Destruction of *Alicyclobacillus acidoterrestris* Spores in Apple Juice on Stainless Steel Surfaces by Chemical Disinfectants

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ABSTRACT

A study was conducted to determine the effects of three commercially available disinfectants on the reduction of *Alicyclobacillus acidoterrestris* spores in single-strength apple juice applied to stainless steel surfaces. Apple juice was inoculated with *A. acidoterrestris* spores, spread onto the surface of stainless steel chips (SSC), dried to obtain spore concentrations of approximately $10^4$ CFU/cm², and treated with disinfectants at temperatures ranging from 40 to 90°C. The concentrations of disinfectants were 200, 500, 1,000, and 2,000 ppm of total chlorine for Clorox (CL) (sodium hypochlorite); 50, 100, and 200 ppm of total chlorine for Carnebon 200 (stabilized chlorine dioxide); and 1,500, 2,000, and 2,600 ppm for Vortexx (VOR) (hydrogen peroxide, peroxyacetic acid, and octanoic acid). For all temperatures tested, VOR at 2,600 ppm (90°C) and CL at 2,000 ppm (90°C) were the most inhibitory against *A. acidoterrestris* spores, resulting in 2.55- and 2.32-log CFU/cm² reductions, respectively, after 2 min. All disinfectants and conditions tested resulted in the inactivation of *A. acidoterrestris* spores, with a maximum reduction of >2 log CFU/cm². Results from this study indicate that *A. acidoterrestris* spores, in single-strength apple juice, may be effectively reduced on stainless steel surface by VOR and CL, which may have practical applications in the juice industry.

*Alicyclobacillus acidoterrestris* is a common and problematic spore-forming spoilage microorganism in both fresh and pasteurized fruit juices (16, 18, 26). *A. acidoterrestris* is a thermophilic acidophilic bacterium, which means it is able to grow in the acidic environments of many fruit juice products at pH values ranging from 2.5 to 6.0 and at elevated temperatures of 25 to 60°C (18, 25). Even a standard hot fill and hold process for fruit juices of 88 to 96°C for approximately 2 min does not destroy commonly found levels of *A. acidoterrestris* spores (3). *Alicyclobacillus* species are soilborne microorganisms and most likely enter processing plants on fruit and vegetables contaminated by the soil during harvest (31). In addition to soil, water used as an ingredient for juice production has been reported as a source of *A. acidoterrestris* contamination (15).

Microbial colonization of food processing surfaces has been extensively investigated (23, 33). It is defined as the attachment of bacterial cells or spores to solid surfaces conditioned with nutrients sufficient for cell growth or spore germination (34). Microbial colonization of food processing equipment typically occurs with the formation of biofilms of vegetative bacteria. However, surviving spores present in a postpasteurized juice may also attach to the surface of clean processing equipments. Spore attachment is influenced by the cell’s surface charge (8), hydrophobicity (5), extracellular polysaccharides (7), and environmental conditions (21). In general, increased surface hydrophobicity enhances bacterial attachment (5). For example, *Bacillus* spores, which have a hydrophobic surface due to their outer coat proteins, show enhanced attachment on hydrophobic surfaces compared to vegetative cells (5, 9, 28). Therefore, spores adhere to stainless steel more easily than do vegetative cells (19) and have greater resistance to environmental stresses such as sanitizers and heat (22). Microbial attachments on industrial food processing surfaces are undesirable because they increase the risk of cross-contamination between food products (10). To prevent this, the bacteria and their spores need to be either physically removed from the surface or destroyed.

Surface contamination through microbial attachment can be controlled by disinfection processes. There is limited information on the efficacy of chemical disinfectants for the inactivation of *A. acidoterrestris* spores. Orr and Beuchat (16) reported reductions of ca. 2.2, 0.4, and 0.1 log CFU of *A. acidoterrestris* spores per ml in a five-strain mixture when spores were suspended in water solutions of 200 ppm of chlorine, 500 ppm of acidified sodium chlorite, and 0.2% H₂O₂, respectively, for 10 min at 23°C. When treated with either 1,000 ppm of chlorine or 4% H₂O₂, the number of spores was reduced by >5 log.

