Effects of Electrolyzed Oxidizing Water on Inactivation of *Bacillus subtilis* and *Bacillus cereus* Spores in Suspension and on Carriers

Chunling Zhang, Baoming Li, Ravirajsinh Jadeja, and Yen-Con Hung

Abstract: Spores of some *Bacillus* species are responsible for food spoilage and foodborne disease. These spores are highly resistant to various interventions and cooking processes. In this study, the sporicidal efficacy of acidic electrolyzed oxidizing (EO) water (AEW) and slightly acidic EO water (SAEW) with available chlorine concentration (ACC) of 40, 60, 80, 100, and 120 mg/L and treatment time for 1, 2, 3, 4, 5, and 6 min were tested on *Bacillus subtilis* and *Bacillus cereus* spores in suspension and on carrier with or without organics. The reduction of spore significantly increased with increasing ACC and treatment time ($P < 0.05$). Nondetectable level of *B. cereus* spore in suspension occurred within 2 min after exposure to both EO waters containing 120 mg/L ACC, while only SAEW at 120 mg/L and 2 min treatment achieved >6 log reductions of *B. subtilis* spore. Both types of EO water with ACC of 60 mg/L and 6 min treatment achieved a reduction of *B. subtilis* and *B. cereus* spores to nondetectable level. EO water with ACC of 80 mg/L and treatment time of 3 min on carrier test without organics addition resulted in reductions of *B. subtilis* spore to nondetectable level. But, addition of 0.3% organics on carrier decreased the inactivation effect of EO water. This study indicated that EO water was highly effective in inactivation of *B. subtilis* and *B. cereus* spores in suspension or on carrier, and therefore, rendered it as a promising disinfectant to be applied in food industry.

Keywords: acidic electrolyzed water, carrier test, slightly acidic electrolyzed water, spore, inactivation

Practical Application: EO water can be used to disinfect food or food contact surface contaminated with *Bacillus* spores.

Introduction

Bacillus species could form endospores which could survive in the absence of exogenous nutrients in a metabolically dormant state for many years when confronted with limited or depleted nutrients in the environment (Driks 2002). Dormant bacterial spores are much more resistant than their vegetative cells to a broad range of stresses, including heat, radiation, desiccation, and a variety of chemicals (Nicholson and others 2000; Logan 2005; Ryu and Beuchat 2005; Setlow 2006). Spores could return to life rapidly in the presence of appropriate nutrients and lead to disease or food spoilage by germinating and resuming vegetative growth (Setlow 2006). A variety of diseases caused by spores of *Bacillus* were reported (Mallozzi and others 2010). As a spore former, *Bacillus cereus* is ubiquitous in the environment and can be isolated from soil, water and vegetation, and it is also a common component of transient gut microbiota in humans. Two primary types of foodborne gastrointestinal illnesses, emetic, and diarrheal syndrome, are usually caused by *B. cereus* which germinated from their corresponding spores (Sudhaus and others 2014). It was reported that hospital linens contaminated by *B. cereus* exhibited the property of causing nosocomial bacteremia (Sasahara and others 2011). Far less common than *B. cereus*, *Bacillus subtilis* is also responsible for many outbreaks of illnesses. In a kindergarten related outbreak in Croatia which occurred in 2000; out of 25 exposed children, 12 exhibited symptoms of nausea, headache, and vomiting after eating breakfast, in which milk contaminated by toxigenic *B. subtilis* (Pavic and others 2005). In addition, baked products such as bread and crumpets are also liable to be involved in a number of *B. subtilis* outbreaks (Adams and Moss 2008).

Various spore inactivation chemicals and methods such as sodium hypochlorite, hydrogen peroxide, peracetic acid, wet heat, and UV radiation are commonly used to kill spores (Setlow and others 2013; Moeller and others 2011; Sudhaus and others 2014). However, unlike bacterial vegetative cells, spores of bacteria are difficult to inactivate because of its high resistance to various stresses. Common disinfecting treatments are usually less effective in inactivation of spore than its vegetative form. Therefore, there is a need for the development of effective, nontoxic, and environmentally friendly disinfection method to reduce or eliminate bacterial spores.

In recent years, electrolyzed oxidizing (EO) water has been a popular alternative to harsh chemical disinfectants because of its generally recognized as safe (GRAS) status and environmentally friendly nature (Huang and others 2008). EO water is produced by electrolysis of diluted sodium chloride solution in a chamber with anode and cathode electrodes separated by a membrane. EO water with a low pH value (<2.7) and a high oxidation reduction potential (ORP, >1050 mV) is one of the main EO water types known as acidic electrolyzed water (AEW), which consists of chlorine gas, hypochlorous acid and hydrochloric acid (Jadeja and others 2013). Slightly acidic electrolyzed water (SAEW) with a pH of 5.0 to 6.5 is produced by electrolysis of hydrochloric acid or a mixture of anode solution and cathode solution, and it

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contains mostly in the form of hypochlorous acid which has greater bactericidal effect than that of hypochlorite ion and chlorine gas. EO water has been widely used to inactivate or eliminate foodborne pathogens (Zhang and others 2011; Hung and others 2010; Kim and Hung 2012; Jadeja and others 2013; Pangloli and Hung 2013; Jadeja and Hung 2014; Wang and others 2014), viruses (Hao and others 2013; Tamaki and others 2014), bacterial spores (Kim and others 2000; Tang and others 2011) and biofilm (Ozaki and others 2012; Sun and others 2012).

