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Morphophysiological responses of detached and adhered biofilms of *Pseudomonas fluorescens* to acidic electrolyzed water

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ABSTRACT

Pseudomonas spp. have emerged as the main spoilage bacteria, with many strains easily forming biofilms on food-contact surfaces and causing cross-contamination. The efficacy of disinfectants against bacteria is usually tested with planktonic cells; however, the disinfection tolerance of biofilms, especially detached biofilms, remains unknown. Here, we investigated the tolerance responses of detached and adhered biofilms of *Pseudomonas fluorescens* to acidic electrolyzed water (AEW) by determining tolerance responses by plate counting, comparing them using a Weibull model, and verifying changes in bacterial morphology by scanning electron microscopy. The experimental data and the responses calculated using Weibull a (scale) and b (shape) parameters agreed well (R^2 values: 0.974–0.999), and we found that AEW exhibited effective antimicrobial activity against *P. fluorescens,* with adhered biofilms were more resistant than detached biofilms and planktonic cells. Additionally, AEW increased the bacterial membrane permeability and decreased the membrane potential, intracellular ATP concentrations, and intracellular pH while also triggering the disruption of extracellular polymeric substances. These results demonstrated that the morphophysiological responses of detached and adhered biofilms differed significantly and provided information on disinfectant-resistance strategies potentially beneficial to the development of novel disinfection approaches.

1. Introduction

Microbial spoilage accompanied by deficient preservation can cause the waste of roughly one-third (~1.3 billion tons annually) of food produced for human consumption worldwide (Brautigam et al., 2014). Spoilage microorganisms are transferred to food at the stages of production, processing, distribution, or consumption and can lead to cross-contamination associated with many cases of food recall and spoilage losses. *Pseudomonas fluorescens* is a Gram-negative, non-spore-forming, oxidase-positive organism that produces the majority of heat-stable lipases and proteases and is causative of off-flavors and unpleasant textural changes in the food industry (Wiedmann et al., 2000). The spoilage profiles of *P. fluorescens* have been widely surveyed (Ge et al., 2017). As a common habitant of poultry, fish, dairy, and other fresh products, *P. fluorescens* is recognized as among the most important spoilage bacteria due to its short generation time, resistance to heat treatment, and ability to form biofilms, especially at low temperatures (Aswathanarayan and Vittal, 2014; Dogan and Boor, 2003). Therefore, it is important to control and prevent this bacterium from contaminating food and the food-processing environment.

When spoilage bacteria were attached to solid-phase surfaces in food-processing environments, such as equipment surfaces, conveyer belts, and containers, they proliferate and form microcolonies that eventually develop into complex structures called biofilms. These bacterial communities produce extracellular polymeric substances (EPS) to form a protective layer enabling cell aggregation and cohesion, which is quite different from planktonic cells. The cells in biofilms can survive stressors commonly encountered within food-processing environments (e.g., refrigeration, acidity, salinity and disinfection) and subsequently reattach to suitable surfaces, thereby increasing the risk to food safety (Wang et al., 2016a,b; Fernández et al., 2014; Toté et al., 2010). Similar to many other microorganisms, *P. fluorescens* can also form biofilm in vitro on stainless steel and glass (Dagang et al., 2016).

Environmental changes, including increased shear stress and alteration of EPS cohesion by disinfection, can trigger biofilm detachment

Abbreviations: ACC, available chlorine concentration; AEW, acidic electrolyzed water; EPS, extracellular polymeric substances; ORP, oxidation-reduction potential; PI, propidium iodide; SEM, scanning electron microscopy; TSA, trypticase soy agar; TSB, trypticase soy broth

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as part of the biofilm life cycle and guarantee the survival of the community when conditions are no longer suitable for proliferation (Shi et al., 2017; Simões et al., 2008). The amount of the community that detaches from surfaces ranges from single cells to large clusters containing more than 1000 cells. The detached biofilms are also hazardous because they can behave similarly to adhered biofilms and survive disinfection challenges due to neutralization or the partial consumption of the disinfectant by EPS, especially shortly after detachment. Cells in detached biofilms retain the ability to adhere to surfaces and form biofilms for at least 48 h after detachment. In contrast to the abilities of permanently planktonic cells, this ability facilitates the colonization of new surfaces (Berlanga et al., 2014).

Regular disinfectants used to kill planktonic cells might not be effective at disinfecting cells in the form of biofilms. In a factory, conventional disinfection is performed using a strong oxidizing agent such as sodium hypochlorite. Although these compounds are inexpensive and commonly used in various industrial applications, health concerns exist due to the carcinogenic and mutagenic potential of disinfection byproducts, such as trihalomethanes and other compounds (Simon et al., 2014), and microorganisms exhibiting resistance to such agents can survive and grow under increasingly higher concentrations of disinfectant. A recent candidate for disinfection includes acidic electrolyzed water (AEW), which is generated by the anodic electrolysis of dilute NaCl solutions. Its physicochemical properties include having a low pH, an available chlorine concentration (ACC), and a high oxidation-reduction potential (ORP). AEW has the potential to be an environmentally friendly sanitizing agent in food sanitization, medical sterilization, agriculture and other fields (Wang et al., 2014). Substantial research has been conducted focusing on its antibacterial activity, including its disinfection efficacy on bacteria grown on various food surfaces, such as vegetables (Hao et al., 2015), fruits (Graça et al., 2011), and meat (AlHoly and Rasco, 2015), and on various food-contact surfaces, such as stainless steel (Li et al., 2017), plastic (Pagedar and Singh, 2015), and glass (Handojo et al., 2009), and determined by measuring reductions in the number of viable cells. Another research area concerns the mechanism by which AEW disinfects planktonic cells in vitro, focusing on the physicochemical properties of AEW and bacterial disinfection targets (Hao et al., 2012, 2016; Zeng et al., 2010). However, few studies have addressed the treatment of detached biofilms with disinfectants and the differences in the levels of resistance between the two types of biofilms.

This study aimed to 1) evaluate the performance of planktonic cells and detached and adhered biofilms formed by *P. fluorescens* exposed to AEW and 2) investigate the morphophysiological differences between detached and adhered biofilms. This paper offer a better understanding of the resistance of biofilms to disinfectants and provide an experimental basis for the development of novel disinfection approaches.