To date, there is little information regarding the effects of chemical disinfectants applied at elevated temperatures for inactivating spores of *A. acidoterrestris* in apple juice on stainless steel surfaces. The application of this study...
could improve the sanitation of postpasteurization equipment such as coolers, piping, and filling equipment through the use of chemical disinfectants applied at elevated temperatures during cleaning operations. Thus, the objective of this study was to investigate the effects of chemical disinfectants on the survival of spores of *A. acidoterrestris* applied on stainless steel surfaces in apple juice.

**MATERIALS AND METHODS**

**Strain and production of spores.** *A. acidoterrestris* strain N-1100, isolated from apple juice concentrate, was obtained from the Grocery Manufacturers Association culture collection. The N-1100 strain was chosen for this study because it has been shown to be more resistant to chemical disinfectants (16). Bacterial cells were grown at 43°C for 2 days in *Alicyclobacillus* broth (ALIB) at pH 3.5. ALIB was prepared from its constituent ingredients according to the method of Evancho and Walls (6). *Alicyclobacillus* agar (ALIA; pH 3.7) and *Bacillus acidoterrestris* thermophilic agar (pH 4.0) (18, 20). Preparation of *A. acidoterrestris* spores was done according to the method of Orr and Beuchat (16). After reaching 80 to 90% sporulation, as determined by microscopic examination, 5 ml of sterile distilled water was added onto the surface of each plate. Spores were gently scrapped off with a sterile hockey stick and transferred into a sterile centrifuge tube. Pooled suspensions from 15 plates were centrifuged at 3,000 × g for 15 min at room temperature. The supernatant fluid was discarded, and the resulting spore pellet was resuspended in 5 ml of sterile distilled water. This procedure was repeated twice. The final pellet was resuspended in 50 ml of sterile distilled water. Spore crop suspensions were stored at 4°C until used. To determine the spore crop titer, 1 ml was transferred into 9 ml of 0.1% peptone water (Fisher Scientific, Fair Lawn, NJ) with 0.5% sucrose (PS; pH 4.0) (11), heated for 10 min at 80°C, serially diluted in PS, and plated (0.1 ml) on duplicate ALIA plates (16). Plates were incubated at 43°C for 2 to 3 days, and colonies were enumerated by standard plate counting techniques. Spore suspensions contained approximately 10⁹ CFU/ml. This suspension was used in studies to determine the efficacy of disinfectants in the destruction of *A. acidoterrestris* spores.

**Preparation of disinfectants.** Solutions of the disinfectants were prepared up to the maximum concentrations according to manufacturer's recommendation, using sterile distilled water. Federal regulations (21 CFR Part 178) permit the use of sanitizing solutions containing sodium hypochlorite in an amount not to exceed 200 ppm chlorine, and if higher concentrations are used, the surface must be rinsed with portable water after sanitizing. The disinfectants included a commercial brand of sodium hypochlorite (Clorox [CL], 6%; The Clorox Co., Oakland, CA); stabilized chlorine dioxide solutions (2%, Carnebon 200 [CAR]; Engelhard Corp., North Kingston, RI); and Vortexx (VOR), containing 6.9% hydrogen peroxide, 4.4% peroxyacetic acid, and 3.3% octanoic acid (Ecolab, Inc., St. Paul, MN). The following concentrations of disinfectants were used in this study: 500, 1,000, and 2,000 ppm of total chlorine followed by the water rinse and 200 ppm with no water rinse for sodium hypochlorite; 50, 100, and 200 ppm of total chlorine for stabilized chlorine dioxide; and 1,500, 2,000, and 2,600 ppm for VOR.