As discussed above, bacterial spores are more resistant to various treatments than their vegetative cells. Very few studies discuss sporicidal capacity of different types of EO water and in the presence of organics. This study was designed to evaluate the effectiveness of available chlorine concentration (ACC) and treatment time of AEW and SAEW for killing *B. subtilis* and *B. cereus* spores in suspension and on carriers.

Materials and Methods

Bacterial strains

Bacillus subtilis (ATCC 19659) and *Bacillus cereus* (ATCC 14579) were obtained from American Type Culture Collection. Nutrient agar (Neogen, U.S.A.) supplemented with 50 mg/L manganese sulfate (NAMS agar, Alfa Aesar, England) was used as a sporulation medium (Kim and others 2000). Two hundred microliters of overnight grown *B. subtilis* cultures in 9 mL of tryptic soy broth (TSB, Difco, Becton Dickinson, Md., U.S.A.) were spread evenly on the surface of NAMS agar using sterile bent glass rods. Plates were sealed with parafilm and incubated at 37 °C for 72 h. *B. cereus* cultures in TSB were grown at 30 °C following the procedure described above. Spores were harvested by adding 20 mL of sterile physiological saline on the surface of each plate and gently rubbing with a sterile glass rod. Suspensions of spores were collected into 50 mL sterile plastic tubes and vortexed for 30 s to make sure spores mixed completely. The white dreg on the surface of suspension was discarded and remaining suspension was centrifuged at 2600 × *g* (4 °C) for 20 min. The supernatant was decanted and the resulting cell pellets were suspended in 30 mL of cold sterile physiological saline (4 °C), which was centrifuged at 2600 × *g* (4 °C) for 20 min. This procedure was repeated twice. The final pellets were suspended in sterile physiological saline (for suspension test) or phosphate buffered solution (PBS, for carrier test) (ASTM 2011). and stored at 4 °C until used. For inoculation experiment, refrigerated spore suspension was brought to room temperature and diluted to about 10^7 CFU/mL. Ten milliliters of diluted spore suspension was heated in a water bath at 80 °C for 5 min to kill vegetative cells before being used (Kim and others 2000).

EO water preparation

AEW and SAEW were produced using an EAU EO water generator (EAU, Model #P30HST44T, Ga., U.S.A.) by electrolyzing 0.03% NaCl solution. After the generator running stabilized, EO water was collected into a sterile, air-tight container and the pH, ORP and ACC were measured immediately before each sporicidal experiment. The pH and ORP of EO water were measured using an ACCUMET pH meter (AR50, Fisher Scientific, Pittsburgh, Pa., U.S.A.) with pH electrode and ORP electrode. The ACC was determined by a DPD-FEAS method (Hach Co., Loveland, Co., U.S.A.). AEW and SAEW with chlorine concentration of 40, 60, 80, 100, and 120 mg/L were prepared by diluting with deionized water.

Suspension sporicidal test

One milliliter of prepared spore suspension was added into 15 mL sterile plastic tube containing 9 mL AEW or SAEW and mixed completely by vortex (G-560, Fisher scientific, U.S.A.) for 5 s. After the desired treatment time, 1 mL suspension was collected and mixed with 1 mL 2× D/E (Dey/ Engley) neutralizing broth (Difco, Becton Dickinson, Sparks, Md., U.S.A.) to quench remaining free chlorine. Counts of viable spores were obtained by spread plating 0.1 mL of appropriate serial dilution of cultures onto sterile tryptic soy agar (TSA, Difco.). The plates of *B. subtilis* spore were incubated at 37 °C (30 °C for *B. cereus* spore) for 24 h prior to counting.

