Received: 21 September 2016 | Revised: 15 January 2017 | Accepted: 17 January 2017

DOI: 10.1111/jfpp.13304

ORIGINAL ARTICLE

WILEY Journal of Food Processing and Preservation

Inactivation of *Bacillus cereus* biofilms on stainless steel by acidic electrolyzed water

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 61401525; Natural Science Foundation of Guangdong Province, Grant/Award Number: 2015A030313598; Training Program for Outstanding Young Teachers in Higher Education Institutions of Guangdong Province, Grant/Award Number: YQ2015092; Youth Innovation Talent Project of Guangdong Province; Guangzhou Science and Technology Planning Project, Grant/Award Number: 201509010005

Abstract

The removal and inactivation of the biofilms remain challenging in the food processing environments. The objective of this study was to examine the efficacy of acidic electrolyzed water (AEW), a novel and green disinfectant, in the inactivation of *Bacillus cereus* biofilms on stainless steel surfaces. The surviving cell population in the biofilms was less than the detection limit upon inactivation by AEW (pH 2.73) for 15 min. AEW showed good storage stability. The inactivation efficiencies of AEW (>99%) against the biofilms remained high after AEW was stored in a closed and dark system at 35°C for 9 days. The presence of bovine serum albumin had a significantly negative effect on the inactivation efficiency of AEW against the biofilm cells. AEW displayed much higher inactivation efficacies against *B. cereus* biofilms than chemically modified water with the same pH, available chlorine concentration (ACC), or oxidation reduction potential (ORP).

Practical applications

Electrolyzed water (EW) has a lot of advantages such as low cost, being environmentally friendly and reduced health concerns, compared with the traditional disinfectants. Acidic EW (AEW) has proved to have a variety of antimicrobial activities. AEW may be a promising non-thermal sanitizer for disinfecting food contact surfaces, particularly those contaminated by the highly recalcitrant *Bacillus cereus* biofilms.

1 | INTRODUCTION

With increasing concerns of food safety and quality, great efforts have been made to develop new preservatives and processing technologies. In the last decade, electrolyzed water (EW), which is produced through electrolysis of a dilute salt solution (e.g., 0.05%–0.2% NaCl), has attracted growing interest as a new and ecofriendly sanitizer in the medical, agricultural, and food industries. Compared with traditional chemical disinfectants, EW has many advantages such as low cost, being environmentally friendly and decreased health concerns (Al-Haq, Sugiyama, & Isobe, 2005). EW, especially acidic EW (AEW), was found to have antimicrobial activities against most food-borne pathogens such as *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enteritidis* (Huang, Hung, Hsu, Huang, & Hwang, 2008). Therefore, AEW might be a potential non-thermal sanitizer for the decontamination of food contact surfaces and fresh ready-to-eat foods (Chiu, Duan, Liu, & Su, 2006; Fabrizio & Cutter, 2005; Hung, Tilly, & Kim, 2010). Presently, EW has been approved as a food additive in Japan (Hricova, Stephan, & Zweifel, 2008).

Bacillus cereus, a Gram-positive, spore-forming, and rod-shaped bacterium, has been classified as a hazard group II microorganism by the European Commission, due to its high infection abilities (Pexara & Govaris, 2010). In addition, it can produce two types of toxins, thus resulting in emetic and diarrheal food poisoning. The food poisoning caused by *B. cereus* was often associated with the consumption of cooked rice, pasta and other starchy foods as well as dried milk products and infant foods (Becker, Schaller, von Wiese, & Terplan, 1994; Ehling-Schulz, Fricker, & Scherer, 2004). It was reported that 20.9% samples were contaminated by *B. cereus* in 230 samples from a variety of infant and baby foods (Kim et al., 2011).

