Food Control 79 (2017) 380–385

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Bactericidal action of slightly acidic electrolyzed water against *Escherichia coli* and *Staphylococcus aureus* via multiple cell targets

Xinyu Liao [a, b], Xiaoting Xuan [c], Jiao Li [a, b], Yuanjie Suo [a, b], Donghong Liu [a, b], Xingqian Ye [a, b], Shiguo Chen [a, b], Tian Ding [a, b, *]

[a] Department of Food Science and Nutrition, National Engineering Laboratory of Intelligent Food Technology and Equipment, Zhejiang University, Hangzhou, Zhejiang 310058, China
[b] Key Laboratory for Agro-Products Postharvest Handling of Ministry of Agriculture, Zhejiang Key Laboratory for Agro-Food Processing, Hangzhou, Zhejiang 310058, China
[c] Institute of Agricultural Products Processing, Ningbo Academy of Agricultural Sciences, Ningbo, Zhejiang 315000, China

ARTICLE INFO

Article history:
Received 8 February 2017
Received in revised form
29 March 2017
Accepted 31 March 2017
Available online 3 April 2017

Keywords:
Slightly acidic electrolyzed water
Escherichia coli
Staphylococcus aureus
Sublethal injury
Inactivation mechanism

ABSTRACT

This study combined plate counting method and fluorescent techniques (membrane integrity and potential, intracellular enzyme activity, and intracellular ROS level) to investigate the lethal and sublethal effects of slightly acidic electrolyzed water (SAEW) on *Escherichia coli* and *Staphylococcus aureus*. Also, the inactivation mechanism of SAEW was further explored through multiple cell targets (outer membrane and intracellular components). The results within 30 s SAEW treatment displayed 6.02 and 5.83 log reductions obtained for *E. coli* and *S. aureus*, respectively. The maximum sublethally injured cell proportions induced by SAEW exposure were 0.34 and 0.40 $\log_{10}$ CFU/mL for *E. coli* and *S. aureus*, respectively. According to the data from experiments of various acting cellular sites by fluorescent techniques, SAEW damaged the microbial membrane integrity and membrane potential severely. Also, it posed inactivation effect on the activity of intracellular esterase enzymes. Therefore, SAEW showed disinfection behavior with multiple cellular targets, including both cell barriers and intracellular components. Furthermore, SAEW did not result in accumulation of reactive oxygen species (ROS) inside microbial cell, indicating SAEW conducted a ROS-independent behavior on microbial inactivation and the chemical oxidants (e.g., hypochlorous acid) played major role in microbial intracellular oxidation processing. The result in this study will help to further understand the disinfection mechanism underlying SAEW on microorganisms and make SAEW inactivation targets more explicit.

1. Introduction

Food safety has been regarded as a critical theme in food industry at the present period. Efficient inactivation of foodborne pathogens has become the most important element for rendering food safety for public health assurance. In recent years, various pathogen inactivation techniques have been proposed and developed, including ozone (Kumar, Williams, Sumner, & Eifert, 2016), irradiation (Osaili & Al-Nabulsi, 2016), pulsed electric field (Zeng, Han, & Zi, 2010; Zhao, Yang, Shen, Zhang, & Chen, 2013), electrolyzed water (Ding et al., 2015; Xuan, Ding et al., 2017; Xuan, Fan et al., 2017; Zhao, Zhang, & Yang, 2017), ultrasound (Li et al., 2017), high pressure (Bi et al., 2015; Li, Xu, Zhao, Wang, & Liao, 2016; Liao, Zhang, Hu, & Liao, 2010), cold plasma (Liao, Liu et al., 2017; Liao, Xiang et al., 2017).

Slightly acidic electrolyzed water (SAEW) is regarded as an alternative disinfectant, which can be produced through electrolysis of a mixture solution of diluted hydrochloric acid and sodium chloride in a generator chamber without separating membrane. Due to its near neutral pH (5.0–6.5), strong antimicrobial efficacy and environmental friendliness, SAEW has received more attentions from researchers in recent years. So far, numerous studies have explored and proved strong bactericidal effect of SAEW on wide range of microorganisms, including *S. aureus*, *E. coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Bacillus subtilis*, yeasts, and so on (Al-Holy & Rasco, 2015; Hao et al., 2011; Issa-Zacharia, Kamitani, Miwa, Muhimbula, & Iwasaki, 2011; Koide, Takeda, Shi, Shono, &

* Corresponding author. Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou, Zhejiang 310058, China.
E-mail address: tding@zju.edu.cn (T. Ding).

http://dx.doi.org/10.1016/j.foodcont.2017.03.050
0956-7135/

Atungulu, 2009; Quan, Choi, Chung, & Shin, 2010; Xuan, Ding et al., 2017; Xuan, Fan et al., 2017). In general, hypochlorous acid (HOCl) in SAEW is thought to be major bactericidal compound (Koide, Shitanda, Note, & Cao, 2011). Nevertheless, the mechanisms underlying the microbial inactivation by SAEW are still not fully understood. In our previous study, we have demonstrated that the lethal effect of SAEW on *S. aureus* is related to the leakage of intracellular potassium, TTC (2,3,5-triphenyl tetrazolium chloride)-dehydrogenase activity inactivation and ultrastructure disruption (Ding et al., 2016). The cellular targets and inactivation pathways of SAEW on bacteria still required further investigations.

Furthermore, the damage on microorganisms induced by SAEW is not necessary enough to death, and the sublethal injury might occur. Once the conditions become more suitable, these damaged cells might resuscitate, regrow and even be pathogenic as normal ones did, posing potential risk on food safety and public health (Manas & Pagan, 2005). However, there was only limited studies concerning about sublethally injured microorganisms induced by SAEW (Xuan, Ding et al., 2017; Xuan, Fan et al., 2017).