2. Materials and methods

2.1. Bacterial cultures and growth conditions

Bacteria were collected from both spoiled chicken carcasses and conveyor belt surfaces in a slaughter plant. *P. fluorescens* (NCM 90) was isolated, verified by its 16S DNA sequences and typical biochemical reactions and then stored at the National Center of Meat Quality and Safety Control (NCM). Prior to each experiment, cultures were stored at −20 °C and then activated on trypticase soy agar (TSA, Hope-Biotechnology, Co. Ltd., Beijing, China) at 28 °C overnight. A single colony from each plate was selected and cultured in 6 mL of trypticase soy broth (TSB, Hope-Biotechnology, Co. Ltd.) at 28 °C for 24 h without shaking. Biofilm formation and the growth of planktonic cells was performed according to procedures described by Jia et al. (2017), with a slight modification. Food-grade stainless-steel coupons (75 × 25 × 1 mm, 304 type, 2b finish; Zhongyi Stainless Steel Materials Co., Ltd., Dongnan, China) were used for biofilm development. Stainless steel, as the main surface material in the modern poultry and meat-processing industries, is highly susceptible to contamination when in contact with carcasses, which increases the likelihood of cross-contamination and enables biofilms to easily adhere to stainless steel surfaces. Prior to use, the coupons were washed with acetone (overnight), as previously described (Tondo et al., 2010). Sterile TSB was then transferred into a sterile glass box containing the stainless-steel coupons, and the initial inoculum concentration was adjusted to approximately $\sim 10^2$ CFU/mL. Half of each stainless-steel coupon was submerged into TSB, and the other half was exposed to the air. The inoculated box was then hermetically sealed with sterilized cling film and incubated at 20 °C for 5 days without agitation in order to allow the biofilm formation. The planktonic cells (no stainless-steel coupon in TSB medium) were incubated in the same manner.

2.2. Preparation of AEW

AEW was prepared by electrolyzing different concentrations of NaCl solution and deionized water using different electricity in an electrolyzed water generator (XL-150; Baoji Xinyu Optics-Mechanics-Electricity Co., Ltd., Baoji, China). The pH, ORP, and ACC of the AEW were measured immediately before each bactericidal experiment. The pH and ORP of AEW samples were measured using a pH/ORP meter (Thermo Fisher Scientific, Pittsburgh, PA, USA) using an electrode and an ORP electrode. The ACC of AEW samples was determined using a digital chlorine test kit (model 16900; Hach Co., Ltd., Loveland, CO, USA). The physicochemical properties and preparation conditions of each AEW sample are shown in Table 1.

2.3. AEW treatment of detached and adhered biofilms

For the sampling of adhered biofilms, a single stainless-steel coupon containing biofilms was rinsed three times with sterile 0.9% NaCl solution to remove unattached cells and according to procedures described by Cai et al. (2018). The coupons were subsequently immersed in 20 mL of sterile 0.9% NaCl as a control or AEW solutions for 5, 10, 15, 20, 25, and 30 min (treatment groups). Subsequently, the coupons were immersed in 40 mL of neutralizing buffer solution (phosphate-buffered saline) containing 0.8% sodium thiosulfate (pH 7.2) to stop the bactericidal effects of AEW. Surviving cells were collected by scraping the coupons and transferred to tubes containing sterile 0.9% NaCl solution. Serial dilutions with 0.9% NaCl solution were plated onto TSA and incubated at 28 °C for 24 h.

After rinsing three times, the biofilms were gently scraped from

Table 1
Physicochemical properties of acidic electrolyzed water.

NaCl concentration (%)	Electricity (A)	ACC (mg/L)	ORP (mV)	pH
2.0	16	20.3 ± 0.5c	1116.0 ± 2.5c	2.91 ± 0.01a
3.5	18	40.3 ± 0.5b	1152.7 ± 1.7b	2.78 ± 0.00b
4.6	19	61.3 ± 0.5a	1167.7 ± 0.9a	2.69 ± 0.00c

Values are expressed as the mean ± standard deviation (n = 5). ACC, available chlorine concentration; ORP, oxidation-reduction potential. a-c: within a column, values with different lowercase letters indicate values that are significantly different ($P < 0.05$).

stainless-steel coupons into tubes containing sterile 0.9% NaCl solution as detached biofilms, followed by their exposure to 4.5 mL of sterile 0.9% NaCl solution as a control or AEW for 5, 10, 15, 20, 25, and 30 min (treatment groups). Neutralizing buffer solution was subsequently used to stop bactericidal effects. Serial dilutions with 0.9% NaCl solution were plated onto TSA and incubated at 28 °C for 24 h.

2.4. AEW treatment of planktonic cells

Lethality tests were also performed using *P. fluorescens* suspensions for comparison. A 5-day *P. fluorescens* culture was washed, and cells were suspended in sterile 0.9% NaCl solution to yield cell concentrations of ~8 log CFU/mL to 9 log CFU/mL. A 0.5-mL aliquot of the cell suspensions was then exposed to 4.5 mL of AEW for 5, 10, 15, 20, 25, and 30 min, after which the 0.5-mL aliquot of the AEW-treated planktonic culture was immediately transferred to 4.5 mL of neutralizing solution. Control samples were treated with sterile 0.9% NaCl solution instead of AEW. All cell counts were determined in five independent experiments by growth on TSA after 24 h at 28 °C.

2.5. Inactivation efficacy and kinetic modeling

Survival curves were plotted as the logarithm decrease [log (N/N0)] in surviving cells versus contact time. To determine the inactivation kinetics, the Weibull model was used to fit the data according to the following equation (Buzrul et al., 2005):

$$\mathrm{Log}\frac{N}{N0} = -at^{b} \tag{1}$$

In Eq. (1), N and N0 are the number of bacterial cells at times t and 0 following exposure to AEW, respectively, and a and b are the scale and shape factors, respectively. These parameters (a and b) might differ if log [N(t)/N0] versus time, t, is used instead of [N(t)/N0] versus time, t, because of the differences in these two forms of representation regarding the weight that is assigned to the central part and tail regions of the distribution. Because the distribution tail is of particular interest in microbial-inactivation processes, the logarithmic form of the relationship should be used as a measure of the efficacy of these processes. Such a model presents the main advantage of remaining very simple while being sufficiently robust to describe both downward concave survival curves ($b > 1$), which report increases in the hazard, and upward concave curves ($b < 1$), suggesting that the hazard is constant. For the linear model originating from the first order polynomial, $b = 1$ describes a decrease in the hazard (Brodowska et al., 2017).