Carrier test

The efficacy of EO water on inactivation of spores on carrier was evaluated using a standard method of carrier test (ASTM 2011). Stainless steel disks of 1.9 × 1.9 cm were soaked in acetone solution for 1 h and followed by soaked in 1M NaOH for 30 min to degrease them. Disks were then washed with deionized water and sterilized by autoclaving at 121 °C for 15 min. For experiment where organic matter was included with the inoculum, 5% bovine serum albumin, 5% tryptone and 0.4% bovine mucin in PBS was prepared separately and then added to the spore suspension at the ration of 5: 7: 20 to obtain the inoculum with 0.3% organics (ASTM 2011). For comparison purpose, carrier test with no organics addition was conducted with 0.3% PBS in the inoculum. The test suspension was vortexed for 5 s to evenly distribute spores and then 20 μL of *B. subtilis* spore inoculum was pipetted onto the center of disk without spreading the inoculum. Three disks were used for each treatment, whereas 3 disks with only 20 μL of PBS was used as blank control. The inoculated disks were placed in a biosafety cabinet with air flow for 2 h to dry. Each disk with inoculum was covered with 100 μL of EO water and was treated at room temperature (22 °C) for 1, 2, and 3 min, respectively. At the end of the treatment time, test disks were collected and transferred into 50 mL tubes containing 10 mL 2× D/E neutralizing broth to terminate the reaction. Tubes were votexed for 1 min to ensure that all spores were successfully eluted from the disk. The viable spore population was obtained by spread plating 0.1 mL of 10-fold serial dilution onto TSA plates and incubated at 37 °C for 24 h before enumeration.

Statistical analysis

All results were obtained from 3 independent trials. Statistical analysis was performed using SAS software 9.2 (Institute, Inc., Cary, N.C., U.S.A.). Least significance difference of means tests was used for multiple comparisons at the 0.05 probability level.

Results and Discussion

Efficacy of EO water with different ACC on inactivation of *B. subtilis* and *B. cereus* spores

Log reductions of the *B. subtilis* and *B. cereus* spores after AEW and SAEW treatments are presented in Figure. 1. As can be seen from Figure. 1A, log reduction significantly increased when *B. subtilis* spores were treated with both AEW and SAEW with increasing ACC from 40 to 120 mg/L ($P < 0.05$). Compared to the initial *B. subtilis* spore population (7.86 log CFU/mL), 0.36, 1.41, 2.30, 3.82, and 4.45 log reductions were observed after AEW treatment, while 0.47, 2.17, 3.79, 5.02, and 6.86 log reductions were obtained after SAEW treatment, respectively. Especially for ACC at 120 mg/L, *B. subtilis* spores treated with SAEW for 2 min

reached a nondetectable level (log reduction at 6.86 as the detection limit is 1.0 log CFU/mL), while only 4.45 log reductions were achieved by AEW treatment. Additionally, with the exception of ACC at 40 mg/L, significant difference were observed between reduction obtained with AEW and SAEW at all other ACC ($P < 0.05$). In this study, SAEW showed higher effectiveness than AEW on inactivation of *B. subtilis* spore.

A previous study reported that inactivation effect of *B. subtilis* spore by chlorine solution (sodium hypochlorite solution) treatment varied with pH (5.6 in contrast to 8.2) of the solution, since an EO water at pH of 5.6 will have most of free chlorine in HOCl form whereas solution at pH 8.2 will have most of the free chlorine in hypochlorite ion (OCl^-) (Cho and others 2006). It has been previously established that HOCl has a higher bactericidal effect than any other forms of chorine such as OCl^- and Cl_2 and therefore chlorine solution at pH 5.6 was higher effective in spores' inactivation than that at pH 8.2. In a previous study, the sporicidal effect of EO water on *B. subtilis var. niger* ATCC 9732 achieved 7.1 log reductions for AEW treatment at 160 mg/L ACC for 60 min or 191 mg/L for 20 min at initial spore counts of 7.1 log CFU/mL (Tang and others 2011). However, in our study, 4.45 log reductions of *B. subtilis* was obtained with AEW treatment at 120 mg/L ACC for 2 min, while SAEW at the same treatment condition achieved nondetectable level. A possible explanation for this difference that *B. subtilis var. niger* ATCC 9732 required higher ACC and longer treatment time to obtain the similar reduction could be that the resistance of *B. subtilis var. niger* ATCC 9732 to various chemicals is much higher than other *B. subtilis* strains. In a study by Sudhaus and others (2014), it was reported that sensitiveness of spores of different strains is greatly different, even though the study only refer to *B. cereus* spore.

Bacterial spores are more resistant to disinfectants than vegetative cells, so much higher available chlorine and longer treatment time are required to achieve targeted reductions. For *B. subtilis* spore inactivation, 6.86 log reductions were obtained by SAEW with ACC of 120 mg/L for 2 min treatment in this study, while in a previous study, only 0.5 mg/L available chlorine for 30 s was required to reach a similar log reduction for *B. subtilis* vegetative cells (Zhang and others 2016).