Generally, the products are contaminated via two approaches: (1) starting materials; (2) secondary contamination during food processing. Most of secondary contamination cases could be attributed to the presence of the biofilms on the food processing surfaces, since the biofilm cells were much more resistant to the commonly used sanitizers and disinfectants than the planktonic cells. According to the US National Health Institute and the Centers for Disease Control and Prevention, more than 65% of all microbial diseases involved the biofilms (Jahid & Ha, 2012). Therefore, the biofilm inactivation is an important, yet challenging task in the food industry. Fortunately, use of novel antimicrobial products and technologies created many opportunities for controlling and removing the biofilms in the food processing environments (Simoes, Simoes, & Vieira, 2010). AEW, as a novel disinfectant, has been reported to be effective to eradicate and inactivate the biofilms (Arevalos-Sanchez, Regalado, Martin, Dominguez-Dominguez, & Garc A-Almend Rez, 2012; Arevalos-Sanchez et al., 2013; Ayebah, Hung, & Frank, 2005; Kim, Hung, Brackett, & Frank, 2001). However, up to date, only several bacteria (e.g., *L. monocytogenes* and *Staphylococcus aureus*) were involved (Hricova et al., 2008). To our knowledge, the inactivation of AEW against *B. cereus* biofilms has not been reported yet. The objective of this study was to examine the inactivation effectiveness of AEW against *B. cereus* biofilms on stainless steel surfaces.

2 | MATERIALS AND METHODS

2.1 | Preparation of stainless steel coupons

Stainless steel coupons (type 304, 1.2 cm × 1.2 cm) were the gifts from Nanan Industry Co., Ltd. (Guangzhou, China). The coupons were washed with tap water, and treated ultrasonically in acetone for 15 min (40 kHz, 250 W) to remove grease; then they were rinsed in 75% alcohol for 30 min, followed by soaking in deionized water; finally, the coupons were dried at room temperature, and autoclaved at 121°C for 15 min.

2.2 | Microbial strain, culture conditions, and biofilm formation

B. cereus (CMCC 63303) was purchased from Guangdong Microbiology Culture Center (Guangzhou, China). After activation, *B. cereus* was cultured in the broth medium (peptone 10 g, beef extract 3 g, NaCl 5 g, deionized water 1,000 ml, pH 7.0) at 37°C. Cultures of *B. cereus* (0.1 ml, 10^8 CFU/ml) were added into 10 ml of the broth medium where the sterile coupons were immersed, and incubated at 37°C for 24 hr.

2.3 | Preparation of AEW and characterization of its properties

AEW was prepared through the electrolysis of 0.1% NaCl solution in a commercial EW generator (CE-7300-4, Saiai Environmental Protection and Technology Development Co. Ltd., Guangzhou, China). Its pH and oxidation reduction potential (ORP) were measured using a dual scale pH/ORP meter (Fisher Scientific, Pittsburgh, USA). The available chlorine concentration (ACC) was determined by an iodometric method with 5 mM of sodium thiosulfate standard solution (Kim et al., 2001).

2.4 | Preparation of chemically modified water

Chemically modified water was prepared through adjusting one of the three properties (pH, ORP, and ACC) of deionized water using acids or/and dilute sodium hypochlorite solutions.

2.5 | Inactivation of the biofilms with AEW and chemically modified water

The biofilms on stainless steel coupons were rinsed gently in sterile potassium phosphate buffer (PPB; 50 mM, pH 7) for 2 times to remove unattached cells. Then they were inactivated by 10 ml AEW or chemically modified water at 37°C for a designed period, followed by gentle rinse in sterile PPB (50 mM, pH 7) for 2 times. After the treated coupons were put into 10 ml sterile PPB (50 mM, pH 7.0) and subjected to ultrasonic treatment (40 Hz, 250 w) for 15 min, *B. cereus* populations suspended were determined by plating method using nutrient agar and incubated for 24 hr at 37°C. The detection limit of this technique was 0.54 log CFU/cm^2. All treatments were conducted in triplicate.

2.6 | Scanning electron microscopy (SEM) analysis

SEM images were taken on a Philips FEI-XL30 scanning electron microscope (The Netherlands). The samples were coated with Au/Pd prior to acquiring images.

2.7 | Statistical analysis

The results of microbiological tests were transformed into log values. Data were reported as means ± standard deviation from triplicate determinations. Comparisons of means were performed using One-Way ANOVA on SPSS Statistics 20 (IBM, USA).

