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Growth Characteristics of Aciduric Sporeforming Bacilli Isolated from Fruit Juices

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ABSTRACT

Two aciduric, aerobic, sporeforming bacteria were isolated from pasteurized juices. The gram-positive, catalase-positive rods produced spores that were located subterminally in a swollen sporangium. The cultures had an optimal pH of 3.5–4.0 for growth and preferred potato dextrose agar over many of the rich media usually used for cultivating sporeforming bacteria. Spore inocula grew well in apple juice and white grape juice. Red grape juice was inhibitory, perhaps because of the concentrations of certain phenolic compounds. The spores were sufficiently heat resistant (D_{90} values of 16 to 23 min and z-values of 7.2 to 7.7°C) to survive commercial pasteurization processes.

Key Words: Aciduric spores, spoilage, fruit juices.

Most bacterial spores cannot germinate and grow in fruit juices and other acidic foods that have a pH under 4.0 (*1*). As a result, pasteurization processes utilizing temperatures of 85 to 95°C are often adequate for this class of foods. A few publications, however, have indicated that some rare sporeforming strains can grow at a pH of 3.7 or lower, and have been responsible for spoilage of acidified vegetables (*9*), peaches and pears (*10*), and wine (*3,5*).

Several years ago we isolated a sporeforming *Bacillus* from commercial pasteurized apple juice and from a hot-filled apple-cranberry beverage. Our isolates resemble cultures responsible for spoilage of apple juice in Germany (2) and of a juice blend in the United States (*6*), in that they prefer a pH under 5 and do not grow on the rich media that support the growth of most bacteria. In this report, data will be presented which show some of the factors that affect growth and death of these bacilli and thus affect their ability to spoil various fruit-juice beverages.

MATERIALS AND METHODS

Source of cultures.

The two cultures, VF and WAC, were obtained respectively from 16 oz. and 11.5 oz. containers of apple juice and apple-cranberry juice blend. Neither beverage was obviously spoiled (not turbid nor gaseous) although the apple juice had been submitted to our laboratory because of an off-odor. The beverages were passed through 0.45 µm membrane filters which then were placed on the surface of potato dextrose agar (PDA), pH 3.5 (since yeast contamination was suspected originally). The plates were incubated 5 to 7 days at 30°C. The incidence of contamination was not high. For example, filtration of 236 ml of apple juice through the membrane yielded only 76 colonies. Microscopic examination revealed the growth to be bacterial.

Spore crops.

The two isolates were used to inoculate the surface of PDA plates, pH 3.5, to obtain confluent growth. The spores were harvested after an incubation of 5 to 7 days at 43°C. After the culture was scraped from the plates, it was suspended in sterile water and then frozen.

Growth trials.

A 10^{-1} dilution of the stock spore suspension was heated 60 min at 60°C to activate dormant spores and to destroy vegetative cells. In a typical trial, 4.9 ml of a test broth was inoculated with 0.1 ml of the heated spores to give an initial population of about 10^6/ml. The culture tubes were incubated at 43°C. At 0 h and after subsequent incubation periods, appropriate decimal dilutions were plated on pH 5.6 PDA. The plates were incubated 5 days at 43°C before colonies were counted. Growth was expressed as the log10 of the final population minus $\log_{10}$ of the initial count ($\log N/N_0$). Negative values reflected death rather than growth of the organism.

The incubation temperature of 43°C was used because it yielded maximal colony counts and permitted good sporulation. A higher temperature might have afforded more rapid growth.

In general, the fruit juices were commercial samples purchased from local markets. Most of the grape juices, however, were prepared from fruit grown in our experimental vineyards. By using our own grapes, we could be assured that the juice did not contain added microbial inhibitors such as sulfites. The white grapes were crushed and pressed at room temperature while pigment was extracted from the red grapes by heating crushed fruit 15 min at 100°C prior to pressing.

Soluble solids were measured with a refractometer; a glass electrode was used to determine pH.