2.6. Measurement of membrane integrity

To evaluate the membrane integrity of *P. fluorescens* biofilms following exposure to AEW, the BacLight LIVE/DEAD membrane permeability kit (Invitrogen, Carlsbad, CA, USA) was used according to manufacturer guidelines. The kit comprises two dyes, propidium iodide (PI) and SYTO 9, that stain nucleic acids. *P. fluorescens* cultures were prepared as described and harvested at an initial concentration of 10^6 CFU/mL. Following exposure to AEW or the control solution, each mL of cells was incubated in 3 μL of a mixture of SYTO 9 and PI for 15 min at room temperature in the dark, followed immediately by flow cytometric analysis. *P. fluorescens* cells were analyzed following 15 min and 30 min of exposure to AEW for detached and adhered biofilms, respectively. The periods of 15 min and 30 min represent the exposure times required for the complete bactericidal activity of AEW against detached and adhered biofilms. A flow cytometer (BD AccuriC6; BD Accuri Cytometers, Ann Arbor, MI, USA) fitted with a 488-nm excitation laser was used for membrane-integrity analyses. Green fluorescence was detected on channel FL1 with a 525-nm bandpass filter, and red fluorescence was detected on channel FL3 with a 620-nm bandpass filter. The results are expressed as the reduction in the ratio of green/red fluorescence from the control value.

2.7. Measurement of membrane potential

Membrane potential was determined using the BacLight bacterial membrane potential kit (Thermo Fisher Scientific, Waltham, MA, USA). The assay uses 3,3′-diethyloxacarbocyanine [$DiOC_2(3)$], a dye that exhibits green fluorescence in all bacterial cells but whose fluorescence shifts toward red as the dye molecules self-associate at higher concentrations in the cytosol, which occurs in cells with larger membrane potentials relative to other cells (Novo et al., 2015). *P. fluorescens* cultures were prepared as described and harvested at an initial concentration of 10^6 CFU/mL. Prior to staining, 1 mM EDTA was added to significantly improve the staining results for the Gram-negative strains. Briefly, in 1 mL of bacterial suspension, 10 μL of 3 mM $DiOC_2(3)$ was added and mixed for 15 min at 25 °C in the dark, and the membrane potential was estimated according to the ratio of red/green $DiOC_2(3)$ fluorescence. At the concentration of dye used, the green fluorescence primarily caused by emission from single dye molecules varies along with the size of the bacterial cell but is largely independent of membrane potential, whereas red fluorescence due to emission from dye aggregates is dependent upon both size and membrane potential. Therefore, the ratio provides a cell-size-independent measurement of membrane potential.

2.8. Measurement of intracellular ATP concentrations

Intracellular ATP concentrations were determined according to the instructions of BacTiter-Glo microbial cell viability assay (Promega Biotec, Madison, WI, USA). In this assay, the luminescence signal was proportional to the amount of ATP present. *P. fluorescens* cells in both biofilms treated with AEW were harvested at an initial concentration of 10^6 CFU/mL, after which 100 μL of reagent was added to 100 μL of medium containing cells for a 96-well opaque-walled plate. The contents were mixed briefly on an orbital shaker and incubated for 5 min at 25 °C, followed by the recording of luminescence using a multimode microplate reader (SpectraMax M2e; Molecular Devices, San Jose, CA, USA). For comparison, the intracellular ATP concentration of cells receiving no AEW treatment was used as a control.

2.9. Measurement of intracellular pH (pHi)

P. fluorescens cells in both biofilms treated with AEW were harvested at an initial concentration of 10^6 CFU/mL, and the pHi was determined following AEW treatment according to a modified version of the method described by Wang et al. (2016c) using the pHi-sensitive dye BCECF-AM (Dojindo Co., Tokyo, Japan). The cells were resuspended in 5 mL of HEPES buffer [138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM glucose, and 20 mM HEPES (pH 7.4)] containing 3 μM BCECF-AM, followed by incubation for 30 min at 37 °C, washing once in HEPES buffer to remove extracellular dye, and resuspension in 5 mL of the HEPES buffer. A pHi standard curve was established by combining the BCECF-AM dye with an intracellular pH calibration buffer kit (Thermo Fisher Scientific) according to manufacturer guidelines. Determination was performed using an excitation wavelength of 490 nm and an emission wavelength of 530 nm with the multimode microplate reader (SpectraMax M2e; Molecular Devices).

2.10. EPS chemical analysis

Both loose EPS (L-EPS) and bound EPS (B-EPS) were extracted using the modified EDTA method according to previously reported protocols (Cao et al., 2011; Pan et al., 2010). Briefly, cells were centrifuged three times with 0.9% NaCl, and the supernatants were combined and then filtered through a 0.22-μm membrane, resulting in filtrate representing L-EPS. For B-EPS, the sediment was then suspended in 0.9% NaCl to a final cell density of ~5 mg wet-cell-biomass/mL. An equal volume of 2% Na_2-EDTA (53.7 mM) in 0.9% NaCl was added to the cell suspension, and the mixture was incubated at 4 °C for 3 h. The cells were pelleted by centrifugation at 14,000*g* and 4 °C for 20 min, and supernatants were filtered through a 0.22-μm membrane. Both L-EPS and B-EPS were purified using a dialysis membrane of 3.5 kD in deionized water. The amounts of protein and carbohydrate in the filtrate were analyzed, with carbohydrate content measured using the phenol-sulfuric acid method with glucose as the standard, and protein content measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific).

2.11. Visualization of the P. fluorescens cells by scanning electron microscopy (SEM)

SEM images were taken of *P. fluorescens* cells treated with AEW and untreated control cells that had been fixed with 2.5% glutaraldehyde (v/v) overnight. Following fixation for 90 min in 1% osmic acid (v/v), the fixed cells were subjected to graded alcohol dehydration [50%, 70%, 80%, 95%, and 100% (twice); 10 min each] and then coated with gold (Wang et al., 2013). Images were obtained with a Hitachi S−3000N SEM instrument (Hitachi, Tokyo, Japan) at 15,000 × magnification.

2.12. Statistical analysis

Curve Expert software (v.1.4 for Windows; https://www.curveexpert.net/) was used for nonlinear regression analysis and to determine the parameters of the Weibull model. The results are presented as the mean ± standard deviation and were analyzed by a one-way Duncan's test using SPSS (SPSS Inc., Chicago, IL, USA) to assess whether differences were significant ($P < 0.05$).