3 | RESULTS AND DISCUSSION

3.1 | Effect of pH on the bactericidal efficiency of AEW

The populations of the survival biofilm cells were determined after dipping in AEW with various pH values at room temperature for 15 min

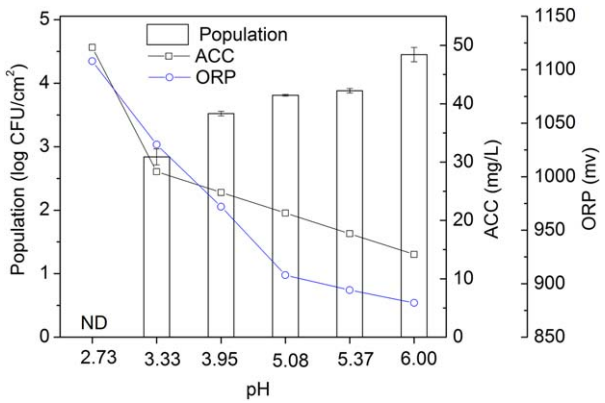

FIGURE 1 Effect of pH on AEW-mediated inactivation of *B. cereus* biofilms

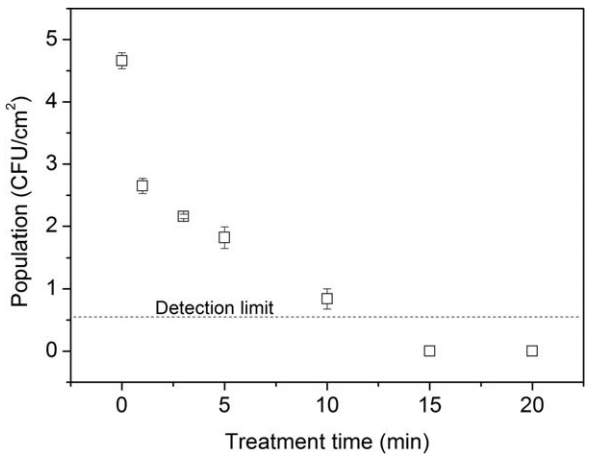

FIGURE 2 Effect of treatment time on AEW-mediated inactivation of *B. cereus* biofilms

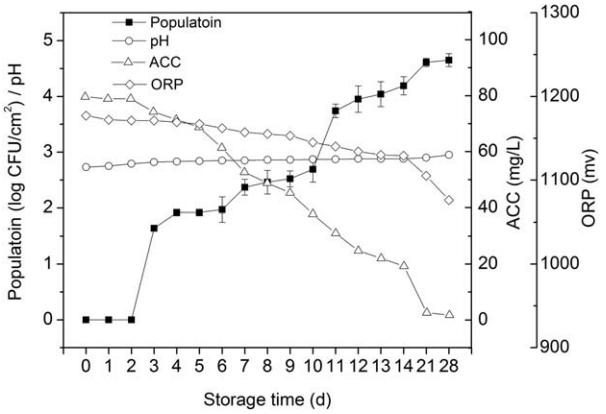

FIGURE 4 Effect of storage time on AEW-mediated inactivation of *B. cereus* biofilms

(Figure 1). As shown in Figure 1, the survival biofilm cell populations decreased significantly ($p < .05$) with decreasing AEW pH. For instance, the survival cell population was reduced to approximately 4.45 log CFU/cm^2 after inactivation by pH 6.00 AEW, while it was less than the detection limit of the conventional plating method (0.54 log CFU/cm^2) in the case of pH 2.73 AEW. Similarly, Park et al. reported that the efficacies of AEW in the inactivation of *E. coli* and *L. monocytogenes* showed a clear dependence on its pH (Park, Hung, & Chung, 2004). The low pH AEW was proposed to reduce bacterial growth and increase the sensitivity of the bacteria to active chlorine compounds. In addition to pH, both active chlorine and ORP have been widely reported to be responsible for the bactericidal activities of AEW (Hricova et al., 2008). Therefore, the ACC and ORP of AEW with different pH were measured. Figure 1 shows that low pH AEW has the higher ACC. It was reported that active chlorine compounds (HOCl, Cl_2, and OCl^-) would destroy cell membranes and nucleic acids of microorganisms as well as key enzymes (Hricova et al., 2008), resulting in the inactivation of bacterial cells. In addition, the relative proportions of these active chlorine compounds were pH dependent. HOCl and Cl_2 were dominant in AEW when its pH was less than 5.0; nonetheless, OCl^- that has a lower bactericidal activity than HOCl had a higher content than HOCl and Cl_2 at pH > 5.0. It might partially explain the much higher efficiency of low pH AEW in the activation of *B. cereus* biofilms. On the other hand, the ORP drastically decreased with the increment of pH, likely due to the Nernst Law (Oldham & Mayland, 1994). The high ORP would change the electron flow in the cells, and lead to the damage of cell membranes and sulfhydryl compounds on the cell surfaces (Liao, Chen, & Xiao, 2007). Hence, the high ORP might also make a significant contribution to the high bactericidal activity of low pH AEW. AEW of pH 2.73 was used in the subsequent studies.