Log reductions of *B. cereus* spore after EO water treatment at different ACC are shown in Figure. 1B. Unlike the data shown in Figure. 1A, there was no significant difference between AEW and SAEW on inactivation of *B. cereus* spore at different ACC. In addition, log reductions of *B. cereus* spore increased significantly with increased ACC for both AEW and SAEW treatment ($P < 0.05$). At the initial *B. cereus* spore level of 7.57 log CFU/mL, 2.29, 3.62, 4.42, 5.13, and 6.57 log reductions were obtained after AEW treated with ACC of 40, 60, 80, 100, and 120 mg/L, while with SAEW treatment, 2.19, 3.35, 4.10, 4.99 and 6.57 log reductions were observed, respectively. Moreover, treatment with both AEW and SAEW at ACC of 120 mg/L for 2 min, *B. cereus* spore inactivation achieved nondetectable level (no colony was observed on TSA). For *B. cereus* spore, similar trend of sporicidal efficacy was obtained by AEW and SAEW treatment. Compared the log reduction of *B. cereus* and *B. subtilis* spores by AEW and SAEW treatment (Figure 1A and B), the susceptibility of both types of spore to AEW and SAEW was much different. *B. cereus* spores were slightly easier to be inactivated by EO water than *B. subtilis* spores at the same ACC, and moreover, there was no significantly different between AEW and SAEW in *B. cereus* spore disinfection. The reason for this result could be that the cell structure of *B. cereus* spore was different than *B. subtilis* spore (Atrih and Foster 2001; Leuschner and Lillford 2001), and was equally susceptive to AEW and SAEW. The disinfection result from a previous study reported 5 min was required to completely inactivate *B. cereus* spores by acidic EO water with ACC of 43 mg/L (Vorobjeva and others 2004). Our results indicated *B. cereus* spore treated with AEW with ACC of 60 mg/L for 2 min achieved 3.62 log reductions and this result is in agreement with the result reported by Kim and others (2000), in which *B. cereus* spore treated with AEW with ACC of 56 mg/L for 2 min, obtained a similar log reduction (3.5 log reductions).

As discussed above, sporicidal effect may be affected due to variation among different strains. In addition, even with the same strain and under the same experimental conditions, the results could vary depending on the medium and how the spores were prepared (Atrih and Foster 2001). It was reported that supplementation of sporulation medium with cysteine, cystine, or

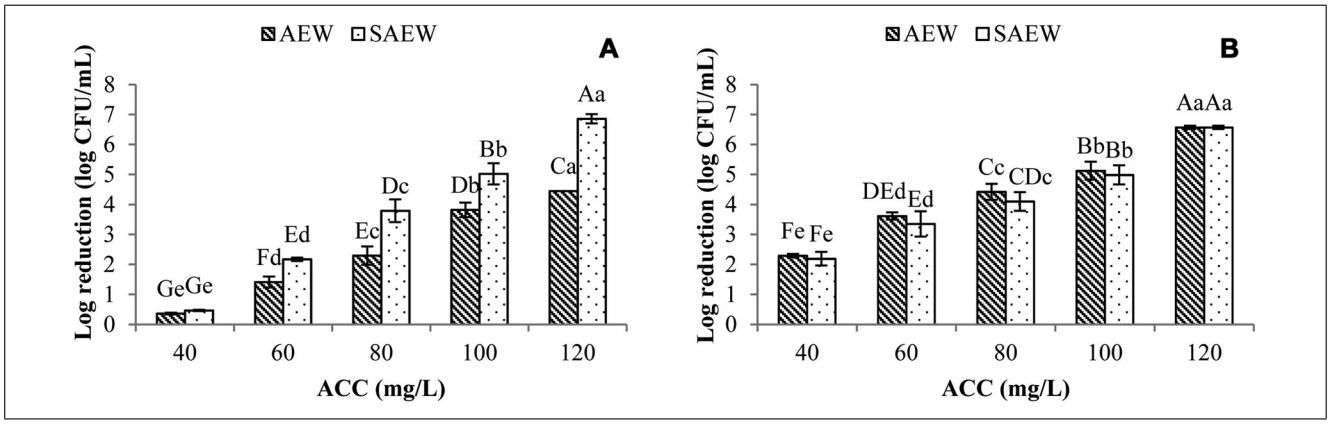

Figure 1–Efficacy of AEW and SAEW with different ACC on inactivation of *B. subtilis* (A) and *B.cereus* spores (B).
Acidic electrolyzed water (AEW) at pH 2.62 to 2.90, ORP 1118 to 1188 mV, slightly acidic electrolyzed water (SAEW) at pH 5.81 to 5.99, ORP 920 to 956 mV. Treatment time: 2 min. Initial *B. subtilis* spores concentration: 7.86 ± 0.16 log CFU/mL, initial *B. cereus* spores concentration: 7.57 ± 0.06 log CFU/mL. Bars labeled with no common uppercase letters in the same available chlorine concentration (ACC) treatment are significantly different ($P < 0.05$), bars labeled with no common lowercase letters for the same type of EO water treatment are significantly different ($P < 0.05$). Detection limit is 1.0 log CFU/mL.

Table 1–Effects of AEW and SAEW on inactivating *B. subtilis* spores on carrier with or without organics.