3. Results

3.1. Inactivation kinetics of P. fluorescens cells susceptible to AEW

The inactivation curves of planktonic cells, as well as detached and adhered biofilms, were fitted with the Weibull model (Fig. 1), and parameters were predicted using the equation for determining the efficacy of AEW (Table 2). Observed values are shown as black dots, and the first value can be assumed to be zero (when t = 0, log (N/N0) is 0). The experimental data revealed that inactivation of *P. fluorescens* cells treated with AEW significantly increased in a concentration-dependent manner. Moreover, after exposure to 20 mg/L AEW, the numbers of viable planktonic cells and viable cells in detached biofilms were reduced by 7.3 log after 5-min and 20-min treatments, respectively. Additionally, we achieved a ~6.7 log CFU/cm^2 reduction in adhered biofilms with 30 min of treatment, indicating an incomplete killing of cells in adhered biofilms. Compared with 20 mg/L AEW treatment, 40 mg/L AEW showed a more complete bactericidal effect on planktonic cells and detached biofilms, with the same log reduction value of 7.3 at 5 min and 15 min, and the number of viable cells in adhered biofilms showing a log reduction value of 6.7 for a 25-min treatment. Viable cells were detected during the long immersions of stainless-steel coupons into AEW (from 25 to 30 min). Although the log reduction value was the same for treatment with 60 mg/L AEW, complete lethality against planktonic cells and detached biofilms required less time (5 and 10 min) using this treatment as compared with that required for 20 mg/L and 40 mg/L AEW treatments. The number of viable cells in adhered biofilms was reduced by 6.7 log CFU/cm^2 following a 30-min treatment with 60 mg/L AEW. Table 2 shows estimation of the N0, a, and b parameters, with the R^2 value indicating the goodness of fit for *P. fluorescens* planktonic cell and biofilm kinetics. Because the R^2 values were between 0.974 and 0.999, the agreement between the experimental data and the values calculated for the Weibull model through the variation of a and b parameters was good for both biofilm- and planktonic cell-inactivation kinetics. Planktonic cells showed lower resistance than detached and adhered biofilms at the same AEW concentration according to the higher scale (a) and lower shape (b) parameters of the planktonic cell kinetics. Furthermore, the shapes of the survival curves were very similar and characterized by an initial drop, followed by a tailing due to disinfectant resistance.

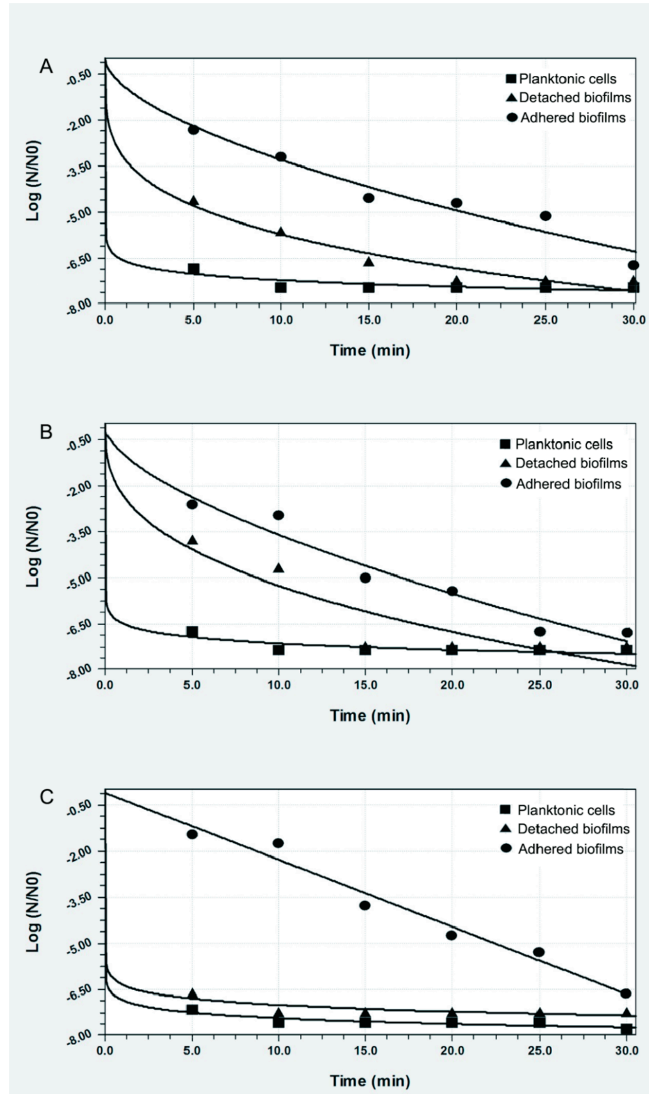

Fig. 1. Survival curves of *Pseudomonas fluorescens* treated with (A) 20 mg/L, (B) 40 mg/L, and (C) 60 mg/L acidic electrolyzed water. The figure shows the mean values of six independent experiments fitted by the Weibull model.

3.2. Membrane integrity analysis

The times required by different AEW treatments to kill the highest concentrations of *P. fluorescens* with the corresponding bactericidal activity differed (Table 3). Because membrane permeabilization can occur naturally following cell death, 15 min and 30 min were used as the minimum periods of bactericidal activity in order to directly evaluate the effects of AEW on *P. fluorescens* membrane integrity. This parameter can be indicative of the membrane integrity of the cells in *P. fluorescens* biofilms, because the red fluorescing dye can only enter cells with damaged membranes.

As shown in Fig. 2, longer contact time with and higher concentrations of AEW caused a constant increase in the inactivation percentage for both biofilms, which corresponds to more cell staining with PI and indicates that more of the cell membrane was compromised. AEW at concentrations of 20 mg/L, 40 mg/L, and 60 mg/L decreased

Table 2
Weibull model parameters for inactivation of *P. fluorescens* planktonic cells, detached biofilms and adhered biofilms treated by acidic electrolyzed water.

Treatment (mg/L)	Cell state	Parameters			
		N0	a	b	R^2
20	PC	8.154 ± 0.054	6.562 ± 0.021	0.042 ± 0.062	0.999
	DB	7.936 ± 0.139	3.316 ± 0.141	0.249 ± 0.240	0.996
	AB	8.081 ± 0.061	0.868 ± 0.010	0.583 ± 0.079	0.988
40	PC	8.074 ± 0.185	6.482 ± 0.510	0.042 ± 0.009	0.999
	DB	7.936 ± 0.139	2.288 ± 0.037	0.362 ± 0.071	0.974
	AB	8.081 ± 0.061	0.855 ± 0.008	0.622 ± 0.102	0.988
60	PC	8.301 ± 0.241	6.863 ± 0.679	0.033 ± 0.010	0.999
	DB	7.936 ± 0.139	6.345 ± 0.340	0.043 ± 0.014	0.999
	AB	8.057 ± 0.287	0.273 ± 0.066	0.941 ± 0.056	0.993

Values are expressed as the mean ± Standard deviation (n = 5). PC, planktonic cells; DB, detached biofilms; AB, adhered biofilms.

Table 3
Protein and carbohydrate content of extracellular polymeric substances from *P. fluorescens* detached biofilms and adhered biofilms treated with 40 mg/L acidic electrolyzed water.

