amino acids and betaine pass into the effluent. The Dowex 1 column can be eluted with acetic or hydrochloric acid to recover the amino acids or can be eluted first with aqueous carbon dioxide to recover the neutral amino acids and other compounds having similar ion exchange properties.

The fraction containing acids can be used for identifying and determining specific acids. The effluent from the column of Duolite A-4 contains carbohydrates and other neutral substances.

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CITRUS BY-PRODUCTS

Lactic Acid Production by Fermentation of Citrus Peel Juice

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Citrus peel juice is produced in large quantities during the manufacture of canned citrus juices. A study was undertaken to determine the possibility of producing lactic acid by fermentation of this waste juice. Fermentations were conducted at 45° C. in the presence of excess calcium carbonate to neutralize continuously the acid formed. A naturally occurring lactobacillus isolated from a fermenting grapefruit juice was able to accomplish a 90%-efficient conversion of sugars to lactic acid in a period of 4½ to 5 days only in the presence of accessory nutrients. Growth factors supplied by yeast autolyzate or malt sprouts were particularly beneficial. Analyses of the fermentation runs were considerably simplified by the use of a cation exchange method for determining the lactic acid produced during fermentation in the presence of calcium carbonate.

IN THE CITRUS FRUIT processing industry, utilization of peel juice is becoming a major factor in maintaining economical plant operations. Preparation, composition, and current uses of peel juice have been adequately covered by Braverman (*2*). This report deals with the selection of a suitable lactobacillus organism which, in the presence of added growth factors, is capable of vigorous and efficient fermentation of peel juice. Furthermore, the need was felt for a simple and rapid method adaptable to routine determinations of lactic acid produced during fermentation in the presence of calcium carbonate. A cation exchange method was developed for this purpose; the principle involves the exchange of calcium ions from the calcium lactate in the clarified fermentor liquor for the hydrogen ions of the exchange resin. The acid thus formed is titrated with a standard alkali solution and calculated as lactic acid.

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Materials

Peel Juice. Citrus peel juice was obtained directly from the canning line, adjusted to pH 6.0 by the addition of phosphoric acid and filtered to remove insolubles. During the off-season, citrus molasses was diluted with tap water to a Brix density of approximately 13°, adjusted to pH 6.0 with a 10% sodium phosphate solution, and filtered.

Yeast Autolyzate. Ground yeast cakes, 1 kg., were autolyzed with 100 ml. of ethyl acetate at room temperature. After complete liquefaction, the mixture was stirred mechanically for 1 hour, during which time it was maintained neutral to litmus by the addition of a 10% sodium phosphate solution. Toluene, 80 ml., was vigorously stirred into the mixture which was then incubated for 24 hours at 35° C. Neutralization and incubation were repeated always making certain of an excess of toluene to prevent putrefaction. The autolyzate was clarified by the addition of egg white and after being heated on a steam bath for 30 minutes, the mixture was filtered hot through a Büchner funnel with the aid of kieselguhr. The solids were extracted with an equal volume of water and filtered. The combined extracts were concentrated on a steam bath to a Brix density of 25° and stored under toluene in the refrigerator.

Tomato Extract. A quantity of tomatoes was macerated in the Waring Blendor and filtered through a Büchner funnel with the aid of kieselguhr. The extract was vacuum concentrated to a Brix density of 15° and stored, under toluene, in the refrigerator.

Culture Maintenance Medium. All cultures were maintained in a nutrient agar composed of glucose (2%), peptone (0.5%, Difco-Bacto), yeast autolyzate (10% v./v.), and agar (1%). Ten-milliliter portions were poured into lipless test tubes (150 × 15 mm.) containing a pinch of calcium carbonate, plugged with cotton, and sterilized at 15 pounds for 20 minutes. Before setting, the contents of the tubes were shaken to disperse the finely suspended calcium carbonate through the medium. In this way, growth of the lactobacillus cultures was easily detected by clearing of the medium. The cultures were maintained by weekly stab transfers followed by incubation at 45° C. until good growth oc-

curred (18 to 24 hours) and refrigerated until the next transfer.

