FOOD BIOLOGICAL CONTAMINANTS

Isolation of *Alicyclobacillus acidoterrestris* from Fruit Juices

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K agar, a novel isolation medium developed for the food industry, was compared with other acidified media for isolation of *Alicyclobacillus acidoterrestris* spores. Spores were inoculated into apple juice, orange juice, and a fruit juice blend and then isolated on the following media: K agar, pH 3.7; semi-synthetic medium, pH 4.0; orange serum agar, pH 3.5; and minimal salts medium, pH 4.0. Media were incubated at 24, 35, 43, and 55°C. Highest recovery of spores was obtained with either K agar or semi-synthetic medium, incubated at 43°C. The effect of heat shocking spores at different times was also determined; heat shocking at 80°C for 10 min was considered appropriate. Peptone, previously shown to inhibit *A. acidoterrestris*, was not inhibitory when present in K agar. A collaborative trial with 9 laboratories was undertaken to determine the repeatability and reproducibility of counts on K agar. K agar prepared from individual components was compared with dehydrated K agar prepared by International BioProducts (Redmond, WA). There were no significant differences between log mean counts for the 2 media for each of the juices analyzed at both the high and the low inoculum levels. Repeatability and reproducibility values were not significantly different either within juices, within trials, or across all samples tested in both trials. K agar is suitable for isolation of *A. acidoterrestris* spores from fruit juices.

Acidophilic sporeformers were first isolated in 1967 from hot springs in Japan (1) and later from soil (2). Those from hot springs had a higher optimum growth temperature and were termed *Bacillus acidocaldarius* (3). Strains isolated from soil were termed *B. acidoterrestris* (4). In 1992, the creation of a new genus, *Alicyclobacillus*, was proposed, to comprise the species *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus* (5). Comparative rDNA sequence analyses showed that the 3 strains were sufficiently different from other *Bacillus* spp. to warrant reclassification in a new genus. Also, *Alicyclobacillus* spp. are unique in their fatty acid profiles, containing ω-alicyclic fatty acid as the major natural membranous lipid component. *Alicyclobacillus acidoterrestris* has been reported to cause juice spoilage, manifested as an off flavor and light cloudiness (6, 7).

A number of media have been used to isolate or grow *A. acidoterrestris*, including potato dextrose agar and orange serum agar (2, 7–12). A study was undertaken to compare K agar (pH 3.7) with media typically used to isolate bacteria from acid products (13, 14). Media included orange serum agar (Difco Laboratories, Detroit, MI); tomato juice agar, special (TJAS; Difco); potato dextrose agar (PDA; Difco), each adjusted to pH 3.5, 4.0, 4.5, and 5.0 using HCl; and dextrose tryptone agar (DTA; Difco) pH 7.4, (14). Higher recovery rates and more rapid growth were observed on K agar.

The objective of this study was to undertake further analysis of the isolation procedure for *A. acidoterrestris*, including comparing K agar with semi-synthetic medium (SSM), pH 4.0 (3), and minimal salts medium with agar (MSM), pH 4.0 (8). In a previous report (15), peptone was shown to inhibit *Alicyclobacillus* spores. Because peptone is an ingredient in K agar, an objective of this study was to determine whether peptone had an impact on spore counts. A collaborative trial with 9 laboratories was undertaken to evaluate the repeatability and reproducibility counts on K agar. K agar prepared from individual ingredients was compared with dehydrated media from International Bioproducts, Redmond, WA. Additionally, a filtration method and a preincubation method were evaluated because low numbers of *A. acidoterrestris* found in juice concentrates (<10 spores/mL) can subsequently cause spoilage when juice is diluted to single strength (16).

Materials and Methods

Part 1. Effect of Heat Shock on Spores of A. acidoterrestris

(**a**) *Source of microorganisms*.—Eleven isolates of acidophilic sporeformers were obtained from industry sources and from Don Splittstoesser, Cornell University (Ithaca, NY; 14).

(**b**) *K agar*.—2.5 g yeast extract; 5.0 g peptone; 1.0 g glucose; 1.0 g Tween 80; 15 g agar; 25% (w/v) malic acid solution, filter-sterilized—use to adjust pH after autoclaving (15 min at 121°C); 990 mL deionized water.

(**c**) *Spore crops*.—Strains were inoculated into OSB, pH 3.5, and incubated at 43°C until turbid (2–5 days). From OSB, 2.0 mL was inoculated onto the surface of potato dex-

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trose agar (pH 3.5, adjusted with tartaric acid) plates (ten 150 × 50 mm plates). Plates were incubated at 43°C for 5–7 days. Spores were harvested by scraping growth from the agar with a sterile glass "hockey stick," and washing the spores 3 times in sterile distilled water. Spores were suspended in sterile distilled water and stored at 4°C.