Treatment (mg/L)	Cell state	Protein	Carbohydrate	Protein/carbo-rate ratio
		(μg/mg-wet-cell-biomass)	(μg/mg-wet-cell-biomass)	hydrate ratio
L-EPS				
Control	DB	548.67 ± 15.07b	121.46 ± 0.48b	4.53 ± 0.16c
	AB	580.40 ± 2.90a	128.61 ± 2.03a	4.51 ± 0.45c
40	DB	257.44 ± 1.21c	52.42 ± 1.00c	5.35 ± 0.95b
	AB	204.35 ± 2.87d	19.72 ± 1.33d	10.46 ± 0.74a
B-EPS				
Control	DB	23.18 ± 1.52B	9.43 ± 0.23B	2.46 ± 0.16A
	AB	46.27 ± 1.27A	17.24 ± 0.21A	2.68 ± 0.14A
40	DB	ND	ND	ND
	AB	ND	ND	ND

Values are expressed as the mean ± Standard deviation (n = 5). ND: not detected. a to d: for L-EPS values with different lowercase letters within a column are significantly different ($P < 0.05$). A to B: for B-EPS, values with different uppercase letters within a column are significantly different ($P < 0.05$). DB, detached biofilms, AB, adhered biofilms; L-EPS, loose extracellular polymeric substances; B-EPS, bound extracellular polymeric substances.

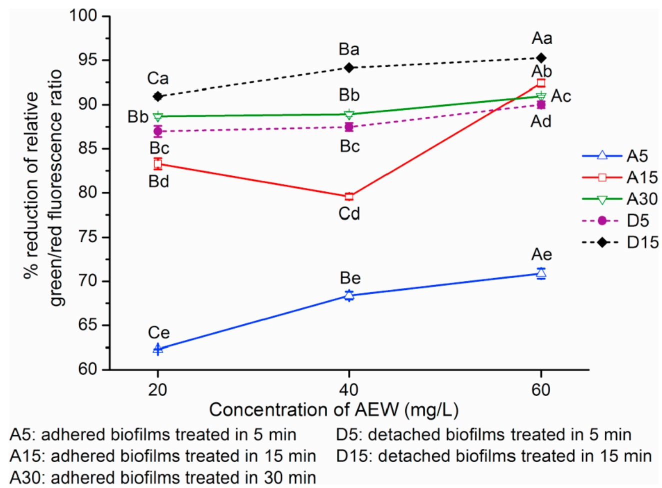

Fig. 2. Effect of treatment with 20, 40, and 60 mg/L acidic electrolyzed water (AEW) on the membranes of *Pseudomonas fluorescens* detached and adhered biofilms. Bars represent standard deviations (n = 3). a–e: for treatments with the same concentration of AEW, values with different lowercase letters are significantly different ($P < 0.05$). A–C: for treatments with only one kind of biofilm, values with different uppercase letters are significantly different ($P < 0.05$).

the membrane integrity of cells in detached biofilms to 13.03%, 12.53%, and 9.99% of their initial value after 5-min treatments, respectively, with reduction percentages significantly increasing ($P < 0.05$) with 15-min treatments; however, the reduction percentage in the membrane integrity of cells in adhered biofilms treated with AEW was significantly lower ($P < 0.05$) than that of cells in detached biofilms.

3.3. Membrane potential analysis

Fig. 3 shows the membrane potential of AEW-treated detached and adhered biofilms and control biofilms. Adhered biofilms had a higher initial membrane potential than detached biofilms. As evidenced by a reduction in the fluorescence ratio during monitoring of the membrane potential, the membrane potential significantly decreased ($P < 0.05$) in all treatment groups, indicating concentration-dependent depolarization. Moreover, detached biofilms showed a lower ($P < 0.05$) membrane potential than adhered biofilms after treatments with all concentrations of AEW. Additionally, increased concentrations of AEW caused a higher degree of cell depolarization.

3.4. Analysis of intracellular ATP concentrations

The effect of AEW on the intracellular ATP concentrations of *P. fluorescens* cells in detached and adhered biofilms is shown in Fig. 4. Following treatment of *P. fluorescens* cells with AEW, intracellular ATP concentrations reflected by a luminescent signal decreased significantly along with increased treatment time ($P < 0.05$). Additionally, intracellular ATP concentrations decreased significantly ($P < 0.05$) relative to those in controls following treatments with different AEW concentrations. Moreover, the luminescence signals of detached biofilms after a 5-min treatment and adhered biofilms after a 15-min treatment were ~50% of the luminescence signal observed in controls, although adhered biofilms consistently displayed higher intracellular ATP concentrations than detached biofilms at these treatment times. Longer treatment times of detached biofilms (15 min) and adhered biofilms (30 min) resulted in luminescence signals suggesting a significant decrease in intracellular ATP concentrations ($P < 0.05$).

3.5. Analysis of pHi

A clear change in pHi was observed after treatment with AEW (Fig. 5). Initial pHi values for the detached and adhered biofilms were similar; however, compared with the pHi of the control, that of *P. fluorescens* exposed to AEW was significantly lower at all concentrations ($P < 0.05$), with higher concentrations of AEW resulting in lower pHi values. After 5-min treatments with 20 mg/L, 40 mg/L, and 60 mg/L AEW, the pHi values of detached biofilms decreased by 0.82, 1.28, and 1.23, respectively. By contrast, 15-min treatments with AEW caused a

