4. Debittering of citrus juice by bacteria entrapped in a dialysis sac. 3 mg (dry weight) of cells was entrapped in a dialysis sac (cf. Materials and Methods). The sac was placed in 4 mL of juice containing 18 ppm of limonin and adjusted to pH 4.5, and after 2 h of incubation the juice was replaced by another batch. This was repeated 5 times. The residual limonin was estimated in each batch by the methods described.

III). It can be seen that the essential oils were those low molecular weight substances responsible for this inactivation. It was found, by using distillation methods (AOAC, 1975), that the oil content in fresh juice was 0.05% while in dialyzed juice it approached 0%. Maruzzella and Lichtenstein (1956) found that essential oils have antibacterial activity. In industry, juice is deaerated and pasteurized before canning. These processes reduce the essential oil content resulting in lack of inhibition of bacterial activity.

Use of Entrapped Bacteria in Dialysis Sacs for Removal of Limonin from Juice. A total of 3 mg (dry weight) of cells was entrapped in a dialysis sac and was transferred to 4 mL of pasteurized juice at pH 4.5 containing 18 ppm of limonin. After 2 h the juice was replaced by another batch, and this was repeated several times. The results are presented in Figure 4. It can be seen that the same dialysis sac can be used for five consecutive transfers through fresh orange juice before the activity has fallen to 50% of the initial. There was no change in the pH and taste of the juice following treatment except for a decrease in bitterness.

In another experiment the quantities were scaled up 125-fold. A total of 300 mg (dry weight) of bacteria was entrapped in a dialysis sac and five sequential transfers to batches of 0.5 L of juice each were made. The results were similar to those of the smaller experiment.

Entrapment of the bacteria in a dialysis sac appears to be a feasible method for industrial use. The separation of cells and juice is easy in a batch reaction, and good flow rates can be achieved in a continuous reaction. There is no danger of microorganisms escaping into the juice, and it is easy to replace inactivated cells. A total of 120 mg (dry weight) of bacteria is needed for the conversion of 1 L of bitter orange juice (at pH 4.5 containing 18 ppm of limonin) to drinkable juice.

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