		Log reduction (no organics)			Log reduction (0.3% organics)		
		Treatment time (min)			Treatment time (min)		
Treatment solution	ACC (mg/L)	1	2	3	1	2	3
AEW	60	C 0.40 ± 0.03c	B 1.48 ± 0.04b	B 3.38 ± 0.40a	B 0.32 ± 0.17b	D 0.63 ± 0.29b	B 1.50 ± 0.37a
	80	C 0.88 ± 0.04b	A 4.04 ± 0.18a	A 4.17 ± 0.15a	B 0.49 ± 0.24c	C 2.03 ± 0.30b	A 3.65 ± 0.29a
	100	B 1.70 ± 0.44b	A 4.17 ± 0.15a	A 4.17 ± 0.15a	AB 0.91 ± 0.17c	B 2.76 ± 0.27b	A 3.85 ± 0.06a
	120	A 2.94 ± 0.21b	A 4.17 ± 0.15a	A 4.17 ± 0.15a	A 1.99 ± 0.34b	A 3.85 ± 0.34a	A 4.17 ± 0.09a
SAEW	60	C 0.57 ± 0.33c	B 2.31 ± 0.23b	A 3.83 ± 0.33a	C 0.21 ± 0.04c	B 0.72 ± 0.14b	B 1.86 ± 0.11a
	80	C 1.05 ± 0.22b	A 3.94 ± 0.30a	A 4.17 ± 0.17a	C 0.41 ± 0.06c	B 1.78 ± 0.38b	A 3.44 ± 0.37a
	100	B 2.18 ± 0.33b	A 4.17 ± 0.17a	A 4.17 ± 0.17a	B 0.82 ± 0.22b	A 3.01 ± 0.29a	A 3.77 ± 0.39a
	120	A 3.17 ± 0.29b	A 4.17 ± 0.17a	A 4.17 ± 0.17a	A 1.93 ± 0.17b	A 3.71 ± 0.39a	A 3.82 ± 0.30a

Acidic electrolyzed water (AEW): pH 2.73 to 2.83, ORP 1165 to 1186 mV; slightly acidic electrolyzed water (SAEW): pH 6.09 to 6.30, ORP 954 to 972 mV. Initial *B. subtilis* spores: 7.17 log CFU/mL. Detection limit in this carrier test is 3.0 log CFU/mL. Means bearing no common uppercase letters on the same column in each treatment are significantly different ($P < 0.05$), means bearing no common lowercase letters on the same row on carrier with or without organics, respectively, are significantly different ($P < 0.05$).

thioproline increased spore resistance to solar UV radiation and to H_2O_2 if the spores were not decoated (Moeller and others 2011). *B. subtilis* spore could become more sensitive to H_2O_2 when there was high Mn level and high Mn/Fe ration in the sporulation medium, but the resistance was unaffected to desiccation, wet or dry heat and ionizing radiation (Granger and others 2011). These variations including medium ingredient, bacterial types, strains and operation conditions would make it difficult to accurately compare the efficacy of various disinfectants on spore inactivation.

Inactivation effect of EO water on *B. subtilis* and *B. cereus* spores with different treatment time

Log reductions of *B. subtilis* and *B. cereus* spores treated with AEW and SAEW at the ACC of 60 mg/L for different treatment time are shown in Figure 2. In this study, spores were treated with EO water for 1, 2, 3, 4, 5, and 6 min, respectively. With increasing treatment time, reductions of both *B. subtilis* and *B. cereus* spores increased significantly ($P < 0.05$). Log reductions of *B. subtilis* spore exposed to AEW from 1 to 6 min were 0.22, 1.13, 2.26, 4.19, 4.94, and 6.72 log CFU/mL, respectively, while 0.32, 1.91, 3.61, 4.98, 6.22, and 6.72 log CFU/mL were observed when exposed to SAEW. Both AEW and SAEW treatment at 60 mg/L ACC reached surviving population at nondetectable level after 6 min for *B. subtilis* spore. Similarly, *B. cereus* spore was completely inactivated after treatment with AEW and SAEW at the 60 mg/L ACC for 6 min as well. It is worth noting that, the reductions of *B. subtilis* spore exposed to SAEW at the same treatment time was always higher than reduction obtained using AEW (Figure 2A). However, there was no obvious difference between AEW and SAEW in reduction of *B. cereus* spore (Figure 2B). This result was in accordance with that showed in Figure 1, in which, SAEW showed higher effective in inactivating *B. subtilis* spore than AEW while no obvious different in *B. cereus* spore treatment.

In a previous study, Sterilox, a special type of EO water with ACC of 240 mg/L, achieved 5 log reductions of *B. subtilis* spore (PS533, PS578, PS2318, and PS2319) in 5 min, and approximately 2 log reductions was observed using 1/10 dilution of Sterilox for 11 min treatment (Loshon and others 2001). The reduction of *B. subtilis* spore treated by O_3 (1.38 mg/L) in sand filtered water was more than 3 logs within 10 min and it increased with increasing exposure time (Choi and others 2007). In the current study, AEW and SAEW with 60 mg/L ACC achieved more than 6 log reductions for both *B. subtilis* and *B. cereus* spores after 6 min treatment.