**Disinfectant treatment of *A. acidoterrestris* spores in apple juice on stainless steel surfaces.** Precut stainless steel chips (SSC; test surface type 304, no. 4 finish, 1.3 by 1.3 cm) were obtained from the G&H Sheet Metal Works, Inc. (Hillside, NJ). The SSC used in this study were moderately rough when compared to other finishes and have distinct flaws that could harbor spores (27). SSC were washed in a warm detergent solution (Suma Ultra, JohnsonDiversey U.S., Cincinnati, OH) for 30 min, rinsed in distilled water, air dried at room temperature, and autoclaved. The sterile SSC were aseptically placed in a petri dish. Single-strength apple juice (pH 3.5; 11.5 Brix, Lucky Leaf) was inoculated with the *A. acidoterrestris* spore suspension at 1% (vol/vol) to obtain an inoculum level of 1⁰ CFU/ml. Aliquots (100 μl) of inoculated apple juice were dispensed and spread onto the surface of SSC to obtain a spore concentration of 1⁰ to 1⁴ CFU/cm². The control SSC were prepared in the same manner as the inoculated SSC; however, sterile water was substituted for the inoculum. All SSC were dried for 2 h in the biosafety hood with the ventilation fan on. Following the drying process, each chip was removed with sterile forceps and rinsed with 10 ml of sterile distilled water to remove nonadhering spores; this was repeated five times. One milliliter of the last rinse was plated in ALIB and incubated at 43°C for 3 days to determine if all nonattached spores had been removed. Inoculated and control SSC were placed in a sterile test tube. Each tube contained 10 ml of a selected disinfectant solution at the tested concentrations. Duplicate inoculated test tubes (one chip per test tube) were exposed to the test disinfectant solutions in solution for 1 or 2 min in a water bath with no stirring at temperatures recommended by the manufacturers: 60°C for sodium hypochlorite and VOR, and 40°C for chlorine dioxide. In addition, all disinfectants were used at 90°C, mirroring standard juice industry sanitation procedures. For sodium hypochlorite treatment at the concentration of 500, 1,000, and 2,000 ppm of chlorine, the SSC were rinsed with sterile water at 60°C for 2 min to remove any residual chlorine. The control samples were prepared in the same way as treated samples; however, sterile water at 60°C was substituted for disinfectant. After exposure, all treated and control SSC were aseptically removed by sterile forceps, without touching the surface, and immediately placed in a petri dish containing 10 ml of Dey-Engley neutralizing broth (pH 7.0; Difco, Becton Dickinson, Sparks, MD) for 1 min (13, 14, 16). Each SSC was removed and placed in a sterile test tube containing 10 ml of PS and 0.5 g of sterile 3-mm-diameter glass beads. The test tubes were vortexed for 2 min to dislodge attached spores.

To enumerate viable spores surviving treatments, the test tubes were heated at 80°C for 10 min and then serially diluted in PS, plated on ALIA (pH 3.7) plates, and incubated at 43°C for 3 days. The reduction in *A. acidoterrestris* spores due to the disinfectants (log CFU per test surface) was calculated by subtracting the number of spores on the chips remaining after selected disinfectant treatment from the number of spores remaining after treatment with sterile distilled water (control samples).

**Statistical analysis.** A three-factor general linear model was employed to assess the effect of disinfectants on the reduction of *A. acidoterrestris* attached to stainless steel. Experimental data was subjected to Minitab 1.4 statistical software (Minitab Inc., State College, PA) for analysis of variance and the least significant differences test to determine significant differences (P < 0.05) between the mean numbers of spore survivors after disinfectant treatments for a given time. Diluted samples were plated in duplicate. The experiment was replicated three times.

**RESULTS AND DISCUSSION**

This study was designed to evaluate the effects of three commercially available disinfectants on the survival rate of...
TABLE 1. Population of spores of A. acidoterrestris applied to a stainless steel surface, surviving treatment with chemical disinfectants at 40 and 60°C, and revived on ALIA

