

Efficacy of Bioside HS 15% and Reflex Against Planktonic *Alicyclobacillus acidoterrestris* at Three Different Concentrations; 50 ppm, 100 ppm, and 200 ppm as Peracetic Acid

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23rd May 2012

Introduction

The contamination of fruit juices by thermophilic acidophilic bacteria (TAB) has become a growing issue for fruit juice producers. The most common TAB is *Alicyclobacillus acidoterrestris*. *A. acidoterrestris* is a spore forming, gram-positive, rod-shaped bacteria that is able to survive normal pasteurization techniques. *A. acidoterrestris* is an aerobic, soil dwelling, non-pathogenic bacteria, but fruit juice contaminated with *A. acidoterrestris* gives off a foul odor reminiscent of a disinfectant¹. Most bacteria cannot survive in acidic environments such as fruit juices, or conventional pasteurization, but *A. acidoterrestris* releases spores which are very difficult to destroy due to their ability to survive at extreme temperatures and pHs. The optimum growth condition for *A. acidoterrestris* is at a temperature range between 30-70°C and pH range between 2-7.² Conventional fruit juice pasteurization techniques (92°C for 10 seconds) are not adequate enough to deactivate the spores. Therefore, producers must seek other preventative measures to control *A. acidoterrestris* contamination. The reason *A. acidoterrestris* grows so well in fruit juices is because it consumes vanillin and converts it to guaiacol, which gives the tainted fruit juice the disinfectant-like aroma.

Purpose

The purpose of this experiment is to determine the efficacy of two different peracetic acid formulations, Reflex and Bioside HS 15%, against vegetative *Alicyclobacillus acidoterrestris* and at 50 ppm, 100 ppm, and 200 ppm as peracetic acid, and to determine if the Reflex at 100 and 200 ppm as PAA has any affect on the *Alicyclobacillus acidoterrestris* spores.

¹ Mirtha Nelly Uboldi et al *Alicyclobacillus* in Orange Juice: Occurrence and Heat Resistance of Spores. Journal of Food Protection, Vol 6, Number 8, August 1999, pp. 883-886

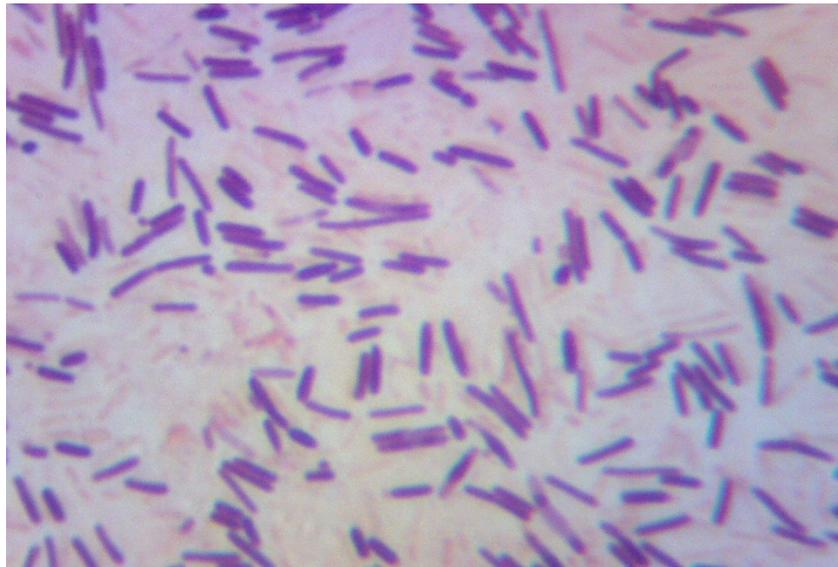
² Su-Sen Chang and Dong-Hyun Kang. *Alicyclobacillus* spp. in the Fruit Juice Industry: History, Characteristics, and Current Isolation/Detection Procedures. Critical Reviews in Microbiology, 30:55-74, 2004.

Methods and Materials

Vegetative Cells

An *Alicyclobacillus acidoterrestris* (ATCC 49025) pellet was reconstituted using orange serum broth (Alpha Bioscience cat no. 015-106). The bacterial solution was plated on potato dextrose agar (Hardy Diagnostics cat no. W60) and incubated at 55°C for 72 hours. After 72 hours there was a moderate amount of bacterial growth on the potato dextrose agar. Five single colonies were selected and streaked on five different potato dextrose agar plates. *Alicyclobacillus acidoterrestris* is a gram positive rod-shaped bacterium that produces a positive catalase test. After 72 hours the second set of plates were examined. The colonies on the potato dextrose agar plate were round, off-white, and slightly raised, which is distinctive for *Alicyclobacillus acidoterrestris* colonies. Confirmation was supported by Gram stain and a positive catalase test. Image 1 is a still image of the Gram stain results on the second growth cycle.