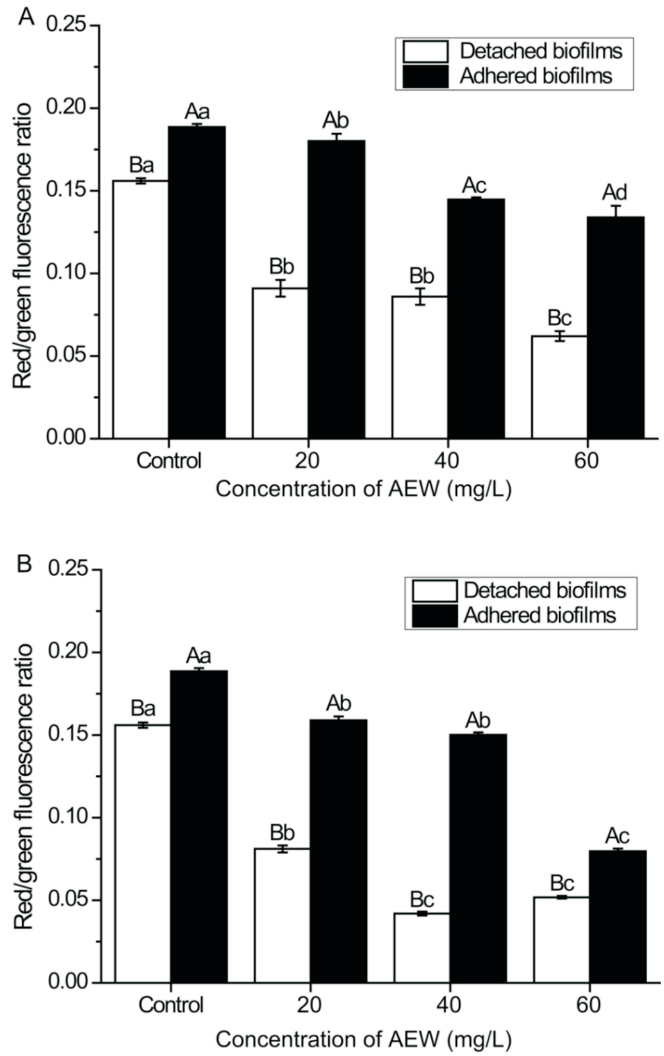

Fig. 3. Effect of control and 20, 40, and 60 mg/L acidic electrolyzed water (AEW) on the membrane potential of *Pseudomonas fluorescens* detached and adhered biofilms after (A) 5 min and (B) 10 min of treatment. Bars represent standard deviations (n = 3). a–d: for treatments with only one kind of biofilm, values with different lowercase letters are significantly different ($P < 0.05$). A–B: for treatments with the same concentration of AEW or the control, values with different uppercase letters are significantly different ($P < 0.05$).

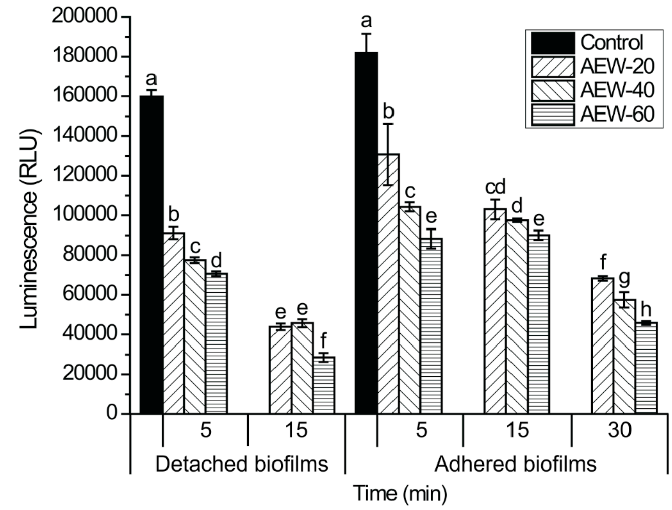

Fig. 4. Effect of treatment with control and 20, 40, and 60 mg/L acidic electrolyzed water on intracellular ATP concentrations of *Pseudomonas fluorescens* detached and adhered biofilms. Bars represent standard deviations (n = 3). a–h: for treatments with only one kind of biofilm, values with different lowercase letters are significantly different ($P < 0.05$).

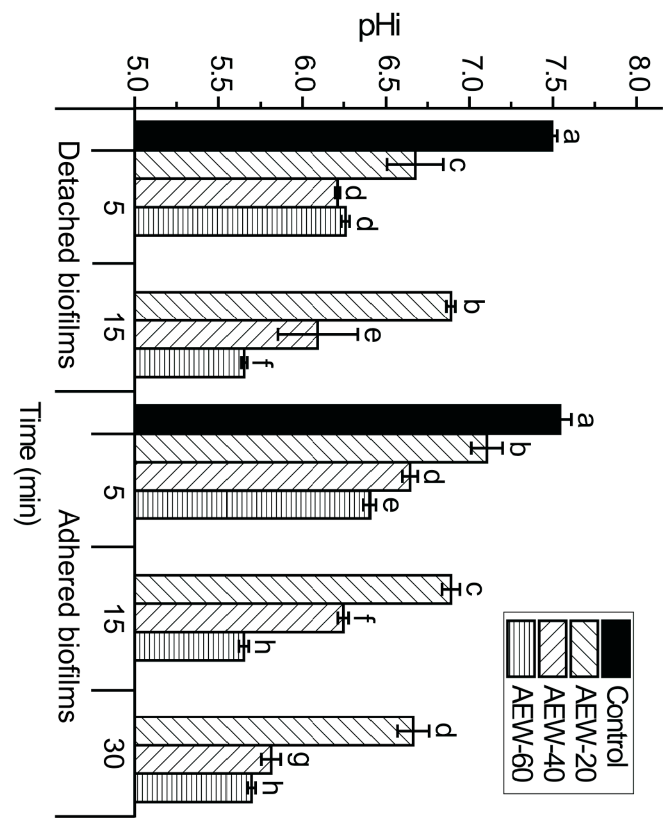

Fig. 5. Effect of treatment with control and 20, 40, and 60 mg/L acidic electrolyzed water on the intracellular pH (pHi) of *Pseudomonas fluorescens* detached and adhered biofilms. Bars represent standard deviations (n = 3). a–h: for treatments with only one kind of biofilm, values with different lowercase letters are significantly different ($P < 0.05$).

significantly greater ($P < 0.05$) reduction in the pHi value, with 20 mg/L, 40 mg/L, and 60 mg/L AEW treatments resulting in 0.6, 1.4, and 1.84 decreases, respectively. Similar to the pHi of detached biofilms, the pHi of adhered biofilms also decreased rapidly and in a similar AEW-concentration-dependent manner. The pHi of adhered biofilms was higher than that of detached biofilms after 5-min AEW treatments, and after 30-min treatments with 20 mg/L, 40 mg/L, and 60 mg/L AEW, the pHi values were reduced by 0.88, 1.73, and 1.88, respectively.

3.6. EPS analysis

Table 3 shows the protein and carbohydrate contents and the protein:carbohydrate ratio in L-EPS and B-EPS treated with 40 mg/L AEW and control samples. For L-EPS, the protein and carbohydrate contents of both biofilms in the control group were similar; however, AEW treatment decreased the protein and carbohydrate contents of detached biofilms by 56.8% and 57.1%, respectively, whereas these contents in adhered biofilms showed an even greater decrease (64.8% and 85.2%, respectively). Simultaneously, the protein:carbohydrate ratio increased from 4.53 to 5.35 for detached biofilms and significantly increased ($P < 0.05$) from 4.51 to 10.46 for adhered biofilms. For B-EPS in control samples, the protein and carbohydrate contents of detached biofilms were lower ($P < 0.05$) than those of adhered biofilms; however, AEW treatment resulted in dramatic decreases in the protein and carbohydrate contents of both biofilms to levels that were undetectable.

