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Short communication

Mitigation of *Alicyclobacillus* spp. spores on food contact surfaces with aqueous chlorine dioxide and hypochlorite

Loretta M. Friedrich [a], Renee Goodrich-Schneider [b], Mickey E. Parish [c], Michelle D. Danyluk [a,*]

[a] *Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Department of Food Science and Human Nutrition, 700 Experiment Station Road, Lake Alfred, FL 33850, USA*
[b] *Department of Food Science and Human Nutrition, Institute of Food and Agricultural Sciences, P. O. Box 110370, Gainesville, FL 32611, USA*
[c] *Department of Nutrition and Food Science, University of Maryland, 0112 Skinner Building, College Park, MD 20742, USA*

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ABSTRACT

The prevalence of *Alicyclobacillus* spp. and other spore-forming spoilage organisms in food handling and processing environments presents a sanitation challenge to manufacturers of products such as juices and beverages. The objectives of this study were to determine the efficacy of chlorine dioxide and sodium hypochlorite in killing *Alicyclobacillus* spores *in situ* and to evaluate the efficacy of various chlorine dioxide and hypochlorite sanitizing regimes on *Alicyclobacillus* spp. spores on stainless steel, wood, and rubber conveyor material. Five or two log CFU/ml spore concentrations were left in aqueous solution or inoculated onto stainless steel, rubber, or wood coupons and challenged with sanitizer for varied time intervals. After treatment, the coupons were placed in sterile sample bags, massaged with neutralizing buffer, and enumerated on Ali agar. Surfaces were also examined before and after treatment by scanning electron microscopy to confirm destruction or removal of the spores. For both five and two log CFU/ml spore concentrations, treatments of 50 and 100 ppm of chlorine dioxide and 1000 and 2000 ppm of hypochlorite, respectively, were the most effective. Of the range of chlorine dioxide concentrations and contact time regimes evaluated for all surfaces, the most effective concentration/time regime applied was 100 ppm for 10 min. Reductions ranged from 0 to 4.5 log CFU/coupon. Chlorine dioxide was least effective when applied to wood. Hypochlorite was not efficient at eliminating *Alicyclobacillus* spores from any of the food contact surfaces at any time and concentration combinations tested. Chlorine dioxide is an alternative treatment to kill spores of *Alicyclobacillus* spp. in the processing environment.

1. Introduction

During the last 20 years, the bacterium *Alicyclobacillus* has been recognized as a significant spoilage organism in beverages. Spoilage by *Alicyclobacillus* results in a flat sour type spoilage with off flavor and cloudiness. The production of guaicol and halogenated phenols is responsible for the primary off flavor, characterized as smoky and medicinal (Cerney et al., 1984; Göçmen et al., 2005). *Alicyclobacillus* is a genus of gram-positive, rod-shaped, spore-forming acidophilic thermophiles. These characteristics combined make *Alicyclobacillus* a challenging spoilage agent in fruit juices and other beverages (Pettipher et al., 1997). The spore-forming nature of these organisms enables them to survive typical pasteurization treatments, then germinate and grow, leading to spoilage in acidic products stored at warm temperature common to many shelf stable products. For example, the D-value at 90 °C can be as high as 1–23 min in orange juice and apple juice respectively (Silva and Gibbs, 2001).

Spoilage caused by *Alicyclobacillus* often results in large economic losses and has become a prime concern in the quality control of acidic beverages. *Alicyclobacillus* spp. are soilborne organisms that may typically enter the processing facility on the surfaces of fruits and vegetables. Food contact surfaces may be contaminated directly by fruit and serves as a point for cross-contamination (Wisse and Parish, 1998). In addition to fruit surfaces and concentrates, *Alicyclobacillus* spp have been isolated from soil (outside processing facilities and in orchards), wash, flume and evaporator water, debris from factory floors and vinegar flies (Groenewald et al., 2009).