Image 1: Gram stain of an *Alicyclobacillus acidoterrestris* colony from the second growth cycle.



Using a sterile plate spreader, the bacteria was transferred from the potato dextrose plates to approximately 550mL sterile phosphate buffer. A 20mL aliquot was removed, serially-diluted, and plated on potato dextrose agar. This served as the control. Immediately thereafter, the remaining bacteria/buffer solution was divided into six 500mL samples. Table 1 is a description of the six test solutions.

Table 1 shows the type and concentration of peracetic acid used in each test solution.

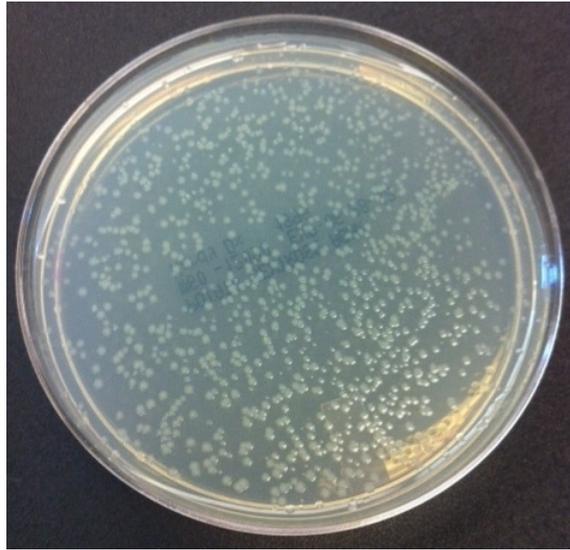
Description	Concentration PAA
Sample solution 1	50ppm from Reflex®
Sample solution 2	100ppm from Reflex®
Sample solution 3	200ppm from Reflex®
Sample solution 4	50ppm from Bioside HS 15%®
Sample solution 5	100ppm from Bioside HS 15%®
Sample solution 6	200ppm from Bioside HS 15%®

The Reflex (lot# 37-12-0515-2) used tested at a peracetic acid (PAA) concentration of 6.27% and a hydrogen peroxide (H₂O₂) concentration of 24.29%. The Bioside HS 15% (lot# 33-12-0514-4) used had a PAA concentration of 15.27% and a H₂O₂ concentration of 22.50%. Prior to the addition of PAA the pH of the test solution was measured at 7.17. Samples 1, 2, and 3 were dosed with 0.40, 0.80, and 1.60 mL Reflex, respectively. Samples 4, 5, and 6 were dosed with 0.16, 0.32, and 0.64 mL Bioside HS 15%, respectively. The PAA concentration was tracked over the 10 minute time interval to ensure that the level of active PAA remained at the target concentration. Aliquots (20mL) were taken at 1, 5, and 10 minutes with the residual H₂O₂ and PAA in the 20mL aliquots being neutralized using a 1% erythorbic acid. Aliquots were then serially diluted and plated on potato dextrose agar plates. The pH of each sample was taken immediately after the addition of PAA and after the 10 minute time interval. Plates were incubated at 55°C for 72 hours upon which they were enumerated.

Spores

Individual colonies were selected from the first set of potato dextrose agar plates and plated on six new potato dextrose agar plates. These 3 plates were incubated at 55°C for 72 hours alongside the six plates used in the vegetative cell sample set. Using a sterile plate spreader the *Alicyclobacillus acidoterrestris* colonies were transferred to a 1000mL sterile buffered solution. The solution was heated at 90°C for 10 minutes in order to obtain spores and destroy the vegetative cells. The solution was allowed to cool to room temperature. A 10mL aliquot was removed, serially-diluted, and plated on potato dextrose agar. The remaining solution was divided into two equal samples, Sample A and Sample B. Sample A and Sample B were dosed with 200 and 100ppm PAA from Reflex. Prior to the addition of PAA the pH was 7.01. Sample A was dosed with 1.60mL Reflex (lot# 37-12-0515-2) and Sample B was dosed with 0.80mL Reflex. Twenty milliliter aliquots were taken at 1, 5, and 10 minutes, neutralized with a 1% erythorbic acid solution, and plated on potato dextrose agar plates. The concentration of PAA was tracked over the 10 minute time interval. Plates were incubated at 55°C for 6 days to allow sufficient time for the surviving spores to germinate. After 6 days the plates were removed from the incubator and enumerated.

Image 2: *Alicyclobacillus acidoterrestris* colonies on potato dextrose agar.