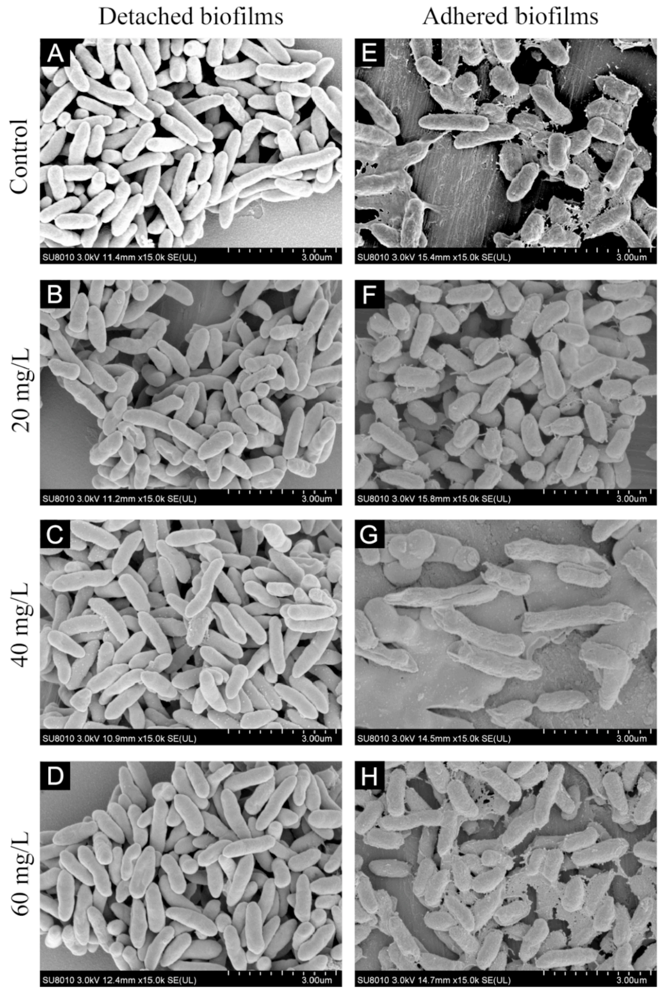

Fig. 6. SEM images of *Pseudomonas fluorescens* detached (A, B, C, and D) and adhered biofilms (E, F, G, and H) after treatment with control and 20, 40, and 60 mg/L acidic electrolyzed water.

3.7. SEM analysis

To observe the characteristic morphology of *P. fluorescens* cells, SEM images of *P. fluorescens* biofilms treated with 20 mg/L, 40 mg/L, and 60 mg/L AEW and control cells were produced. Representative SEM images of *P. fluorescens* biofilms forming three-dimensional cell aggregates are shown in Fig. 6. Overall, *P. fluorescens* cells were rod-shaped and ~1.5 mm in length. The cells of adhered biofilms produced more EPS on the contact surfaces, with some embedded cells, whereas the detached biofilms showed smooth cell surfaces with less obvious EPS secretion. Following AEW treatment, aggregates of cells held together by EPS remained, and *P. fluorescens* cells exhibited a small number of surface indentations and cell distortions; however, there were significant differences in adhered biofilms after 40 mg/L AEW treatment, with cell-surface indentations evident after a 30-min treatments and relative to the control and biofilms receiving other treatments.

4. Discussion

Biofilms as a known source of bacterial cross-contamination in food-processing environments have become a public health issue and an economic concern. Critical factors, such as temperature, time, and contact surfaces commonly encountered during food processing, are responsible for the attachment of bacteria and biofilm formation. Most studies concerning the control of biofilms focus on those grown under standard laboratory conditions but not applicable to food-processing environments. In workshops or slaughterhouses, the actual temperature is likely ~20 °C during working hours because of temperature fluctuations resulting from frequent entries and exits. Also, the low initial number of cells (2 log CFU/mL) was inoculated and incubated for 5 days to simulate the growth of residual bacteria after disinfection, with the amount of viable bacteria survived usually about 1–2 log CFU. Because thorough cleaning and disinfection is likely performed every 5 days in order to reduce costs, in the present study, the *P. fluorescens* incubated for 5 days were tested.

The Weibull model has been chosen to characterize disinfectant behavior and tolerance responses because this model is more flexible and robust over a wide range of treatments based on the time and concentration and relative to a log-linear model. The results showed that all values of the shape parameter b were < 1.0, which agreed with previous findings regarding biofilms (Vaid et al., 2010). Disinfectants should reduce cell numbers by 5 log in the presence of a neutralizer (Jahid and ha, 2014). In the present study, the results demonstrated that low concentrations of AEW reduced planktonic cell and detached-biofilm cell numbers by 5 log, whereas higher AEW concentrations and more time caused greater reductions in adhered-biofilm cell number (by 5 log; Fig. 1). AEW was active on planktonic cells and both biofilms, although significantly higher resistance was observed in both biofilms relative to that of planktonic cells. Adhered biofilms were the most resistant to AEW at all concentrations, and as the concentration increased from 20 mg/L to 60 mg/L, the increased levels induced a faster reduction in the cell numbers of both biofilms. These results were supported by the work of Steed (2006), who concluded that cells grown in adhered biofilms were the most resistant and that cells grown in detached biofilms and exposed to AEW in suspension displayed intermediate susceptibility. It is generally assumed that cells in detached biofilms quickly transform into their planktonic counterparts (Behnke and Camper, 2012); however, a recent study showed that detached biofilms were physiologically distinct from their adhered or planktonic counterparts, represented a transitional phenotype between the adhered and planktonic state and exhibiting different gene-expression profiles (Ding and Tan, 2016), all of which resulted in negative effects concerning resistance to AEW disinfection in the present study. Moreover, cells that have experienced these conditions have a distinct transcriptome profile, which might provide evidence of corresponding regulatory roles in response to disinfectant treatment (Carvalhais et al., 2014). In contrast to adhered biofilms that are already intrinsically tolerant to disinfectants, detached biofilms might act as counterparts released to the surrounding environment in order to maintain disinfectant-tolerant profiles (Cerca et al., 2014).

Changes in bacterial number over time in a sample are usually determined to assess the lethal effects of disinfectants. Although a definite number of bacteria was counted in a given volume by flow cytometry, it was difficult to examine cell viability. Based on previous work, we used a combination of measuring membrane potential and membrane integrity as a useful indicator of the physiologic state of the cell membrane and cell function. Cells with intact membranes and DNA are capable of metabolic activity and reproduction, whereas those without an intact membrane cannot maintain or generate a stable membrane potential and can be classified as dead based on the exposure of their internal structures to the environment (Mikula et al., 2012). In the present study, we investigated membrane integrity using the dyes PI and SYTO 9, which have been widely used to investigate the effects of biofilm inhibitors or disinfectants on various bacteria (Gandhi and Shah, 2015; Otto et al., 2010; Reck et al., 2011). We observed a significant increase ($P < 0.05$) in the number of cells with damaged membranes following treatment with different AEW concentrations, with increases in concentration resulting in increased membrane damage in the cells of both biofilms. Notably, we observed that changes in membrane integrity were more significant in detached biofilms than in adhered biofilms ($P < 0.05$), which was consistent with CFU-counting results; however, the extent of cell-membrane damage determined from fluorescence staining was smaller than that obtained by CFU counting. Therefore, the ratio of green:red fluorescence in cells in the biofilms relative to the control ratio represented conservative estimate of biofilm damage in *P. fluorescens*.