Due to the varied processing equipment, line configurations and subsequent thermal processing, juice processing plants have few standardized sanitation protocol. For example, in citrus juice production, incoming fruit is destemmed and washed with acid fruit wash products through brush washers, with a potable water rinse. Conveyor equipment is often washed and sanitized on an as-needed

* Corresponding author. Tel.: +1 863 956 1151x1252.
E-mail address: mddanyluk@ufl.edu (M.D. Danyluk).

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basis, depending on individual protocols. Generally, clean-in-place (CIP) procedures are employed for juice contact surfaces such as piping, tanks and evaporators. CIP cleaning and sanitizing occurs on an intermittent basis (8–48 h) with dilute (1.5–2%) solutions of sodium hydroxide at temperatures ranging from 50 to 60 °C over a wide range of times. The caustic treatment is followed by a thorough rinse with potable water, typically with the determination of pH to ensure the complete removal of any residue. Clean-out-of-place equipment (COP) is generally soaked, then scrubbed with approved cleaners, generally alkaline in nature, which are rinsed in potable water at ambient temperatures. Post-thermal treatment systems (chillers, filler surge tanks, fillers) are typically sanitized after cleaning with hot, potable water (90 °C) to prevent cross-contamination with thermally-treated juice. Aseptic systems such as aseptic packaging systems and aseptic storage tanks have a specific cleaning and sterilization protocols of their own and were not considered in this project. While current sanitizing practices, along with pasteurization and/or evaporation, have apparently been adequate to control most microbial spoilage and pathogenic organisms, it is unlikely that these protocols are sufficient for the control of spore-forming organisms such as *Alicyclobacillus* spp.

Chlorine dioxide is a strong oxidizing and sanitizing agent and may have a practical application for sanitizing surfaces within the food industry. Lee et al. (2004) have shown that aqueous chlorine dioxide at 40–120 ppm is effective in killing spores of *Alicyclobacillus acidoterrestris* on apple surfaces. In contrast, Orr and Beuchat (2000) determined reduction in *Alicyclobacillus* spore populations after 1 min exposure to 500 ppm chlorine dioxide did not inactivate spores more than 1-log unit on apple surfaces. Differences may be due in part to the inoculation and recovery techniques used, and the variety of apple used.

Typical sanitation regimes (aqueous chlorine between 50 and 200 ppm) applied to food contact surfaces, have been reported to give reductions of between ca. 0.4 and 2.2 log CFU/ml spores of *Alicyclobacillus* in suspended water solution (Orr and Beuchat, 2000). However, when Podolak et al. (2009) treated *A. acidoterrestris* spores in apple juice on stainless steel surfaces with chlorine dioxide and hypochlorite under conditions similar to those seen in clean-in-place (CIP) operations, they achieved a ca. 2 log CFU/cm^2 reductions. Presently, there are no sanitizing regimes shown to effectively eliminate *Alicyclobacillus* spores on a variety of potential food contact surfaces.

Information regarding the effectiveness of chlorine dioxide in killing spores on food contact surfaces is needed to aid in the development of optimal sanitation conditions for beverage processors. The objectives of this study were to (i) determine the efficacy of aqueous chlorine dioxide and sodium hypochlorite in killing *Alicyclobacillus* spores in solution and (ii) evaluate the efficacy of aqueous chlorine dioxide and hypochlorite in reducing spore numbers of *Alicyclobacillus* on food contact surfaces.

2. Materials and methods

2.1. Selection of test strains

A mixture of five strains of *Alicyclobacillus* spp. isolated from fruit concentrates or soils were used. Strains studied (identified by their strain reference number) and their sources included: *A. acidoterrestris* (MDD 268; frozen concentrated orange juice from the Netherlands); *A. acidoterrestris* (MDD 267; frozen concentrated orange juice from Brazil); *A. acidoterrestris* (MDD 180; soil from a citrus grove in Brazil); *Alicyclobacillus acidocaldarius* (MDD 266; frozen mango concentrate from Mexico); and *A. acidocaldarius* (MDD 265; frozen coconut cream from the Dominican Republic). All strains were identified by 16S rDNA sequencing and analysis with NCI-BLAST.