Conventional plating count methods might fail to take sublethally injured and viable but non-culturable states (VBNC) of microorganisms into consideration, leading to inaccurate results (Barer & Harwood, 1999). Flow cytometery (FCM) coupled with fluorescent staining provides powerful tool for obtaining and analyzing different physiological activities (e.g., membrane integrity and potential, intracellular enzyme activity, intracellular pH, ROS level) of microorganisms induced by stressors in single cell level (Diaz, Herrero, Garcia, & Quiros, 2010; Li et al., 2016; Zhao et al., 2011). Our previous work has successfully employed flow cytometer combined with fluorescent dyes of propidium iodide (PI) and carboxyfluorescein diacetate (cFDA) to explored the damage on *E. coli* and *S. aureus* induced by ultrasound (Li, Xu et al., 2016; Li, Ahn et al., 2016).

The aim of this work is to investigate the lethal and sublethal actions of slightly acidic electrolyzed water (SAEW) on Gram-negative *E. coli* and Gram-positive *S. aureus* with the use of traditional plating methods and fluorescent techniques, helping to provide significant insights to future research on the inactivation pathway of SAEW.

2. Methods and materials

2.1. Bacterial strains and growth conditions

Gram-negative *Escherichia coli* ATCC 25922 and Gram-positive *Staphylococcus aureus* ATCC 25923 (Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China) were transferred to 100 mL nutrient broth (NB) (Base BioTech Co., Hangzhou, China) and incubated in an air bath incubator with a reciprocal shaker (TS-2102C; Tensuc, Shanghai, China) for 24 h at 150 rpm, 37 °C. Five mL of stationary-phase cultures were harvested by centrifugation at 2,320 × *g*, 4 °C for 10 min (TGL-20M centrifuge; Kaida Scientific Instruments Co., Ltd., Changsha, Hunan, China). The cell pellet was washed twice and resuspension by 5 mL of 0.85% sterile saline solution to obtain a final bacterial concentration of approximately 10^9 CFU/mL, which were determined using plating count method on tryptone soya agar (TSA) (Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China).

2.2. SAEW preparation

SAEW was produced by a flow type electrolysis chamber (Rui Andre Environmental Equipment Co., Ltd., Beijing, China) provided with an electrolytic cell without the separating membrane between the anode and cathode electrodes. In this study, SAEW (pH 6.1, ORP 863.5 mV, available chlorine concentration-ACC 30 mg/L) was generated by electrolysis of a mixture containing dilute hydrochloric acid (0.01%) and sodium chloride (0.03%) at a voltage of 220 V for 1 min. The pH and ORP values of SAEW were estimated by a dual scale pH/ORP meter (PB-10, Sartorius Co., Germany) equipped with a pH electrode and an ORP electrode. The ACC was measured by using a digital chlorine test kit (detection range, 0–300 mg/L) (RC-3F, Kasahara Chemical Instruments Co., Saitama, Japan).

2.3. SAEW treatment

One mL of *E. coli* and *S. aureus* suspension (around 10^9 CFU/mL) was transferred into 9 mL SAEW and mixed thoroughly for 0, 15, 20, and 30 s, respectively. Then, the residual SAEW was neutralized with a sterile buffer solution containing 0.85% saline solution and 0.5% sodium thiosulphate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Subsequently, cell viability was analyzed by plate counting method and fluorescent techniques.

2.4. Plate counting method

The bacterial count of *E. coli* and *S. aureus* was determined after SAEW treatment by plate counting method. Serial 10-fold dilutions of each sample were performed with 0.85% sterile saline solution to appropriate concentrations. Then, 1 mL of diluted sample was poured into non-selective medium (TSA) and selective medium (TSA supplemented with 2% (w/w) and 7% (w/w) sodium chloride for *E. coli* and *S. aureus*, respectively) (Zhao et al., 2013). The pates were then incubated at 37 °C for 48 h under atmospheric conditions. Each experiment was carried out in triplicate.

2.5. Fluorescent staining procedure

2.5.1. Membrane integrity

Membrane integrity was estimated by propidium iodide (PI) (Sigma-Aldrich Co., USA). PI probe preparation and staining methods were referred to (Li, Xu et al., 2016; Li, Ahn et al., 2016).

2.5.2. Esterase enzyme activity

Carboxyfluorescein diacetate (cFDA) (Sigma-Aldrich Co., USA) was employed for assessment of esterase enzyme activity. cFDA probe preparation and staining methods were referred to (Li, Xu et al., 2016; Li, Ahn et al., 2016).

2.5.3. Membrane potential

Membrane potential was estimated by BacLight™ Bacterial Membrane Potential Kit (B34950, Molecular Probes, Invitrogen, Grand Island, NY). $DiOC_2$ (3) (3,3′-diethyloxacarbocyanine iodide) was a dye, changing from green to red fluorescence as the membrane potential increasing. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) could destroy membrane potential via eliminating membrane proton gradient. Ten μL $DiOC_2$ (3) (3 mM) was added into 1 mL sample and mixed thoroughly. As for control, 10 μL CCCP (500 μM) was added before the addition of $DiOC_2$ (3). The mixture was then incubated for 30 min at room temperature (23 ± 2°C). The samples were then centrifuged at 2,320 × *g* for 10 min and washed with 1 mL of 0.85% sterile saline solution to remove excess $DiOC_2$ (3).

2.5.4. Intracellular ROS level

ROS assay kit (S0033, Beyotime, Shanghai, China) was used to estimate the intracellular ROS level. DCFH-DA (2,7-Dichlorodihydrofluorescein diacetate), main component in ROS assay kit, could be hydrolyzed by esterase enzyme into nonfluorescent DCFH (dichlorofluorescin), which could continue to be