(**d**) *Comparison of heat shock regimens for spores in juices*.—Three heat shock regimens were compared for isolation and enumeration of spores inoculated into apple juice. A composite of 4 strains of *A. acidoterrestris* was inoculated into apple juice at a level of ca 10 spores/mL. Juices (3 × 10 mL tubes per heat shock) were heat-shocked at 60°C for 10 min, 80°C for 10 min, and 100°C for 5 min, and then plated in duplicate on K agar and incubated at 43°C for up to 5 days. Tubes were immersed in a water bath adjusted to the appropriate temperature, to a depth above the level of juice in the tube. The temperature was checked by using an extra tube filled with a similar sample of juice, fitted with a thermometer. Once the juice reached the appropriate temperature, the timer was started, and tubes were held until the time period was complete. Tubes were cooled in an ice bath immediately after the heat shock treatment. Three replicate experiments were undertaken.

(**e**) *Effect of heat shock time at 80°C*.—A composite of spores (4 strains) was inoculated into apple juice at a level of ca 10^3 spores/mL. Juices (3 × 10 mL tubes) were heated at 80°C for 5, 10, 15, 20, 25, and 30 min as described above (*Part 1*, [**d**]). Tubes were plunged into ice, and then plated onto K agar in duplicate. Three replicate experiments were undertaken.

(**f**) *Data analysis*.—Data were analyzed for significant differences using a one-way analysis of variance (SigmaStat, SPSS Science, Chicago, IL).

Part 2. Comparison of Isolation Media

(**a**) *Strains*.—Spore crops were prepared as described above (*Part 1*, [**c**]). A cocktail of 4 strains of spores was used for this experiment.

(**b**) *Comparison of isolation procedures*.—Apple juice, orange juice, and a fruit juice blend containing mainly white grape juice (100% fruit juice) were used in this study. Juices were inoculated with spores at a level of 10^2 spores/mL. Spores were isolated by a pour plate method on the following media: minimal salts medium with agar (MSM), pH 4.0 (8); semi-synthetic medium (SSM), pH 4.0 (3); orange serum agar (OSA, Difco), pH 3.5 (adjusted with HCl); and K agar, pH 3.7. Isolation was determined with and without a heat shock at 80°C for 10 min. Media were incubated at 24, 35, 43, and 55°C for up to 2 weeks.

(**c**) *Data analysis*.—Data were analyzed for significant differences using a one-way analysis of variance (SigmaStat).

Part 3. Effect of Peptone on A. acidoterrestris

(**a**) *Strains*.—Spore crops were prepared as described above (*Part 1*, [**c**]). A cocktail of 11 strains of spores was used for this experiment, and ATCC strain 49028, a reference strain, was tested separately.

(**b**) *Experimental protocol*.—K agar was prepared with and without peptone (Bacto Peptone, Difco). Spores were heat-shocked at 80°C for 10 min, and then serially diluted in 0.1% (w/v) peptone water and spread-plated on each of the agars, in duplicate. Plates were incubated at 43°C for 3 days.

(**c**) *Data analysis*.—Data were analyzed for significant differences using *t*-tests (SigmaStat).

Part 4. Collaborative Trial

(**a**) *Collaborators*.—Nine laboratories collaborated in the trial. The objective of the study was to evaluate the repeatability and reproducibility of the National Food Processors' Association (NFPA)'s isolation procedure for *A. acidoterrestris*. K agar prepared from individual ingredients was compared with dehydrated media from International Bioproducts (IBP). A filtration method and a preincubation method were also evaluated.

(**b**) *Strains*.—Spore crops were prepared as described above (*Part 1*, [**c**]). A cocktail of 11 strains of spores was used for this experiment.

(**c**) *Juice samples*.—Apple juice (pH 3.8, 11.5° Brix), apple juice concentrate (pH 3.3, 72° Brix), white grape juice (pH 3.9, 16 ° Brix), and orange juice (pH 3.8, 11.8° Brix) were used as test samples for the collaborative trial. Two independent trials were undertaken.