Two C_{18} Sep-Paks™ (Waters Associates) connected in series were used to remove phenolic fractions from grape juice (*7*). For the removal of acidic phenolics, 10 ml of juice was adjusted to pH 2.2 with hydrochloric acid (HCl) and then passed through C_{18} Sep-Paks™ that had been preconditioned with HCl. Neutral phenolics were removed by adjusting the juice to pH 7 with sodium hydroxide (NaOH) and then passing it through C_{18} Sep-Paks™ pretreated with methanol.

The samples of catechin, epicatechin and quercetin, neutral phenolics that were tested for inhibitory activity, were obtained from Sigma Chemical Co., St. Louis, MO. The catechin-gallate

was isolated from grapes using the procedure of Lee and Jaworski *(8)*.

Heat resistance.

A capillary-tube procedure was used in the heating trials *(9)*. A 10^{-1} dilution of the stock spores in the test juice was heat activated 60 min at 60°C. Then 0.04 ml of the suspension was flame sealed in 1.7 × 100 mm capillary tubes. After heating in a water bath for various times and temperatures, five replicate tubes were cooled, surface treated in 70% ethanol, and then crushed in 20 ml sterile water to release the spores. Appropriate dilutions were plated on PDA, pH 5.6; the plates were incubated 5 days at 43°C.

Incidence in commercial juices.

Juices purchased from markets were passed through 0.45 μm membrane filters, which then were incubated on PDA pH 3.5 for 14 days at 43°C. The volume of juice that was filtered varied with the size of the container and the suspended solids content of the juice, the latter because the filters often became plugged. Bacterial colonies were recognized by microscopic examination.

RESULTS AND DISCUSSION

General properties.

Both isolates were gram-positive, catalase-positive rods whose spores were located subterminally in swollen sporangia. Spores were first detected after an incubation of 24 h at 43°C on PDA, pH 3.5 or 5.6. The spores of VF were retained in the sporangium whereas those of WAC were more prevalent as free spores. Spore inocula of VF grew over a temperature range of 16 to 55°C, while the minimum temperature for strain WAC was about 25°C. Both isolates appeared to be obligate aerobes in that no growth was obtained in apple juice that had been steamed prior to inoculation to drive off dissolved oxygen.

Agar media.

As illustrated with isolate VF (Table 1), the spores of both strains grew over a wide pH range, although the recoveries were much lower at a neutral pH. In other trials in which Bacto mycological agar was used, similar results were obtained with both strains over the pH range of 3 to 5.5. When the pH was reduced by the addition of phosphoric, citric, or tartaric acids the three acidulants gave comparable results (data not shown).

The cultures were unique in that they did not grow in rich media that support the growth of many fastidious bacteria. Thus, no growth occurred in nutrient agar, trypticase soy agar, brain heart infusion agar, or veal infusion agar. No growth also was obtained in these media when they were acidified to pH 3.5 with tartaric acid. Growth was obtained, on the other hand, in the glucose, yeast extract, salts medium of Darland and Brock *(4)* over a pH range of 3.3 to 4.9.

TABLE 1. *Recovery of strain VF when spores were cultured on PDAs of different pH values.*

pH[a]	CFU/ml × 10^6
7.0	1.6
6.0	79
5.0	79
4.0	120
3.5	120
3.0	28
2.5	<0.1

[a] Adjusted with HCl or NaOH.

Fruit juices.

When heat-activated spores were inoculated into various commercial juice beverages, some were found to support growth, while only death occurred in others (Table 2). Tomato juice permitted the most growth, followed by apple juice. The higher pH of tomato juice along with its lower soluble-solids content may explain why it was the more favorable growth medium. A pH under 3.0 may be the reason why growth did not occur in some of the juices. Some differences were obtained between the two strains: VF grew in citrus juices and the tropical juice blend, while the WAC spores did not.

Our studies with various grape juices showed several factors that determined whether growth or death would occur. The soluble-solids concentration, for one, was important in that growth did not occur in the presence of the high sugar levels commonly found in grapes (Table 3). The Riesling grape juice of 21.6° Brix almost completely prevented growth, while reducing the sugar concentration to 16° Brix resulted in maximal growth. The fact that no growth also occurred when the juice was diluted with sugar solution indicated that the results were due to the high soluble-solids content rather than to the presence of other inhibitors in white juice. In similar studies with juice from the Seyval grape, 19.2° Brix was inhibitory, while maximal growth was obtained in 18.2° Brix juice (data not shown).