2.2. Inoculum preparation

Prior to the experiment, a loopful of frozen stock culture of each *Alicyclobacillus* strain was inoculated by streaking onto *Alicyclobacillus* agar (AA; Wisse and Parish, 1998) and incubated at 45 °C until 90% sporulation was achieved (5–10 days). The formulation for AA includes in g/L distilled water: 0.4 $(NH_2)SO_4$; 1.0 $MgSO_4 \times 7H_2O$; 0.5 $CaCl \times 2\ H_2O$; 6.0 KH_2PO_4; 2 glucose; 4 soluble starch; and 4 yeast extract. The pH is adjusted to 3.5 with a (1 N) H_2SO_4 solution before autoclaving. An equal volume of, Bacto-agar (Difco Laboratories, Detroit, MI) was prepared to a final concentration of 3.5%. The two solutions are autoclaved and tempered to 50 °C before mixing and pouring (Wisse and Parish, 1998). Sporulation was determined using a hemacytometer. Spores were then harvested by depositing 1–2 ml of sterile water onto the agar surface and gently rubbing with a glass rod. Pooled suspensions from 15 plates of each strain were centrifuged at 4000 × *g* 10 min at 23 °C and subsequently washed six times in sterile distilled water. Spores in the final pellet were resuspended in 50 ml of sterile water and enumerated following serial dilutions in sterile water, plating onto AA and incubating at 45 °C for 48 h. Equal volumes of a 1×10^7 CFU/ml stock of each strain were combined to construct the 5-strain culture cocktail. The spore cocktail was stored at −20 °C until needed. Prior to use, to determine the viable number of spores in the cocktail, the spore cocktail was thawed at ambient temperature for approximately 15 min and diluted to the appropriate concentration in sterile deionized water and enumerated by plating onto AA, and incubating at 45 °C for 48 h. An appropriate dilution of the spore cocktail in sterile deionized water was used as the inoculum.

2.3. Preparation of chlorine dioxide and hypochlorite

Chlorine dioxide sanitizer (100 ppm) was prepared according to the manufacturer's instructions (ZEP Manufacturing Co., Atlanta, GA) in sterile deionized water and diluted to 0, 10, 20, and 50 ppm solutions in sterile deionized water. The level of free chlorine dioxide was measured with the LaMotte 1200 colorimeter DPD chlorine dioxide and verified with the included test strip (ZEP Manufacturing Co.); pH was also measured (Table 1).

A hypochlorite solution was also prepared (from household bleach; Clorox company, Oakland, CA, 6% sodium hypochlorite) at concentrations of 0, 100, 200, 500, 1000, and 2000 ppm in sterile deionized water. The levels of free hypochlorite, using the LaMotte 1200 colorimeter DPD chlorine, and the pH was measured (Table 1).

2.4. Chemical treatment of spores

The spore cocktail, diluted in sterile water (0.1 ml), was added to 4.9 ml of each concentration of chlorine dioxide (0, 5, 10, 50, and 100 ppm) and hypochlorite (0, 100, 200, 500, 1000, and 2000 ppm) to achieve concentrations of approximately 2 and 5 log CFU/ml and stirred continuously at ambient temperature. At timed intervals of 0, 5, 10, 20, and 30 min for the low spore level and 0, 2, 5, 10, 15, 20, 25, 30, and 60 min for the high concentration, 1 ml of the sample was removed and placed immediately into 9 ml of Dey–Engley (DE)

Table 1
pH of treatment solutions.

Sanitizer	Concentration (ppm)				
	5	10	20	50	100
Chlorine dioxide	3.6	2.8	2.7	2.5	2.3
	100	200	500	1000	2000
Sodium hypochlorite	7.3	8.8	10.0	10.4	10.47