3.2 | Effect of treatment time on the bactericidal efficiency of AEW

The surviving cell populations in the biofilms were recorded after inactivation at room temperature by pH 2.73 AEW for different time (Figure 2). Figure 2 shows that the populations of the survival biofilm cells decrease significantly ($p < .05$) with the prolongation of the inactivation time, which is in good agreement with the previous results (Arevalos-Sanchez et al., 2012; Kim et al., 2001). For example, the surviving population decreased from the initial 4.66 log CFU/cm^2 to 2.65 log CFU/cm^2 after dipping in AEW for 1 min. It was worth noting the biofilm cell population on stainless steel surface was less than the detection limit after inactivation for 15 min. It was also verified by comparing SEM images of the biofilm cells on stainless steel before and after 15 min inactivation (Figure 3). The time required for complete inactivation of the biofilms appeared to be much longer

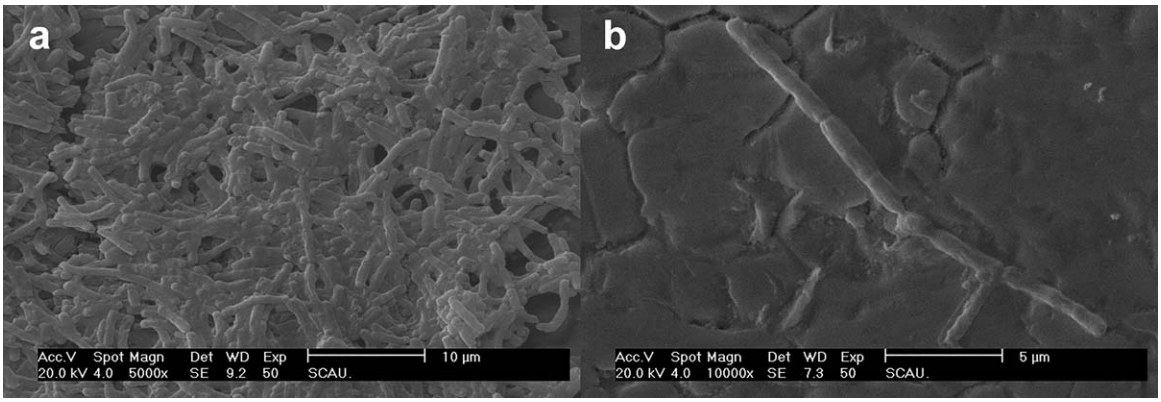

FIGURE 3 SEM images of the biofilm cells before (a) and after inactivation (b)

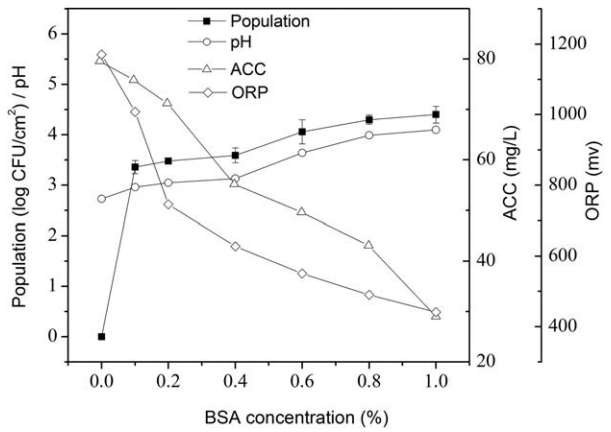

FIGURE 5 Effect of the presence of BSA on AEW-mediated inactivation of *B. cereus* biofilms

compared with that for complete inactivation of planktonic bacteria (Ding, Rahman, & Oh, 2011; Issa-Zacharia, Kamitani, Morita, & Iwasaki, 2010), possibly due to the higher resistance of the former to environmental stress (Davies, 2003; Mah & O'Toole, 2001).