When grape juices were diluted to soluble-solids concentrations that would not be inhibitory, it was observed that death occurred in all of the red juices, whereas the

TABLE 2. *Growth of heat-activated VF and WAC spores in various commercial fruit-juice beverages.*

Juice	°Brix	pH	Growth[a] Log N/N_0 VF	WAC
Apple, brand A	11.4	3.5	1.3	1.8
brand B	11.0	3.5	1.1	1.6
Apple-grape-cherry blend	14.8	3.0	-1.4	-0.9
Apple-orange-pineapple	14.8	2.9	-0.1	0.3
Apple-raspberry-grape	12.2	2.8	-1.5	-1.1
Apple-red grape	12.4	3.7	-0.4	-1.0
Cranberry cocktail	14.0	2.4	-1.4	-1.3
Grape red, brand A	15.8	3.3	-0.7	-0.7
brand B	13.6	2.9	-1.2	-1.5
Grape-apple-cherry blend	12.4	3.7	0.7	-0.8
Grapefruit	10.4	3.2	0.3	-1.3
Orange	12.0	3.6	1.2	-0.8
Pineapple	13.4	3.3	0.4	-0.7
Pineapple-orange	12.8	3.4	0.3	-0.6
Prune	18.8	3.7	-0.6	-0.1
Tomato	7.0	4.0	2.3	2.4
Tropical fruit blend	13.6	3.7	1.2	-1.3

[a] Two-day incubation at 43°C.

TABLE 3. *Growth in Riesling grape juice diluted with water or 21.6% (wt/vol) glucose solution.*

% Riesling	Diluent	Final °Brix	Growth VF[a] Log N/N_0
100	None	21.6	0.1
75	Water	16.2	2.0
50	Water	10.8	2.0
25	Water	5.4	2.3
50	Glucose soln.	21.6	0.1
25	Glucose soln.	21.6	0.4

[a] Two days at 43°C.

white juices, with the exception of chardonnay, supported growth (Table 4). In other studies, red grape juice was mixed with white grape juice or apple juice to determine the concentrations that would be inhibitory. A 1:4 dilution of 20° Brix Concord juice in apple juice caused death of strain WAC and partially inhibited growth of strain VF (data not shown). In another trial, a 1:16 dilution of 20.4° Brix Gamay Noir juice in white Seyval grape juice caused death of Strain WAC, but at this dilution had no detectable inhibitory activity against Strain VF.

The fact that red grape juice was more inhibitory than white juice suggested that certain phenolic compounds might be involved. In general, red grape juice contains higher concentrations of phenolics than do white juices. To study this possibility, red Gamay Beaujolais juice was treated with C_{18} Sep-Pak™ cartridges, which had been preconditioned to remove neutral or acidic compounds. Removal of acidific phenolics did not convert the red grape juice into a favorable growth medium and thus it was concluded that compounds such as *trans*-caffeoyl tartaric acid and *cis*-coumaroyl tartaric acid were not the inhibitors. When neutral phenolics were removed, on the other hand, spore inocula were able to grow in the Gamay Beaujolais juice. To determine which of the neutral phenolics were inhibitory, various concentrations of some of the more prevalent compounds in grape juice were added to diluted apple juice. The results (Table 5) showed that catechin-gallate was inhibitory to both strains when present at a relatively high concentration of 1,000 mg/l. It is likely that other phenolics including anthocyanin pigments also are inhibitory and that the presence of a combination of compounds may exert an additive or synergistic effect.

The decrease in viable counts in red grape juice and other beverages may have resulted because the heat-activated spores germinated in the juice menstruum, but then subsequent outgrowth or vegetative cell division was blocked and, as a result, death occurred. Evidence for this was obtained when activated spores were incubated 1 h at 43°C and then reheated 15 min at 70°C to destroy any spores that had germinated and thus had lost their heat resistance. Spores incubated in diluted Seyval (white) and Gamay Beaujolais (red) juices showed respectively 82% and 85% germination, while those incubated in distilled water exhibited no loss of heat resistance, and thus no evidence of germination. The fact that the red and white juices yielded similar percentages indicates that some step other than germination was affected by the components of the red juice.