Effects of ACC and treatment time on inactivation of *B. subtilis* spore on carriers with and without organics

Food contact surface contaminated with microorganisms may cause cross-contamination during food processing. Reductions of *B. subtilis* spore on carrier treated with AEW and SAEW with different ACC and treatment time are presented in Table 1. At each given treatment time or ACC, log reductions increased with

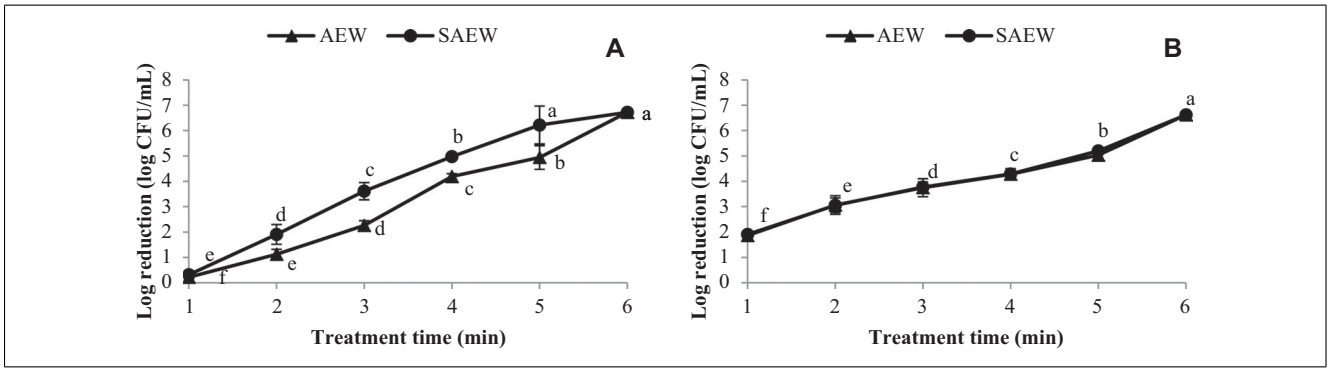

Figure 2–Effect of treatment time on inactivation of *B. subtilis* (A) and *B. cereus* spores (B) by AEW and SAEW.
Acidic electrolyzed water (AEW) at available chlorine concentration 60 mg/L, pH 2.84 ± 0.02, ORP 1142 ± 21 mV, slightly acidic electrolyzed water (SAEW) at available chlorine concentration 60 mg/L, pH 5.89 ± 0.16, ORP 930 ± 35 mV. Initial *B. subtilis* spores concentration: 7.72 ± 0.04 log CFU/mL, initial *B. cereus* spore concentration: 7.62 ± 0.05 log CFU/mL. Data point on the figure with no common letters are significantly different ($P < 0.05$). Detection limit is 1.0 log CFU/mL.

increasing ACC and treatment time. When the spores were treated by either AEW or SAEW for 1 min, significant increase in log reduction was observed when available chlorine increased from 80 to 120 mg/L ($P < 0.05$). It was further observed that *B. subtilis* spores could be inactivated to nondetectable level from carrier without organics by both AEW and SAEW at 80 mg/L ACC for 3 min or 100 mg/L ACC for 2 min.

The sporicidal efficacy of EO water also can be influenced by the presence of interfering substances. As free chlorine can react with or get neutralized by organics, resulting in decrease in the biocidal effect of chlorine-based agents. Therefore, effect of 0.3% organics (containing tryptone, bovine serum albumin and bovine mucin) in the spores' culture prior to inactivation test was conducted. The effect of EO water on inactivating *B. subtilis* spore on carrier with 0.3% of organics also increases with increasing ACC and treatment time. Compared to carrier test which was without organics addition, the effect of AEW and SAEW on inactivating *B. subtilis* spore on carrier with 0.3% of organics addition was always lower. Nondetectable level of *B. subtilis* spore in carrier test with organics addition was achieved by using AEW with 120 mg/L ACC for 3 min, while only 80 mg/L available chlorine of AEW was required at the same treatment time for carrier test without organics addition. It was in agreement with a previous report that the biocidal efficacy of sodium hypochlorite on carrier test was greatly affected by the presence of food residue (Hilgren and others 2007). For maintaining sporicidal activity, disinfectant must possess sufficient concentration of available chlorine and be able to penetrate the cell membrane to exert their action. Organic compounds such as ammonia reacted with free chlorine, leading to production of chlorine derivatives which have lower disinfection efficacy than free chlorine (Rule and others 2005). In addition to neutralize the chlorine, the presence of organics in the treatment medium could also protect cell membrane from permeabilization and increase the resistance of bacteria to chlorine by stabilizing the bacterial membranes and not allowing disinfectant to enter cell (Virto and others 2005). For carrier test, AEW presented similar sporicidal effect as SAEW, while AEW showed lower effective in inactivation of *B. subtilis* spore in suspension test than SAEW. A possible explanation for this observation could be that some residual PBS ions on the carrier exerting a buffering function and change the pH of AEW to a level similar to the pH of SAEW.