(**d**) *Sample preparation*.—(*1*) *Apple/ orange/white grape juices*: Juice (600 mL) was split into 2 equal portions. One portion was inoculated with a cocktail of 11 strains of spores at a low level (ca 40/mL), and one portion was inoculated at a high level (ca 400/mL). Each collaborator received two 15 mL aliquots from each portion of juice. Negative controls were prepared from uninoculated juices. Each collaborator received one 15 mL uninoculated control per juice. (*2*) *Apple juice concentrate for filtration and pre-incubation study*: Apple juice concentrate (600 mL) was inoculated with a low level of spores (ca 1/mL). Each collaborator received four 15 mL aliquots of inoculated apple juice concentrate and one 15 mL aliquot of uninoculated apple juice concentrate as a negative control.

(**e**) *Shipping*.—All samples were shipped on the same day as they were prepared, in prechilled containers in an upright position. Enough frozen gel packs were used to maintain refrigeration temperatures for 24 h. Samples were shipped by priority overnight delivery.

Collaborator Procedures

(**a**) *Media preparation*.—K agar was prepared from individual ingredients and by using IBP dehydrated K agar according to manufacturer's instructions.

(**b**) *Plating on K agar (negative control, juices)*.—Juices were heat-shocked at 80°C for 10 min, and then pour-plated using both K agar prepared from individual ingredients (for 3 plates) and IBP K agar for the other 3 plates. Plates were incubated at 43°C for 3 days.

(**c**) *Filtration study*.—Apple juice concentrates were heat-shocked at 80°C for 10 min, and then mixed (10 mL) with 50 mL sterile distilled water in a sterile flask, in dupli-

cate. Each 60 mL volume was filtered through a 0.45 μm membrane. Membranes were placed on K agar and incubated at 43°C for 3 days. All colonies on membranes were counted and expressed as count per 10 mL.

(**d**) *Pre-incubation study*.—Apple juice concentrates (10 mL) were heat-shocked at 80°C for 10 min, and then mixed with sterile distilled water (50 mL), in a sterile flask, in duplicate. Flasks were incubated at 43°C for 48 h. A loopful of juice was streaked onto 2 K agar plates to check for growth.

(**e**) *Data analysis*.—Precision estimates were obtained as described in the AOAC INTERNATIONAL guidelines (17). Log mean counts within laboratories and between laboratories were compared using software provided by AOAC (Calculation of AOAC Performance Parameters—Balanced/Unbalanced Replicates, Rev. 12/10/97). The microorganism, matrix, mean microorganism count, standard deviations for repeatability (S_r) and reproducibility (S_R), and relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R) for each juice were tabulated. Repeatability values (r) and reproducibility values (R) were calculated as $2.8 \times S_r$ and $2.8 \times S_R$, respectively. Log mean counts and repeatability and reproducibility values for the 2 media for each juice for each trial were compared using a *t*-test. Data on repeatability and reproducibility were then combined and an analysis of variance was undertaken. Outlying data were identified by the Cochran test and the Grubbs test as recommended by AOAC, and were discarded.

Results and Discussion

Effect of Heat Shock

At low inoculum levels, heat shocking at 80°C for 10 min gave significantly higher counts ($P < 0.05$) than heat shocking at 60°C for 10 min or 100°C for 5 min (Table 1). When spores were inoculated at a higher level (10^3 spores/mL) and were heat-shocked at 80°C for varying lengths of time, there were no significant differences in counts obtained at each heating time (Table 1). Splittstoesser et al. (15) observed that heat shocking at 60°C for 30 min doubled the viable counts when compared with no heat shock, whereas Pettipher et al. (11) showed no differences in the count per mL when a heat treatment of 80°C for 10 min was compared with no heat treatment. Of the regimens considered, heat activation at 80°C for 10 min appears to be appropriate.

Comparison of Isolation Procedures

Good recovery of spores was observed on K agar from all juices tested (Table 2). Counts were significantly higher on K agar and SSM than on OSA at all temperatures tested ($p < 0.05$). Pettipher et al. (11) found OSA to be the optimum isolation medium of 5 tested; however, they did not indicate the pH of the isolation media. Highest counts on K agar were observed when plates were incubated at 43°C; counts were either higher or not significantly different from counts on SSM at this temperature when isolated from apple juice or the juice blend. Spore counts from orange juice were significantly higher on SSM than on K agar ($p < 0.05$), except at 35°C without a heat shock, where there was no significant difference (Table 2). Colonies were visible on K agar at 43°C in 3 days as compared with 4 days at 35 and 55°C and 7–11 days at 24°C. Spores germinated and grew on SSM at 43°C within 2–3 days, but in some cases, colonies spread over the medium, making counting difficult. Heat shocking the juices resulted in significantly higher counts ($p < 0.05$) in almost all instances; however, numerically, the differences were not very large (<0.5 log). MSM with agar proved unsuitable for isolation of