Peel Juice–Yeast Autolyzate Medium. This medium contained 230 ml. of peel juice, 50 ml. of yeast autolyzate, and tap water to make a total volume of 500 ml. Ten-milliliter portions of this solution were placed into test tubes (150 × 20 mm.) containing an excess of calcium carbonate, plugged with cotton, and sterilized at 15 pounds for 20 minutes. This medium was used to grow inoculum starters for all fermentation runs.

Isolation of Lactobacillus Culture. Of several lactic acid bacteria examined, including *Lactobacillus delbrückii* (ATCC 9649), a naturally occurring one was found to give best results. This organism was isolated from a sample of fermenting grapefruit juice as follows: 1 ml. of a spontaneously fermenting grapefruit juice was added to 10 ml. of a nutrient glucose solution (culture maintenance medium without agar) and incubated for 24 hours at 45° C. A stab transfer was made into the same medium with agar, followed by a similar incubation period. By repeated subculturing at 45° C. a typical lactobacillus organism was obtained showing no evidence of gas production. The microorganism was isolated as a pure culture by plate dilutions in Brewer-type anaerobic dishes (*3*). (No growth could be obtained aerobically.) It grew as white, round, slightly elevated colonies and was able to produce acid from glucose, fructose, sucrose, and lactose without evidence of gas production. Microscopic examination revealed typical lactobacillus rods occurring singly and in chains of four or five units. It was easily cultured on peel juice–yeast autolyzate medium (liquid or in agar stabs) and showed a catalase negative reaction.

Table I. Recoveries of Added Lactic Acid after Cation Exchange[a]

Lactic Acid Added, Mg.	Titer after Ion Exchange[b], Ml. 0.1N NaOH/Ml. Supernatant	Lactic Acid Recovered, Mg.	Recovery, %
0	5.24	...	...
106	6.42	106	100
212	7.66	218	103
530	11.1	530	100
530	10.5	461	87

[a] $CaCO_3$ added in all but last test.
[b] Average of two determinations which were within ±1% of each other.

Table II. Increase in Acidity during a Lactic Acid Fermentation of Citrus Peel Juice as Measured by the Cation Exchange Method

Fermentation Time, Hr.	Increase in Titer, Ml. 0.1N NaOH/Ml. Sample	Lactic Acid, G./100 Ml.
0	...	...
24	4.2	3.78
46	6.1	5.49
71	6.5	5.85
96	7.1	6.39
144	7.9	7.11

Total sugars originally present, 7.55 grams/100 ml.
Residual sugars, 0.20 gram/100 ml.

Methods

Columns of Dowex-50, each having a bed depth of 13 cm. and bed volume of 40 ml., were set up for downflow operations. A constant level device which prevented draining and possible channelling in the beds was attached to each of the columns. When exhausted, the beds were regenerated with 2% hydrochloric acid solution followed by thorough rinsing with distilled water.

The fermentation sample to be analyzed was treated with an excess of calcium carbonate and Seitz-filtered to remove insolubles and bacterial growth. (Alternatively, the sample was heated on a boiling water bath for 5 minutes and centrifuged.) An aliquot of the clarified sample—1 ml. was found to be convenient—was passed through the column followed by elution with distilled water. The effluent, approximately 150 ml., was titrated with 0.1*N* sodium hydroxide using phenolphthalein as an indicator. A zero determination was made immediately after inoculation, and the subsequent increase in acidity was calculated as lactic acid. Total acidity can be assumed to be lactic acid when homofermentative organisms are used. Duplicate determinations on each sample were made at the same time through two different columns, so that any irregularity in the behavior of the columns could be detected.

Extent of fermentation—lactic acid yield—was calculated as the ratio of lactic acid produced to the total sugars originally present in the medium and expressed on a percentage basis. Total sugars were estimated after inversion by the method of Shaffer and Hartmann (*8*).

A recovery experiment was carried out as follows: 0, 1, 2, and 5 ml. of a standard lactic acid solution (1.18*N*) were added to 5 ml. of a partially fermented peel juice and diluted to 10 ml. with distilled water. Calcium carbonate was added to all but one of the solutions. After neutralization and centrifugation, 1-ml. portions of the supernatants were analyzed by the cation exchange method as outlined above.