Results and Discussion

Vegetative Cells treated with Reflex and Bioside HS 15%

Table 2 compares the average \log_{10} CFU/mL remaining, \log_{10} reduction, and percent reduction of planktonic *Alicyclobacillus acidoterrestris* treated with 50 ppm PAA from Reflex and Bioside HS 15%. Reflex had better efficacy at 50 ppm at all times compared to Bioside HS 15%. The control averaged a \log_{10} of 6.00 CFU/mL. At one minute the Reflex had a \log_{10} of 4.85 corresponding to a 1.15 CFU/mL (92.921%) reduction. Bioside HS 15% at one minute had a \log_{10} of 5.92 CFU/mL corresponding to a moderate 0.08 CFU/mL (16.82%) reduction. After 10 minutes Reflex had zero remaining CFU/mL while Bioside HS 15% had a \log_{10} of 4.06 CFU/mL corresponding to a 1.94 CFU/mL (98.852%) reduction. Therefore, it can be concluded that Reflex is more efficacious than Bioside HS 15% against planktonic *Alicyclobacillus Acidoterrestris* at 50 ppm.

Table 2: Results from 50ppm PAA of Reflex and Bioside HS 15%

Description	Log ₁₀ Remaining	Log ₁₀ reduction	Percent Reduction
Control	6.00	N/A	N/A
50 ppm Reflex 1 minute	4.85	1.15	92.921
50 ppm Reflex 5 minute	4.54	1.46	96.533
50 ppm Reflex 10 minute	0.00	>5.99	>99.999
50 ppm Bioside HS 1 minute	5.92	0.08	16.824
50 ppm Bioside HS 5 minute	5.81	0.19	35.435
50 ppm Bioside HS 10 minute	4.06	1.94	98.852

Table 3 compares the average Log₁₀ CFU/mL remaining, log₁₀ reduction, and percent reduction of planktonic *Alicyclobacillus acidoterrestris* treated with 100 ppm Reflex and Bioside HS 15%. Reflex had better efficacy at 100 ppm at one and five minute contact times compared to Bioside HS 15%. The control averaged a log₁₀ of 6.00 CFU/mL. At one minute the Reflex had a log₁₀ of 4.74 corresponding to a 1.26 CFU/mL (94.505%) reduction. Bioside HS 15% at one minute had a log₁₀ of 5.17 CFU/mL corresponding to a moderate 0.83 CFU/mL (85.209%) reduction. By 10 minutes both Bioside HS15% and Reflex had zero remaining CFU/mL for a >99.999% reduction. Therefore, it can be concluded that Reflex® is more efficacious than Bioside HS 15% against planktonic *Alicyclobacillus Acidoterrestris* at 100 ppm.

Table 3 Results from 100ppm PAA from Reflex® and Bioside HS 15%

Description	Log ₁₀ Remaining	Log ₁₀ reduction	Percent Reduction
Control	6	N/A	N/A
100 ppm Reflex 1 minute	4.74	1.26	94.505
100 ppm Reflex 5 minute	4.48	1.52	96.980
100 ppm Reflex 10 minute	0	>5.99	>99.999
100 ppm Bioside HS 1 minute	5.17	0.83	85.209
100 ppm Bioside HS 5 minute	5.12	0.88	86.817
100 ppm Bioside HS 10 minute	0	>5.99	>99.999

Table 4 compares the average log₁₀ CFU/mL remaining, log₁₀ reduction, and percent reduction of planktonic *Alicyclobacillus acidoterrestris* treated with 200 ppm PAA from Reflex and Bioside HS 15%. Reflex had slightly better efficacy at 200 ppm at one and five minute contact

times compared to Bioside HS 15%. The control averaged a log₁₀ of 6.00 CFU/mL. At one minute the Reflex had a log₁₀ of 4.68 corresponding to a 1.32 CFU/mL (95.214%) reduction. Bioside HS 15% at one minute had a log₁₀ of 4.80 CFU/mL corresponding to a 1.20 CFU/mL (93.690%) reduction. By 10 minutes both Reflex and Bioside HS 15% had zero remaining CFU/mL which a >99.999% reduction. Therefore, it can be concluded that Reflex is more efficacious than Bioside HS 15% against planktonic *Alicyclobacillus Acidoterrestris* at 200 ppm

Table 4 Results from 200ppm PAA from Reflex® and Bioside HS 15%

Description	Log₁₀ Remaining	Log₁₀ Reduction	Percent Reduction
Control	6.00	N/A	N/A
200 ppm Reflex 1 minute	4.68	1.32	95.214
200 ppm Reflex 5 minute	4.39	1.61	97.545
200 ppm Reflex 10 minute	0.00	>5.99	>99.999
200 ppm Bioside HS 1 minute	4.80	1.20	93.690
200 ppm Bioside HS 5 minute	4.78	1.22	93.974
200 ppm Bioside HS 10 minute	0.00	>5.99	>99.999