Conclusions

B. subtilis and *B. cereus* spores in suspension or on carriers were inactivated effectively by AEW and SAEW in this study. Higher available chlorine and longer treatment time rendered more reductions. EO water with available chlorine of 60 mg/L could achieve the surviving population of *B. subtilis* and *B. cereus* spores in suspension to nondetectable level after 6 min treatment. On the other hand, *B. subtilis* spores could be inactivated to nondetectable level from carrier without organics by either AEW or SAEW with 80 mg/L ACC for 3 min or 100 mg/L for 2 min. When organic matters were present, biocidal efficacy of EO water was reduced. The results of this study indicated that EO water can be used in food industry to disinfect food or food contact surface contaminated with *Bacillus* spores.

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Author Contributions

Author Chunling Zhang designed the study, performed the experiment, analyzed the data and drafted the manuscript. Baoming Li designed the study and interpreted the results. Ravirajsinh Jadeja conducted the experiment and revised the manuscript. Yen-Con Hung designed the experiment, analyzed the data and with overall responsibility of the project.

References

Adams MR, Moss MO. 2008. Bacterial agents of foodborne illness. In: Adams MR and Moss MO, editors. Food microbiology. 3rd ed. Cambridge: The Royal Society of Chemistry Publishing. p 190.

ASTM. 2011. Standard quantitative disk carrier test method for determining bactericidal, virucidal, fungicidal, mycobactericidal, and sporicidal activities of chemicals. West Conshohocken, Pa., U.S.A: ASTM International, E2197–11

Atrih A, Foster SJ. 2001. Analysis of the role of bacterial endospore cortex structure in resistance properties and demonstration of its conservation amongst species. J Appl Microbiol 91(2):364–72.

Cho M, Kim JH, Yoon J. 2006. Investigating synergism during sequential inactivation of *Bacillus subtilis* spores with several disinfectants. Water Res 40(15):2911–20.

Choi Y, Cho M, Lee Y, Choi J, Yoon J. 2007. Inactivation of *Bacillus subtilis* spores during ozonation in water treatment plant: influence of pre-treatment and consequences for positioning of the ozonation step. Chemosphere 69(5):675–81.

Driks A. 2002. Overview: development in bacteria: spore formation in *Bacillus subtilis*. Cell Mol Life Sci 59(3):389–91.

Granger AC, Gaidamakova EK, Matrosova VY, Daly MJ, Setlow P. 2011. Effects of Mn and Fe levels on *Bacillus subtilis* spore resistance and effects of Mn^{2+}, other divalent cations, orthophosphate, and dipicolinic acid on protein resistance to ionizing radiation. Appl Environ Microbiol 77(1):32–40.

Hao XX, Shen ZQ, Wang JL, Zhang Q, Li BM, Wang CY, Cao W. 2013. In vitro inactivation of porcine reproductive and respiratory syndrome virus and pseudorabies virus by slightly acidic electrolyzed water. Vet J 197(2):297–301.

Hilgren J, Swanson KMJ, Diez-Gonzalez F, Cords B. 2007. Inactivation of *Bacillus anthracis* spores by liquid biocides in the presence of food residue. Appl Environ Microbiol 73(20):6370–7.

Huang YR, Hung YC, Hsu SY, Huang YW, Hwang DF. 2008. Application of electrolyzed water in the food industry. Food Control 19(4):329–45.

Hung YC, Tilly P, Kim C. 2010. Efficacy of electrolyzed oxidizing (Eo) water and chlorinated water for inactivation of *Escherichia coli* O157:H7 on strawberries and broccoli. J Food Qual 33(5):559–77.

Jadeja R, Hung YC. 2014. Efficacy of near neutral and alkaline pH electrolyzed oxidizing waters to control *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT 104 from beef hides. Food Control 41:17–20.

Jadeja R, Hung YC, Bosilevac JM. 2013. Resistance of various shiga toxin-producing *Escherichia coli* to electrolyzed oxidizing water. Food Control 30(2):580–4.

Kim C, Hung YC. 2012. Inactivation of *E. coli* O157:H7 on blueberries by electrolyzed water, ultraviolet light, and ozone. J Food Sci 77(4):M206–11.

Kim C, Hung YC, Brackett RE. 2000. Efficacy of electrolyzed oxidizing (EO) and chemically modified water on different types of foodborne pathogens. Intl J Food Microbiol 61(2-3):199–207.

Leuschner RGK, Lillford PJ. 2001. Investigation of bacterial spore structure by high resolution solid-state nuclear magnetic resonance spectroscopy and transmission electron microscopy. Intl J Food Microbiol 63(1–2):35–50.