Volatile acids were determined (*7*) in the acidified samples after a 6-day fermentation period and found to be less than 0.15 gram per 100 ml. calculated as acetic acid.

Fermentation Procedure

An 18- to 20-hour stab culture of the lactobacillus was used to inoculate 10 ml. of peel juice–yeast autolyzate medium containing an excess of calcium carbonate. After 24 hours of incubation, approximately 2.6% of lactic acid formed, which is 50% of the theoretical maximum based on total sugars originally present. This vigorously growing starter was used to inoculate 100 ml. of the medium contained in a 200-ml. bottle to give a surface area of 0.27 sq. cm. per ml. Liquid accessory growth factors (yeast autolyzate and tomato extract) were added on a per cent by volume basis and dried malt sprouts on a per cent by weight basis. All fermentation media were sterilized at 15 pounds for 20 minutes, allowed to cool, and equilibrated at 45° C. before addition of inoculum starters.

The fermentations were run in the presence of excess calcium carbonate which was added to the mash before sterilization. Continuous agitation in these runs was not possible; however, the bottles were shaken intermittently throughout the day and the pH remained at 5.7 ± 0.1. After standing overnight the pH usually fell 0.3 to 0.5 unit.

Results and Discussion

Results of the recovery experiment on the cation exchange method are shown in Table I. They indicate that close to 100% recoveries are possible, except where the lactic acid is not neutralized with calcium carbonate. Complete neutralization of free lactic acid may therefore be an essential step in the procedure. The cation exchange method as used here has consistently given reproducible results with an accuracy of ±1%. Table II shows a typical set of results obtained when a peel juice medium undergoes a lactic acid fermentation. A good correlation is obtained when in-

Table III. Lactic Acid Fermentations of Citrus Peel Juice

Total sugars, G./100 Ml.	Accessory Nutrient	Time of Fermentation, Days	Residual Sugars, G./100 Ml.	Lactic Acid, G./100 Ml.	Fermentation, %
8.00	10% malt sprouts	4	0.52	7.02	87.8
7.60	10% yeast autolyzate	4	0.50	6.66	87.6
7.60	10% tomato extract	5	1.00	6.40	84.2
10.95	10% malt sprouts	4	1.88	8.92	81.5
10.83	5% malt sprouts	4	1.85	8.25	76.2
7.50	Nil	6	1.48	5.50	73.2

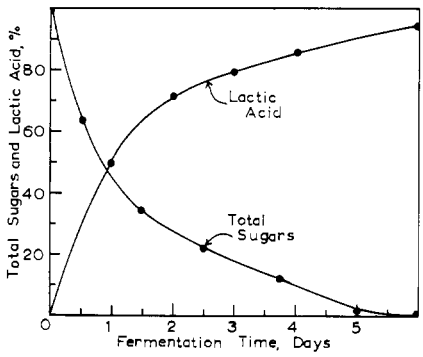

Figure 1. A peel juice–yeast autolyzate (10% v./v.) fermentation as measured by the cation exchange method

Total sugars at zero time was 7.60 grams/100 ml.

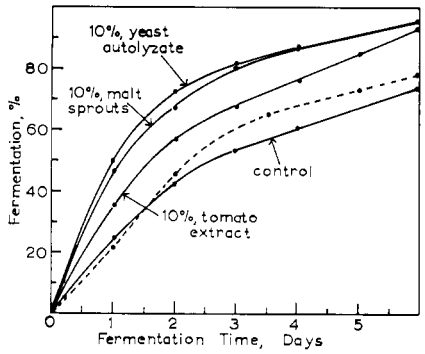

Figure 2. Effect of accessory nutrients on rate of peel juice fermentation using the selected lactobacillus

----A peel juice –yeast autolyzate (10% v./v.) fermentation using *Lactobacillus delbrückii* (ATCC 9649) under similar conditions

Table IV. Growth of Lactobacillus Cultures on Citrus Peel Juice Media after 24 Hours' Incubation at 45° C.