Spores treated with Reflex®

Table 5 compares the average log₁₀ CFU/mL remaining, log₁₀ reduction, and percent reduction of planktonic *Alicyclobacillus Acidoterrestris* spores treated with 100 and 200 ppm PAA from Reflex. The control averaged a log₁₀ of 5.02 CFU/mL. At one minute with 100 ppm PAA (from Reflex) the average log₁₀ was 4.74 CFU/mL which represents a moderate 0.28 CFU/mL (47.5%) reduction. At one minute with 200 ppm PAA from Reflex the average log₁₀ was 4.67 CFU/mL which is only a 0.35 CFU/mL (55.3%) reduction. By 10 minutes the log₁₀ remaining for 100 and 200 ppm PAA was 4.51 and 4.49 CFU/mL respectively. This data suggests that Reflex at 100 and 200 ppm PAA is only mildly efficacious against planktonic *Alicyclobacillus acidoterrestris* spores.

Table 5 Results from the *Alicyclobacillus acidoterrestris* spores treated with 100 and 200 ppm PAA from Reflex

Description	Log ₁₀ Remaining	Log ₁₀ reduction	Percent Reduction
Control	5.02	N/A	N/A
100 ppm Reflex 1 minute	4.74	0.28	47.5
100 ppm Reflex 5 minute	4.59	0.43	62.8
100 ppm Reflex 10 minute	4.51	0.51	69.1
200 ppm Reflex 1 minute	4.67	0.35	55.3
200 ppm Reflex 5 minute	4.57	0.45	64.5
200 ppm Reflex 10 minute	4.49	0.53	70.5

Conclusions

Alicyclobacillus Acidoterrestris Vegetative Cells

- 50 ppm PAA from Reflex is more efficacious against planktonic compared to 50 ppm Bioside HS 15% at contact times of 1, 5, and 10 minutes.
- 50 ppm PAA from Reflex had a log₁₀ reduction of 1.46 CFU/mL which is a 96.533% within a five minute contact time, while Bioside HS 15% at 50 ppm PAA and a contact time of five minutes only had a log₁₀ reduction of 0.19 (35.435%). At ten minutes contact time Reflex had zero remaining CFU/mL while Bioside HS 15% still had a log₁₀ of 4.06 CFU/mL representing a log₁₀ 1.94 CFU/mL reduction.
- At a concentration of 100 ppm PAA Reflex had a log₁₀ reduction of 1.26 and 1.52 CFU/mL at one and five minute contact time, respectively, while Bioside HS 15% had a log₁₀ reduction of 0.83 and 0.88 CFU/mL at one and five minute contact times, respectively. By 10 minute contact time both Reflex and Bioside HS 15% at 100 ppm had zero remaining colonies. This data suggests that Reflex is more efficacious against planktonic *Alicyclobacillus acidoterrestris* than Bioside HS 15% at 1, 5, and 10 minute contact times.
- 200 ppm PAA from Reflex had a log₁₀ reduction of 1.32 and 1.61 CFU/mL at 1 and 5 minute contact time, respectively, while Bioside HS 15% had a log₁₀ of 1.20 and 1.22 CFU/mL at 1 and 5 minute contact time, respectively. At 10 minute contact time there were zero remaining colonies at 200 ppm PAA from both Reflex® and Bioside HS 15%.
- Overall, Reflex was more efficacious than Bioside HS 15% against *Alicyclobacillus acidoterrestris* but, there was not a significant difference in efficacy when comparing 50, 100 and 200 ppm PAA. On the other hand, increasing the contact times significantly increases the efficacy of Reflex.
- While eradication of *Alicyclobacillus acidoterrestris* in the fruit juice itself is probably impractical due to its reactivity with components present in the juice, it would appear that

Reflex would be particularly suitable as a surface and equipment sanitizer. In order to provide sufficient contact time for total eradication, it is recommended that Reflex is used in conjunction with Perafoam, an additive mixed with the product at the point of use. Perafoam provides the Reflex with vertical surface cling properties to prolong the time that it remains in contact with the surface.

Alicyclobacillus Acidoterrestris spores treated with Reflex®

- 100 ppm PAA from Reflex was only slightly efficacious against *Alicyclobacillus acidoterrestris* spores. At one minute contact time the log₁₀ reduction was only 0.28 CFU/mL which is only a 47.519% reduction. By five minutes the log₁₀ reduction was 0.43 CFU/mL which is only 62.846% reduction. After 10 minutes there was only a log₁₀ reduction of 0.51 CFU/mL.
- 200 ppm PAA from Reflex was slightly more efficacious than 100 ppm PAA but still did not show a significant reduction. At 1, 5, and 10 minutes the log₁₀ reduction was 0.35, 0.45, and 0.53 CFU/mL respectively.
- Reflex proved to be slightly efficacious against the very resistant *Alicyclobacillus acidoterrestris* spores in solution at 100 and 200 ppm PAA at contact times of 1, 5, and 10 minutes.