3.3 | Effect of storage time on the bactericidal efficiency of AEW

One of the limiting factors in the use of AEW is the rapid reduction of its antimicrobial activity over time under specific storage conditions (Al-Haq et al., 2005). Len et al. found that the chlorine loss through evaporation followed first-order kinetics under open conditions and active chlorine compounds in EW disappeared totally after 100 hr storage (Len et al., 2002). Therefore, AEW was stored in a closed, static and dark system at 35°C, and the effect of the storage time on the inactivation efficiencies of AEW against *B. cereus* biofilms was studied (Figure 4). Figure 4 shows the surviving populations of the biofilm cells after 15 min inactivation with AEW stored for different time. The biofilm cell population on stainless steel surface was less than the detection limit after inactivation by AEW stored for 3 days. The inactivation efficiencies of AEW (>99%) against the biofilms remained high after it was stored for 9 days. However, AEW stored for 28 days displayed a pretty low bactericidal activity. Moreover, the changes in the physicochemical properties of AEW were examined during storage. As shown in Figure 4, the pH and ORP of AEW were 2.95 and 1076 mV after storage for 28 days, respectively, which were comparable to the corresponding values (2.73 and 1,177 mV, respectively) of the fresh AEW. However, the ACC reduced significantly from the original 79.59 mg/L to 1.74 mg/L, which is consistent with the previous reports (Hsu & Kao, 2004). It suggests that the decreased inactivation efficiencies of AEW during storage may be mainly attributed to the reduction of the ACC, likely because of Cl_2 evaporation and HOCl decomposition (Al-Haq et al., 2005).

3.4 | Effect of the presence of BSA on the bactericidal efficiency of AEW

Figure 5 shows the surviving cell populations in the biofilms after they are inactivated with AEW for 15 min in the presence of bovine serum albumin (BSA). It was found that the inactivation effectiveness of AEW decreased significantly with the increment of BSA concentration, which is in good agreement with the previous results (Oomori, Oka, Inuta, & Arata, 2000; Park, Alexander, Taylor, Costa, & Kang, 2008, 2009). The surviving population was less than the detection limit in the absence of BSA, while it significantly increased to 3.36 log CFU/cm^2 in the presence of 0.1% BSA. Besides, the properties of AEW were characterized in the presence of various concentrations of BSA (Figure 5). The pH increased slightly ($p > .05$) upon the addition of BSA, while ACC and ORP changed markedly. In addition, both ACC and ORP reduced significantly ($p < .05$) with increasing BSA concentration. Previously, several groups also reported that organic matter exerted a significant effect on the ACC of AEW, and that protein or amino acid would react with the active chlorine compounds, resulting in organic chloramines (Ayebah, Hung, Kim, & Frank, 2006; Oomori et al., 2000). The significant changes in the ACC and ORP of AEW might account for its reduced inactivation efficacies against the biofilm cells in the presence of BSA. It suggests that the organic contaminants should be removed prior to the inactivation of the biofilms by AEW.

3.5 | Comparison of the efficacies of AEW and chemically modified water in the inactivation of biofilms

Although AEW has been demonstrated to have high bactericidal and virucidal activities, and moderate fungicidal activities, it is not clear

TABLE 1 Comparison of the inactivation efficacies of AEW and chemically modified water against *B. cereus* biofilms