Heat resistance.

The spores of both strains possess sufficient heat resistance to survive the thermal process that is applied to many juices and other fruit products (Table 6). The D-values and z-values for the two strains were quite similar even though the juices differed in pH and concentration of soluble solids. The effect of these variables on the resistance of the aciduric bacilli is unknown, although spores are often more resistant when heated in solutions of higher pH and Brix.

Incidence.

The fact that commercial thermal processes were unlikely to kill spores of these strains raised the question as to the incidence of viable aciduric bacteria in various fruit juices. To study this question, 33 juice products were obtained from retail markets for culturing. The samples

TABLE 4. *Growth of VF and WAC spores in diluted juice of various grape cultivars.*

Cultivar	° Brix	pH	Growth[a] Log N/N_0 VF	WAC
White juices				
Riesling	10.8	3.4	2.0	1.9
Seyval	9.5	3.2		2.2
Chardonnay	11.3	3.3	-0.9	-1.0
Elvira	7.8	3.4	2.2	2.2
Cayuga White	8.6	2.8	1.8	0.17
Red juices				
Early Burgundy	9.1	3.5		-0.8
Gamay Noir	10.2	3.1		-0.9
Gamay Beaujolais	10.3	3.3	-1.2	-0.72
Pinotage	10.9	3.8	-1.0	-0.57
Cabernet Sauvignon	12.2	3.7	-1.0	-0.63
Concord	10.0	3.5	-1.2	

[a] Two days at 43°C.

TABLE 5. *Growth of spore inocula in 6° Brix apple juice containing different concentrations of added neutral phenolic compounds.*

Phenolic	mg/l	Growth[a] Log N/N_0 VF	WAC
Catechin	100	1.7	2.3
	200	1.7	2.3
	1000	1.9	2.9
Epicatechin	100	1.7	2.3
	200	1.8	2.3
	1000	1.9	2.6
Catechin-gallate	100	1.8	2.1
	200	1.8	2.1
	1000	-1.1	-0.8
Quercetin	100	1.9	2.2
	200	1.8	2.2
	1000	1.8	2.6

[a] Two days at 43°C.

TABLE 6. *Heat resistance of strains VF and WAC in apple and grape juice.*

Strain	Juice	°C	D-value[a]	z-value
VF	Apple, pH 3.5, 11.4° Brix	85	56 ± 14 min	7.7°C
		90	23 ± 7.5	
		95	2.8 ± 0.7	
WAC	Grape, pH 3.3, 15.8° Brix	85	57 ± 13 min	7.2°C
		90	16 ± 4.1	
		95	2.4 ± 0.9	

[a] Average of 5 trials ± standard deviation.

included 8 apple, 7 grape, 3 cranberry, 3 cherry and 12 juice blends. Only 3 of the 33 samples yielded viable spores. One apple juice gave a count of 25 per 300 ml, one grape juice yielded 1 spore per 100 ml and one cherry-flavored juice had a count of 3 per 245 ml. It was concluded that although viable aciduric spores are not common, they can be recovered if one cultures enough samples.

Identity.

Further work is needed before it can be known whether isolates VF and WAC represent previously undescribed species. Cerny, Hennlich and Poralla (*2*) believed that their isolate from spoiled apple juice was a strain of *Bacillus acidocaldarius*, the species first described by Darlan and Brock (*4*). Their identification was based partly on the presence of ω-cyclohexane fatty acids and hopanoids. Our isolates resemble *B. acidocaldarius* in that they grow at a low pH and are fastidious in their nutritional requirements. They differ, however, in their growth temperatures. The *B. acidocaldarius* cultures of Darland and Brock (*4*) grew within the temperature range of 45 to 70°C, while our cultures were less thermophilic with a temperature range of under 20 to about 55°C.

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