AEW treatment reduced the membrane potential by increasing the electrical depolarization of the target cells (Fig. 3). Membrane potential is required for localization of a variety of cell-division proteins, and depolarization can induce the release of autolytic enzymes from their usual sites of activity at the outer layers of the cell wall, thereby resulting in autolysis (Lamsa et al., 2012). In the present study, the untreated control was observed in a polarized state with stable, closed Na^+ and K^+ channels on both sides of the membrane. Because a primary component of AEW is Na^+ ions, we assumed that AEW opened Na^+ channels to increase intracellular Na^+ concentrations in order to promote electrical depolarization, which could also be caused by K^+ efflux (Jayakannan et al., 2013). In addition to the increased movement of ions, changes in pH can affect membrane potential (Bot and Prodan, 2009; Li et al., 2014). In the present study, we found that changing from a neutral to an acidic pH depolarized the cell membrane. In cases of membrane rupture, membrane potential is determined to be zero, and large holes permit inorganic ions to freely cross the membrane. Given that we observed variations in membrane depolarization according to AEW concentration, this suggested the onset of cumulative damage while the bacteria continued to maintain a partial ability to regulate their membrane potential.

Additionally, AEW treatment decreased intracellular ATP concentration. Similar results were reported by Li et al. (2014) and Shi et al. (2017), showing that an antimicrobial agent decreased intracellular ATP concentrations in a concentration-dependent manner. A rapid decrease in ATP concentration in the intracellular environment is a danger signal that indicates an impending threat and weakened metabolic processes. As previously described, AEW enhanced cell-membrane permeability, leading to leakage of intracellular ATP and an altered balance of cations, such as H^+ and Na^+, as active transport of cations requires ATP, which also decreases intracellular ATP concentrations. Additionally, depletion of intracellular ATP might have resulted from AEW causing a reduced rate of ATP synthesis or increasing ATP hydrolysis by the proton-pump ATPase (Li et al., 2014). This suggests that the variable damage to cell membranes and alterations in ATP-related activity might explain the differences observed in intracellular ATP concentration following AEW treatment of detached and adhered biofilms.

The pHi values varied (range: 5.6–9.0) according to the bacterial and culture environment. The pHi is important for controlling multiple cellular processes, including DNA transcription, enzyme activity, and protein synthesis (Chen et al., 2012), and an acidic pHi can greatly enhance the effects of agents that cause apoptosis (Chen et al., 2012). In the present study, changes in pHi positively correlated with AEW concentration in a time-dependent manner. The low pH value of AEW (range: 2.69–2.91) might promote decreases in pHi due to its ability to alter internal pH gradients, which can render bacterial cells more sensitive to active chlorine by sensitizing their cell membrane to the entry of HOCl (Hricova et al., 2008).

The control of biofilms is hindered by EPS, which limits the diffusion of disinfectants into the deepest layers of biofilms (Han et al., 2017). Therefore, an effective disinfection procedure should disrupt or dissolve the EPS to enable the access of disinfectants to viable cells (Shen et al., 2016), as once bacterial cells are liberated from the EPS in the biofilm matrix, they are more vulnerable to disinfectants (Steed, 2006). However, little is known about whether EPS plays a vital role in the different resistance responses of detached and adhered biofilms and whether AEW disrupts EPS to promote cell lethality. Therefore, we preliminarily determined the influence of AEW on EPS characteristics by investigating alterations in protein and carbohydrate contents (Table 3). The efficiency of AEW-mediated inactivation of *P. fluorescens* might vary according to EPS composition. Our experimental results showed that AEW treatment had a remarkable effect on EPS consumption, with significant differences ($P < 0.05$) in the protein and carbohydrate contents of the EPS in both biofilms, especially those of B-EPS. Specifically, we found that the protein content was higher than the carbohydrate content in the EPS of both biofilms, suggesting that AEW consumption is more likely to reduce protein content than carbohydrate content in the EPS of *P. fluorescens*. The amount of EPS produced by this strain, including that with a protein:carbohydrate ratio between 2.46 and 4.51, was lower than those reported previously (the protein and carbohydrate contents of L-EPS from *Pseudomonas fragi* were ~1280–1800 μg/mg and ~66–87 μg/mg, respectively), indicating that environmental, bacterial, and other factors play a vital role in determining EPS composition (Jiao et al., 2010; Wang et al., 2017). The EPS barrier represents a basic mechanism of cell resistance to disinfectants, and differences in EPS composition, quantity, and spatial distribution are important components of disinfectant resistance (Dynes et al., 2009). In the present study, we observed that cells in detached biofilms had insufficient EPS protection according to SEM analysis (Fig. 6). Additionally, we found that detached biofilms with less EPS and a thinner EPS layer relative to adhered biofilms showed more limited diffusion and a greater penetration of AEW. The resulting increases in AEW-specific components, such as HOCl, OCl^-, and Na^+, inside the cells of detached biofilms might have induced a decrease in pHi and depolarization of the membrane potential. Simultaneously, this would also promote active transport of ions, a lower intracellular ATP concentration, and increased leakage of cellular content from detached biofilms, thereby leading to cell distortions and death.

5. Conclusion

In summary, this represents the first report showing a relationship between the nonlinear Weibull model and planktonic cells, detached biofilms, and adhered biofilms. We used the Weibull model to fit the data for three sample types treated with disinfectants, allowing demonstration of the ability of AEW to effectively exert antimicrobial activity against *P. fluorescens* and significantly different morphophysiological responses in the three samples. Our results showed that AEW treatment differentially impaired the cell membrane and disrupted cellular homeostasis in both detached and adhered biofilms. Additionally, differences in EPS between detached and adhered biofilms influenced AEW efficacy. Our findings represent a preliminary examination of the response of detached and adhered biofilms associated with meat-borne spoilage bacteria to disinfectants. Our future research will include investigations of the interaction between EPS and AEW and transcriptome analyses to identify genes involved in the tolerance response of AEW-exposed detached and adhered biofilms and potentially representing important targets for biofilm control.

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