		Properties			Population (log CFU/cm^2)[a]		
Entry	Reagents	pH	ACC (mg/L)	ORP (mV)	1 min	5 min	15 min
1	Deionized water	6.82	0	504	4.66 ± 0.02a	4.64 ± 0.03a	4.61 ± 0.04a
2	AEW	2.73	79.6	1177	2.65 ± 0.03a	1.82 ± 0.04b	ND[b]c
3	Acetic acid solution (5%)	2.73	0	746	3.70 ± 0.05a	3.35 ± 0.04b	3.19 ± 0.03c
4	HCl solution (0.004 M)	2.73	5.8	567	3.57 ± 0.06a	3.49 ± 0.02a	3.34 ± 0.04b
5	NaOCl solution (0.05%)	10.39	79.6	506	3.53 ± 0.03a	3.40 ± 0.02b	3.35 ± 0.05b
6	HCl (0.01 M) + NaOCl (0.05%) solution	2.75	29.7	1177	3.74 ± 0.04a	3.34 ± 0.03b	3.21 ± 0.03c
7	Acetic acid (10%) + NaOCl (0.05%) solution	2.72	34.0	1177	3.72 ± 0.09a	3.32 ± 0.04b	2.92 ± 0.02c

[a] Data are expressed as means ± standard deviation. Same capital letters within each row indicate no significant difference ($p > .05$).
[b] ND: less than the detection limit.

whether pH, active chlorine compounds, ORP of AEW, or their synergistic effects are responsible for its antimicrobial activities (Al-Haq et al., 2005). To gain a deep understanding of the antimicrobial mechanism of AEW, a comparison study of the inactivation efficacies of AEW and chemically modified water with the same pH, ACC or ORP against *B. cereus* biofilms was conducted (Table 1), in which deionized water was used as the control. The surviving populations changed slightly over time when deionized water was used (Table 1, entry 1), indicating that the biofilms were very stable and dipping would not lead to the reduction in the biofilm cells. As shown in Table 1, entry 2, significant decreases in the surviving populations of the biofilm cells were observed upon AEW treatment. After 15 min inactivation by AEW, the population was less than the detection limit. As compared with AEW, chemically modified water with pH 2.73 showed much lower inactivation efficacies against *B. cereus* biofilms (Table 1, entries 3 and 4). For example, the surviving population of 3.34 log CFU/cm^2 was observed after 15 min inactivation by pH 2.73 HCl solution, which is even higher than that (2.65 log CFU/cm^2) after 1 min inactivation by AEW. It suggests that low pH is not the unique contributor to the high bactericidal capacity of AEW. Likewise, the efficacy of NaOCl solution with the ACC of 79.6 mg/L was much lower in the inactivation of the biofilms than that of AEW, since the surviving population was up to 3.35 log CFU/cm^2 after 15 min inactivation by the former (Table 1, entry 5). In addition, chemically modified water with the ORP of 1,177 mV exhibited much lower bactericidal activities than AEW (Table 1, entries 6 and 7). For example, the surviving populations were 3.32 and 2.92 log CFU/cm^2, respectively, after the biofilms were inactivated for 5 and 15 min by chemically modified water containing both 10% acetic acid and 0.05% NaOCl (Table 1, entry 7). The results obtained in Table 1 suggest that the high inactivation efficiency of AEW against *B. cereus* biofilms may be attributed to the synergistic effects of pH, active chlorine and ORP rather than one of the three properties. In addition, active chlorine compounds appeared to play a decisive role in the inactivation of AEW against the biofilm cells.

4 | CONCLUSIONS

In summary, AEW has been demonstrated to be effective for the inactivation of *B. cereus* biofilms for the first time. *B. cereus* biofilms on stainless steel surfaces could be killed totally by AEW at room temperature after 15 min. The low pH and high ORP of AEW might act synergistically with the active chlorine compounds to inactivate biofilms, which rationally accounted for the high bactericidal efficacy of AEW. AEW is a promising alternative to the traditional chemical disinfectants, due to low cost, being environmentally friendly, and reduced health concerns. Use of AEW may open up novel opportunities for controlling and inactivating the biofilms in the food processing.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (61401525), the Natural Science Foundation of Guangdong Province (2015A030313598), the Training Program for Outstanding Young Teachers in Higher Education Institutions of Guangdong Province (YQ2015092), the Youth Innovation Talent Project of Guangdong Province, and Guangzhou Science and Technology Planning Project (201509010005).

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How to cite this article: Li N-W, Liu G-L, Liu J. Inactivation of *Bacillus cereus* biofilms on stainless steel by acidic electrolyzed water. *J Food Process Preserv*. 2017;00:e13304. https://doi.org/10.1111/jfpp.13304