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Disinfection efficacy and mechanism of slightly acidic electrolyzed water on *Staphylococcus aureus* in pure culture

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ABSTRACT

Slightly acidic electrolyzed water (SAEW), considered as a broad-spectrum and high-performance bactericide are increasingly applied in the food industry. However, its disinfection mechanism has not been completely elucidated. This study aims to examine the disinfection efficacy and mechanism of SAEW on *Staphylococcus aureus*, compared with that of sodium hypochlorite (NaClO) and hydrochloric acid (HCl). SAEW treatment significantly reduced *S. aureus* by 5.8 log CFU/mL in 1 min, while 3.26 and 2.73 log reductions were obtained with NaClO and HCl treatments, respectively. A series of biological changes including intracellular potassium leakage, TTC-dehydrogenase relative activity and bacterial ultrastructure destruction were studied following disinfection treatment of *S. aureus*. The results showed that SAEW decreased the relative activity of TTC-dehydrogenase by 65.84%. Comparing intracellular potassium leakage, the SAEW treatment caused a greater percent of protein leakage (108.34%) than the NaClO (18.75%) or HCl (0.84%) treatments. These results demonstrated the potent impact SAEW had on the permeability of cell membranes. In addition, the ranking of partly agglutinated cellular inclusion formation was HCl > SAEW > NaClO. It appeared that HCl, along with its low pH value, are responsible for most of the cytoplasmic disruptions. Overall, this study demonstrated that the disinfection mechanism of SAEW was disrupting the permeability of cell membrane and the cytoplasmic ultrastructures in *S. aureus* cells.

1. Introduction

Staphylococcus aureus, a gram-positive microorganism, is capable of secreting several different kinds of enterotoxins associated with specific staphylococcal infections (Ding et al., 2010). The risks associated with consuming foods contaminated with *S. aureus* have caught the attention of major public and governmental organizations in recent years. To date, there are at least 30 countries on six continents that have reported *S. aureus* infection in humans. In Japan, about 32.5% of the contaminated food showed the presence of *S. aureus* and particularly for raw milk, a 76.3% higher rate of contamination was found (Tian, Ji, Yang, Li, & Liu, 2007). The percentage of bacterial food poisoning cases caused by *S. aureus* was estimated to be 33% and 45% in United States and Canada, respectively (Shao, Dong, Zhao, Kong, & Liu, 2013). Similar poisoning cases have also occurred in China where more than 100 children were infected when they drank contaminated calcium milk produced by the Weiwei Daheng Dairy Co., Ltd. in 2008 (Xia et al., 2012).

In order to produce high-quality, microbiologically safe food for human consumption, numerous disinfection methods have been studied and/or used in the food industry. A few of these methods are the use of chemical disinfectants (hypochlorite, chlorine dioxide, hydrogen peroxide, hydrochloric acid, ozone etc.), physical treatments (heat and irradiation etc.), and their combinations (Koide, Shitanda, Note, & Cao, 2011; Zhang, Wu, Zhang, & Yang, 2011). The mechanisms concerning the traditional disinfection methods have been sufficiently studied by many researchers (Juneja & Sofos, 2002; Komanapalli & Lau, 1996; Setlow et al., 2002; Wang, Chang, Yang, & Cui, 2015). The physical heat treatment method, where the bacteria are subjected to inhospitable

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temperatures, is regarded as the primary factor affecting microbial inactivation, as it can inhibit the activity of intracellular proteins and enzymes, and damage the cellular membranes and nucleic acids (Juneja & Sofos, 2002). Setlow et al. 2002 reported that the disinfection mechanism of strong acid or alkali treatment on *Bacillus subtilis* spores was due to the inactivation of the intracellular enzymes and the disruption of the permeability barrier. To date only a few researchers have investigated the disinfection mechanism of electrolyzed oxidizing water (EOW) (Zeng et al., 2010, 2011). These researchers reported that EOW caused damage to the cell walls, nucleus and outer membranes, which lead to the rapid leakage of intracellular K^+, DNA and proteins, and a decrease in the dehydrogenase activities of *S. aureus*. It is generally accepted that the high oxidation reduction potential (ORP) of EOW could significantly influence EOW's disinfection power by penetrating the outer and inner membranes (Liao, Chen, & Xiao, 2007), and the low pH was also a main factor in EOW's bactericidal efficacy (Waters & Hung, 2014). On the other hand, slightly acidic electrolyzed water (SAEW) has a relatively low ORP value (less than 1000 mV), a pH near neutrality pH 5.0–6.5 and HClO as the main chlorine compound (Cao, Zhu, Shi, Wang, & Li, 2009; Koide, Takeda, Shi, Shono, & Atungulu, 2009; Koide et al., 2011). To date, there are very few studies investigating the disinfection mechanism of SAEW.