Logan NA. 2005. *Bacillus anthracis*, *Bacillus cereus*, and other aerobic endospore-forming bacteria. In: Borriello SP, Murray PR, Funke G, editors. Topley and Wilson's microbiology and microbial infections. 10th ed. London: Hodder Arnold. p 922–52.

Loshon CA, Melly E, Setlow B, Setlow P. 2001. Analysis of the killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox (R). J Appl Microbiol 91(6):1051–8.

Mallozzi M, Viswanathan VK, Vedantam G. 2010. Spore-forming *Bacilli* and *Clostridia* in human disease. Future Microbiol 5(7):1109–23.

Moeller R, Wassmann M, Reitz G, Setlow P. 2011. Effect of radioprotective agents in sporulation medium on *Bacillus subtilis* spore resistance to hydrogen peroxide, wet heat and germicidal and environmentally relevant UV radiation. J Appl Microbiol 110(6):1485–94.

Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64(3):548–72.

Ozaki M, Ohshima T, Mukumoto M, Konishi H, Hirashita A, Maeda N, Nakamura Y. 2012. A study for biofilm removing and antimicrobial effects by microbubbled tap water and other functional water, electrolyzed hypochlorite water and ozonated water. Dent Mater J 31(4):662–8.

Pangloli P, Hung YC. 2013. Effects of water hardness and pH on efficacy of chlorine-based sanitizers for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Food Control 32(2):626–31.

Pavic S, Brett M, Petric N, Lastre D, Smoljanovic M, Atkinson M, Kovacic A, Cetinic E, Ropac D. 2005. An outbreak of food poisoning in a kindergarten caused by milk powder containing toxigenic *Bacillus subtilis* and *Bacillus licheniformis*. Arch Lebensmittelhyg 56(1):20–2.

Rule KL, Ebbett VR, Vikesland PJ. 2005. Formation of chloroform and chlorinated organics by free-chlorine-mediated oxidation of triclosan. Environ Sci Technol 39(9):3176–85.

Ryu JH, Beuchat LR. 2005. Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. J Food Prot 68(12):2614–22.

Sasahara T, Hayashi S, Morisawa Y, Sakihama T, Yoshimura A, Hirai Y. 2011. *Bacillus cereus* bacteremia outbreak due to contaminated hospital linens. Eur J Clin Microbiol Infect Dis 30(2):219–26.

Setlow B, Yu J, Li YQ, Setlow P. 2013. Analysis of the germination kinetics of individual *Bacillus subtilis* spores treated with hydrogen peroxide or sodium hypochlorite. Lett Appl Microbiol 57(4):259–65.

Setlow P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol 101(3):514–25.

Sudhaus N, Nagengast H, Pina-Pérez MC, Martínez A, Klein G. 2014. Effectiveness of a peracetic acid-based disinfectant against spores of *Bacillus cereus* under different environmental conditions. Food Control 39(0):1–7.

Sun JL, Zhang SK, Chen JY, Han BZ. 2012. Efficacy of acidic and basic electrolyzed water in eradicating *Staphylococcus aureus* biofilm. Can J Microbiol 58(4):448–54.

Tamaki S, Bui VN, Ngo LH, Ogawa H, Imai K. 2014. Virucidal effect of acidic electrolyzed water and neutral electrolyzed water on avian influenza viruses. Arch Virol 159(3):405–12.

Tang WW, Zeng XP, Zhao YS, Ye GQ, Gui WC, Ni YM. 2011. Disinfection effect and its mechanism of electrolyzed oxidizing water on spores of *Bacillus subtilis var. niger*. Food Sci Biotechnol 20(4):889–95.

Virto R, Manas P, Alvarez I, Condon S, Raso J. 2005. Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. Appl Environ Microbiol 71(9):5022–8.

Vorobjeva NV, Vorobjeva LI, Khodjaev EY. 2004. The bactericidal effects of electrolyzed oxidizing water on bacterial strains involved in hospital infections. Artif Organs 28(6): 590–2.

Wang JJ, Sun WS, Jin MT, Liu HQ, Zhang W, Sun XH, Pan YJ, Zhao Y. 2014. Fate of *Vibrio parahaemolyticus* on shrimp after acidic electrolyzed water treatment. Intl J Food Microbiol 179C:50–6.

Zhang CL, Lu ZH, Li YY, Shang YC, Zhang G, Cao W. 2011. Reduction of *Escherichia coli* O157:H7 and *Salmonella enteritidis* on mung bean seeds and sprouts by slightly acidic electrolyzed water. Food Control 22(5):792–6.

Zhang CL, Li BM, Jadeja R, Hung YC. 2016. Effects of bacterial concentrations and centrifugations on susceptibility of *Bacillus subtilis* vegetative cells and *Escherichia coli* O157:H7 to various electrolyzed oxidizing water tratments. Food Control 60: 440–6.