Table 1. Effect of heat shock regimen on isolation of spores inoculated into apple juice

Heat shock regimen	Mean plate count, $\log_{10}$ spores/mL (low inoculum)	Mean plate count, $\log_{10}$ spores/mL (high inoculum)
60°C/10 min	1.38	ND[a]
80°C/5 min	ND	3.60
80°C/10 min	1.51	3.53
80°C/15 min	ND	3.60
80°C/20 min	ND	3.60
80°C/25 min	ND	3.49
80°C/30 min	ND	3.44
100°C/5 min	0.3	ND

[a] ND = not determined.

Table 2. Effect of isolation medium, incubation temperature, and heat shock on enumeration (cfu/mL) of *A. acidoterrestris* from inoculated fruit juices

Isolation medium[a]	24°C No HS/HS[b]	35°C No HS/HS	43°C No HS/HS	55°C No HS/HS
		Apple Juice		
K	6/19	105/165	176/224	63/158
SSM	91/219	90/256	98/154	76/158
OSA	2/5	13/19	9/16	1/1
		OrangeJuice		
K	17/32	116/167	155/194	39/108
SSM	60/160	115/323	220/423	188/338
OSA	42/66	36/138	86/140	ND/1[c]
		Juice blend		
K	9/27	120/186	217/212	69/194
SSM	56/100	55/142	50/106	38/103
OSA	3/5	8/19	8/16	ND/5

[a] Data for MSM omitted.

[b] HS = heat shock at 80°C for 10 min.

[c] ND = none detected.

Table 3. Effect of peptone in K agar on recovery of spores of *A. acidoterrestris*

Strains	Mean count on K agar with peptone, log_{10} spores/mL	Mean count on K agar without peptone, log_{10} spores/mL
ATCC 49028	6.33	6.34
Composite	6.57	6.62

A. acidoterrestris; the medium did not solidify well and colonies were transparent, making enumeration very difficult (data not shown). MSM was developed as a broth for continuous culture, not an agar, which may explain these results. Although counts were higher on SSM when isolated from orange juice, SSM must be prepared from numerous individual ingredients, making preparation cumbersome. Therefore, isolating spores on K agar at 43°C was considered the most suitable procedure.

Effect of Peptone

There were no significant differences in counts on K agar with or without peptone, for either the ATCC strain 49028 or the cocktail of 11 strains (Table 3). Colonies of ATCC 49028 on K agar without peptone were more likely to be spreaders rather than forming distinct colonies as on K agar with peptone; therefore, addition of peptone to the agar facilitated enumeration of individual colonies. With the cocktail of strains, no obvious differences were observed in colonial appearance based on the presence of peptone. Based on these results, we conclude that in K agar, peptone did not have an inhibitory effect on the strains tested.

Collaborative Trial Using K Agar

There were no significant differences between log mean counts for the 2 media for each of the juices analyzed at both the high and the low inoculum levels (Tables 4 and 5). Repeatability values also were not significantly different either within juices, within trials, or across all samples tested in both trials. The reproducibility was significantly better when IBP media were used for apple juice ($p < 0.05$) and white grape juice ($p < 0.05$) in Trial 1, but not in Trial 2. When data were combined there were no significant differences, and so this may have been an artifact of the sample size. Therefore, IBP media will give results equally as good as media prepared with individual ingredients, in terms of the plate count and repeatability of the count within laboratories.

For the filtration study, sufficient data for analysis were available only from Trial 2. Usable results (excluding outliers) from 8 laboratories are needed to analyze the data according to

Table 4. Repeatability and reproducibility values for *A. acidoterrestris* isolated from inoculated fruit juices (Trial 1)[a]

Inoculum level, log_{10} spores/mL	K agar	Mean	r	S_r	RSD_r, %	R	S_R	RSD_R, %
Apple juice								
1.60	S	1.51	0.30	0.11	7.04	0.66	0.24	15.57
1.60	IBP	1.62	0.16	0.06	3.47	0.19	0.07	4.20
2.60	S	2.52	0.15	0.05	2.07	0.61	0.22	8.70
2.60	IBP	2.63	0.12	0.04	1.57	0.24	0.09	3.20
Orange juice								
1.60	S	1.66	0.36	0.13	7.7	0.69	0.25	14.85
1.60	IBP	1.72	0.24	0.09	4.99	0.24	0.09	4.99
2.60	S	2.71	0.13	0.05	1.70	0.33	0.12	4.39
2.60	IBP	2.62	0.08	0.03	1.14	0.68	0.24	9.32
White grape juice								
1.60	S	1.54	0.25	0.09	5.69	0.62	0.22	14.43
1.60	IBP	1.68	0.20	0.07	4.28	0.33	0.12	6.98
2.60	S	2.51	0.12	0.04	1.72	0.75	0.27	10.60
2.60	IBP	2.63	0.13	0.05	1.73	0.23	0.08	3.13