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Concentration (ppm)</th>
<th>1 min</th>
<th>2 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clorox(^b) at 60°C</td>
<td>0 (control)</td>
<td>3.84 A</td>
<td>3.87 A</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>2.48 B</td>
<td>2.05 B</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2.46 B</td>
<td>1.97 BC</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1.78 C</td>
<td>1.78 C</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>1.77 C</td>
<td>1.77 C</td>
</tr>
<tr>
<td>Carnebon 200(^c) at 40°C</td>
<td>0</td>
<td>3.98 A</td>
<td>3.87 A</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.85 A</td>
<td>3.62 AB</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.64 A</td>
<td>3.16 AB</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>2.25 B</td>
<td>2.21 B</td>
</tr>
<tr>
<td>Vortex(^d) at 60°C</td>
<td>0</td>
<td>4.02 A</td>
<td>3.94 A</td>
</tr>
<tr>
<td></td>
<td>1,500</td>
<td>2.56 B</td>
<td>2.01 BC</td>
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<td></td>
<td>2,000</td>
<td>2.32 B</td>
<td>1.77 C</td>
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<tr>
<td></td>
<td>2,600</td>
<td>2.00 BC</td>
<td>1.77 C</td>
</tr>
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</table>

\(^a\) Values within a column followed by different uppercase letters are significantly different (\(P \leq 0.05\)).
\(^b\) Sodium hypochlorite.
\(^c\) Stabilized chlorine dioxide.
\(^d\) Hydrogen peroxide, peroxyacetic acid, and octanoic acid.

A. acidoterrestris spores in single-strength apple juice applied to stainless steel surfaces. Populations of A. acidoterrestris spores surviving treatment with CL and VOR at 60°C and CAR at 40°C are presented in Table 1. As the concentrations of chemical disinfectants increased, the reductions in counts of A. acidoterrestris were greater. A maximum spore reduction of >2 log CFU/cm\(^2\) was obtained for treatment with CL at 1,000 ppm, and a 1.7 log CFU/cm\(^2\) reduction was obtained for treatment with CAR at its highest concentration (200 ppm). The increase in the time of disinfection from 1 to 2 min with CL and CAR was a relatively unimportant variable (\(P > 0.05\)) in the reduction of A. acidoterrestris spores. However, concentrations of VOR and time of disinfection correlated significantly (\(P \leq 0.05\)) to the reduction of A. acidoterrestris spores in apple juice. When treated with 2,600 ppm of VOR for 2 min, the number of spores was reduced by 2.2 log.

The effect of disinfectant treatments at 90°C is shown in Table 2. These time/temperature parameters (1 to 2 min at 90°C) were chosen because they reflect typical parameters for hot water circulation of the disinfection on equipment surfaces as a part of the “clean in place” cycle in the juice industry. A similar trend in spore reductions was observed as the temperature of applied disinfectants was increased from 40 to 90°C; hence, reduction was greater as the concentration of disinfectants increased. CL, CAR, and VOR, at all tested concentrations, significantly contributed (\(P \leq 0.05\)) to the reduction of A. acidoterrestris spores. At each increasing concentration of the disinfectants, a higher number of spores were inactivated. At the highest concentration tested (200 ppm), a 2.1-log reduction was achieved at 2 min. At the same concentration (200 ppm), CAR was more effective than CL in eliminating spores of A. acidoterrestris. This is in agreement with the work of Ryu et al. (22), who reported that the peroxyacetic acid–based disinfectants were less effective than chlorine or chlorine-based dioxide in destruction of bacterial spores in biofilms. VOR at the concentration of 2,600 ppm was the most effective in reducing A. acidoterrestris spores (up to 2.6 log) when compared with treatments with CL or CAR. This is likely due to the strong oxidation properties of CL and therefore its increased efficacy as a sanitizer targeting bacterial spores (2). VOR is an equilibrium mixture of hydrogen peroxide, peroxyacetic acids, and octanoic acid. The octanoic acid equilibrated with its peroxidated form results in its enhanced effectiveness, which is thought to be due to the membrane-altering capability of the perooctanoic acid (4). Other researchers have demonstrated that spore-forming microorganisms adhere to food contact surface (9, 12). Ryu et al. (22) studied the attachment of Bacillus cereus on stainless steel surfaces and concluded that the spore formation was affected by nutrient availability, temperature, and relative humidity. A 2% stock solution containing 23% hydrogen peroxide and 4% peracetic acid, combined with a contact time of 5 min at 25°C was shown to effectively reduce the survival of Bacillus spp. (29).