Medium	Selected Lactobacillus	*Lactobacillus delbrückii* (ATCC) 9649
Peel juice, 13° Brix		
Agar	++	+
Liquid	+++	−
Peel juice, 5° Brix		
Liquid	+++	++
Yeast autolyzate, agar	++++	++
Yeast autolyzate, peptone, agar	++++	+++

All media contained excess calcium carbonate.

crease in lactic acid is plotted *vs.* decrease in total sugars on a percentage basis (Figure 1).

The cation exchange method for determining acid production during fermentation in the presence of calcium carbonate considerably reduces the time required for analysis of fermentation runs. Using a single column, a determination can be obtained in 30 minutes, of which some 5 minutes are handling time. Furthermore, the acid produced is determined by a simple acid-base titration. The ease and rapidity of this method contrast with other analytical methods which have been employed for this type of study. The method used by Stiles and Pruess (*9*) requires a 5- to 7-hour extraction period and extreme care must be taken to ensure complete removal of sulfuric acid. The method of Friedmann and Graeser (*4*) involves a considerable outlay of apparatus and reagents, and the final titration of bound bisulfite is variable and influenced by several factors.

Previous investigations of lactic acid fermentation provide a sound basis for the study of a similar fermentation where a different source of fermentable carbohydrates is available (*5, 9, 10*). Those studies emphasized the importance of supplying accessory nutrients to achieve a high yield and rate of acid production. Table III shows that addition of yeast autolyzate or malt sprouts results in an efficient conversion of peel juice sugars to lactic acid. Tomato extract was not as beneficial as yeast autolyzate or malt sprouts, although a higher rate of lactic acid production was obtained than when no accessory nutrient was added. When peel juice containing a higher initial sugar concentration was fermented in the presence of malt sprouts, a corresponding increase in the rate of lactic acid production was obtained, at the expense, however, of a decreased efficiency.

Yeast autolyzate addition was consistently more effective than malt sprouts in stimulating the initial rate of fermentation; however, after 3 to 4 days the lactic acid yields in both cases were comparable (Figure 2). Tomato extract stimulated the initial fermentation rate, although not to the same extent as the yeast or malt adjuncts, but the lactic acid yield at the end of 6 days approached that attained by the latter additions. This result is of some interest, because preliminary experiments had shown that tomato extract was nearly equal to yeast autolyzate in stimulating the rate of acid production from a glucose basal medium. Citrus peel juice may possibly contain some of those factors present in tomato extract which are beneficial to the growth of lactobacillus organisms (*7*).

Residual sugars in the fermentor liquor after 6 days were reduced to 0.10 to 0.20 gram per 100 ml. in those runs where close to 95% of the theoretical maximum lactic acid was produced.

Lactobacillus delbrückii type of organism is usually selected for industrial lactic acid fermentations (*10*). When *Lactobacillus delbrückii* (ATCC 9649) was used to ferment peel juice with added yeast autolyzate (10% v./v.), the initial rate of fermentation and lactic acid yield at the end of 6 days were considerably lower than those given by the selected lactobacillus (Figure 2). Actually, this result was not unexpected because it was observed in a preliminary study that *Lactobacillus delbrückii* was not easily cultured on peel juice media (Table IV). On the other hand, the selected lactobacillus readily acclimatized to these media and grew vigorously whether or not they were fortified with nutrients.

The fermentation progress curves shown in Figure 2 indicate that citrus peel juice can undergo a 90%-efficient lactic acid fermentation in $4^1/_2$ to 5 days when suitable nutrients are added. Braverman (*2*) estimates that each ton of citrus fruit processed in the canning plant can give on the average 350 kg. of peel juice with about 8% of fermentable sugars. If this waste juice undergoes a 90%-efficient lactic acid fermentation, then each ton of fruit processed is capable of yielding 25 kg. of lactic acid. In the 1956–57 season, the state of Florida processed approximately 3,000,000 tons of citrus fruit which would be capable of yielding 150,000,000 pounds of crude lactic acid. Needle and Aries (*6*) predicted that the United States could utilize 200,000,000 pounds of plastic grade lactic acid if it were produced very cheaply. With improvements in refining methods and more efficient plants, citrus peel juice offers a potentially important raw material for producing lactic acid by fermentation.

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