[a] Abbreviations: S = from scratch (i.e., individual ingredients); IBP = International Bioproducts; r = repeatability value; R = reproducibility value; S_r = standard deviation for repeatability; S_R = standard deviation for reproducibility; RSD_r = relative standard deviation for repeatability; RSD_R = relative standard deviation for reproducibility.

Table 5. Repeatability and reproducibility values for *A. acidoterrestris* isolated from inoculated fruit juices (Trial 2)[a]

Inoculum level, $\log_{10}$ spores/mL	K agar	Mean	r	S_r	RSD_r, %	R	S_R	RSD_R, %
				Apple juice				
1.60	S	1.60	0.13	0.05	3.06	0.35	0.13	7.91
1.60	IBP	1.60	0.16	0.06	3.65	0.28	0.10	6.33
2.60	S	2.56	0.10	0.04	1.40	0.29	0.10	4.07
2.60	IBP	2.53	0.08	0.03	1.15	0.21	0.08	3.00
				Orange juice				
1.60	S	1.67	0.06	0.02	1.34	0.29	0.1	6.21
1.60	IBP	1.65	0.14	0.05	2.98	0.30	0.11	6.48
2.60	S	2.68	0.14	0.05	1.85	0.28	0.10	3.76
2.60	IBP	2.65	0.22	0.08	2.92	0.27	0.10	3.65
				White grape juice				
1.60	S	1.62	0.19	0.07	4.11	0.35	0.13	7.81
1.60	IBP	1.65	0.17	0.06	3.6	0.25	0.09	5.39
2.60	S	2.62	0.09	0.03	1.25	0.28	0.10	3.79
2.60	IBP	2.62	0.11	0.04	1.5	0.25	0.09	3.40

[a] Abbreviations: S = from scratch (i.e., individual ingredients); IBP = International Bioproducts; r = repeatability value; R = reproducibility value; S_r = standard deviation for repeatability; S_R = standard deviation for reproducibility; RSD_r = relative standard deviation for repeatability; RSD_R = relative standard deviation for reproducibility.

AOAC procedures. Only 6 sets of usable results were obtained in Trial 1 once outlying data were discarded. The filtration method was a new method for collaborators, which may explain why insufficient results were available from the first trial. Repeatability values were within the range of those obtained for the plate count but reproducibility values were higher (Table 6).

Variable results were obtained for the pre-incubation study. In Trial 1, 7 laboratories reported no growth in either flask, whereas 2 reported growth in one of the 2 flasks incubated. In Trial 2, 4 laboratories reported no growth in either flask, whereas 5 laboratories reported growth, with 4 reporting one flask positive and one reporting both flasks positive. It is not clear why there were variable results; the samples used were from the same batch as those used in the filtration study, the results of which confirmed that *A. acidoterrestris* was present.

Isolation on K agar proved highly repeatable and reproducible. In initial studies, the medium was prepared from individual ingredients; it can now be purchased dehydrated from International BioProducts. Higher recovery was obtained on K agar than on OSA, whereas comparable results were obtained with SSM. The latter medium contains many ingredients and is cumbersome to prepare. Where low numbers of spores were present, use of filtration to concentrate spores gave more consistent results than the use of a pre-incubation method to determine presence or absence of spores. K agar is a suitable isolation medium for *A. acidoterrestris* from fruit juices.

Table 6. Repeatability and reproducibility values for *A. acidoterrestris* isolated from inoculated apple juice concentrate using a filtration procedure (data from trial 2)[a]

Product	Inoculum level, log spores/10 mL	Mean	r	S_r	RSD_r, %	R	S_R	RSD_R, %
Apple juice concentrate	1.60	1.36	0.33	0.12	8.6	0.91	0.33	23.92

[a] Abbreviations: r = repeatability value; R = reproducibility value; S_r = standard deviation for repeatability; S_R = standard deviation for reproducibility; RSD_r = relative standard deviation for repeatability; RSD_R = relative standard deviation for reproducibility.

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