Published reports related to the treatment of microbial attachment formed by A. acidoterrestris on stainless steel surfaces with disinfectants are practically nonexistent. Orr and Beuchat (16) studied the efficacy of disinfectants in killing spores of five different strains of A. acidoterrestris, including the N-1100 strain, which was the most resistant to the sanitizers tested. The researchers obtained reductions of about 2.6 and 5.3 log in the number of A. acidoterrestris

TABLE 2. Population of spores of A. acidoterrestris applied to a stainless steel surface, surviving treatment with chemical disinfectants at 90°C, and revived on ALIA

<table>
<thead>
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<td>3.95 A</td>
<td>3.93 A</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>2.46 B</td>
<td>2.17 B</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2.20 B</td>
<td>2.00 BC</td>
</tr>
<tr>
<td>Carnebon 200(^c)</td>
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<td>3.83 A</td>
<td>3.80 A</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.47 B</td>
<td>2.39 B</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.41 B</td>
<td>1.93 BC</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>2.08 BC</td>
<td>1.76 C</td>
</tr>
<tr>
<td>Vortex(^d)</td>
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<td>3.88 A</td>
<td>3.80 A</td>
</tr>
<tr>
<td></td>
<td>1,500</td>
<td>2.24 B</td>
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<td></td>
<td>2,000</td>
<td>2.10 BC</td>
<td>1.67 c</td>
</tr>
<tr>
<td></td>
<td>2,600</td>
<td>1.69 c</td>
<td>1.25 dc</td>
</tr>
</tbody>
</table>

\(^a\) Values within a column followed by different uppercase letters are significantly different (\(P \leq 0.05\)).
\(^b\) Sodium hypochlorite.
\(^c\) Stabilized chlorine dioxide.
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spores when treated with sodium hypochlorite at concentrations of 200 and 500 ppm, respectively. These reductions were dramatically greater than those obtained with similar treatments with sodium hypochlorite at the same concentrations as those used in our studies. These differences are likely due to the fact that A. acidoterrestris spores in the Orr and Beuchat (16) study were deposited into solutions of disinfectants, while in our study the spores were attached to the surface of stainless steel. Therefore, after the treatment with disinfectants, some of the spores were still attached to the steel surface, and hence, they were not in the surrounding medium. In addition, the attachment formation on steel surfaces may have also acted as an additional protection for spores against disinfectants, especially for the spores in subsurface areas of the attachments (22). Another possible explanation for the greater resistance of spores to disinfectants may be found in the structure of stainless steel, which is one of the most common food contact surfaces used in food processing operations (27). While stainless steel appears smooth to the unaided eye, it is very rough when viewed under a microscope. It is well established that spores firmly attached to a surface or trapped within the biofilm matrix may become more resistant to common disinfectants (12).

In our study, A. acidoterrestris spores were recovered from stainless steel surfaces after the treatment with disinfectants, followed by heating of the neutralized suspension at 80°C for 10 min. Young and Setlow (32) demonstrated that chlorine dioxide inactivated spores of Bacillus subtilis by damaging the inner membrane, resulting in the inhibition of germination and outgrowth of spores. It is reasonable to assume that the same theory might be applied to other bacterial spores, such as A. acidoterrestris spores, because the spores have similar wall structures that protect them from environmental stresses. The exact mechanism of killing Bacillus spores by treatment with wet heat is not fully understood (24, 25). However, it has been well documented that dipicolinic acid in the spore core greatly contributes to the increased resistance of spores to wet heat (1, 17, 30). The absence of dipicolinic acid in spores increases their sensitivity to heat. Inner membrane damage of Bacillus spores by treatment with chlorine dioxide has been implicated in the reduction in wet heat resistance.

In conclusion, the results of this study provide useful information on the effectiveness of disinfectants commonly used in the food processing industry in destruction of A. acidoterrestris spores applied to a food contact surface. These insights will be useful when developing sanitation strategies focused on reducing the spoilage of apple juice associated with A. acidoterrestris applied to stainless steel surfaces.

REFERENCES

23. Sagripanti, J. L., and A. Bonifacino. 1999. Bacterial spores survive...