Therefore, the aim of this study was to investigate the disinfection efficacy and mechanism of SAEW against *S. aureus* in comparison with NaClO and HCl where the main forms of the chlorine compounds are ClO^- and Cl^-. The inactivation of *S. aureus* by SAEW, NaClO, and HCl was determined by plate count while the disinfection mechanism was investigated by measuring changes to intracellular potassium leakage, TTC-dehydrogenase relative activity and cellular ultrastructure as indicators.

2. Materials and methods

2.1. Bacterial strains and culture preparation

The *S. aureus* (ATCC 25923-3) used in this study was purchased from Hope Bio-Technology Co. Ltd, Qingdao, Shandong, China. The stock cultures were transferred into 100 mL nutrient broth (NB, Basebio, HangZhou, China) and incubated in an incubator shaker (TS-2102C, TENSUC, Shanghai, China) for 24 h at 150 r/min, 37 °C. Following incubation, 5 mL of the enriched culture were pooled into sterile centrifuge tube and subsided in a refrigerated centrifuge (TGL20M, Kaida Scientific Instruments Co., Ltd., Changsha, Hunan, China) at 5000 rpm, 4 °C for 10 min. The resulting cell pellet was washed twice and resuspended in 5 mL of 0.85% sterile saline solution. The final population in each culture of *S. aureus* was approximately 10^9 CFU/mL that was confirmed by plating 1 mL portions of appropriately diluted *S. aureus* suspension on plate count agar (PCA, Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China) plates and then incubated at 37 °C for 24 h.

2.2. Preparation of liquid disinfectants

SAEW was produced by a oxidizing redox potential water generator (Beijing Intercontinental Resources and Environmental Protection Technology Co., Ltd., Beijing, China) equipped with an electrolytic cell without the separating membrane between the anode and cathode electrodes. SAEW (pH 6.1, ORP of 893.5 mV, available chlorine concentration ACC of 30 mg/L) used in this study was generated by electrolysis (1 min) of an aqueous mixture containing 0.01% HCl and 0.03% NaCl. The pH and ORP values were measured using a dual scale pH/ORP meter (PB-10, Sartorius Co., Germany) bearing a pH electrode (Sartorius Co., Germany) and an ORP electrode (501, Ruosull Co., Shanghai, China). The available chlorine concentration (ACC) was measured by a colorimetric method using a digital chlorine test kit (Chlormeter Duo, Palintest Co., UK) with a detection range of 0–250 mg/L.

The NaClO (ACC of 30 mg/L, pH 10.8, ORP of 350 mV) solution was prepared by diluting the 5.2% NaClO solution (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) with distilled water. The 0.1% HCl (pH 1.65) was prepared by diluting the 37% HCl (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) reagent with distilled water.

2.3. Disinfection treatment

Nine milliliters of SAEW, NaClO, HCl, and deionized water were added separately into four test tubes. 1 mL of *S. aureus* suspension (about 10^9 CFU/mL) was transferred into each test tube containing the different disinfectants or deionized water and mixed thoroughly for 1 min. Then, 1 mL of mixture in each tube was transferred to a sterile tube containing 9 mL neutralizing buffer solution (0.85% NaCl solution and 0.5% sodium thiosulphate) or 0.85% NaCl solution respectively. Subsequently, all the treated groups (SAEW, NaClO and HCl groups) of samples and deionized water group were prepared for the following measurements such as intracellular potassium leakage, TTC-dehydrogenase relative activity, TEM, and SEM. The bacterial count of *S. aureus* was determined by inoculating 0.1 mL portions or after serial 10-fold dilutions with 0.85% NaCl solution on duplicate PCA plates and cultivated at 37 °C for 24 h. The deionized water group was performed as the control. All the experiments were replicated three times.

2.4. Measurement of intracellular potassium leakage

The intracellular potassium leakage of treated and control samples was determined using flame atomic absorption spectrophotometry (contrAA 300, Analytik Jena AG, Germany) at 766.5 nm (Herbert, Phipps, & Strange, 1971; Ioannou, Hanlon, & Denyer, 2007). Before measurement of samples, the instrument was calibrated by using potassium standars (analytical grade, Sigma–Aldrich, Poole, United Kingdom) of 2.00, 4.00, 6.00, 8.00, and 10.00 mg/L (final concentration) through diluting by 1% dilute nitric acid solution. A linear relationship between potassium concentration and emission was obtained. The filtrate samples were measured by AAS and calculated by the calibration. The leakage rates of intracellular potassium were formulated as follows (Xiong, Liu, Liu, & Li, 2010):

$$The\ \ leakage\ \ rates\ \ (\%) = (the\ \ leakage\ \ amount - control)\ \ /control\ \ \times\ \ 100\%$$

2.5. Measurement of TTC-dehydrogenase relative activity

TTC (2,3,5-triphenyl tetrazolium chloride) is a low molecular weight compound that can pass through the cell walls and membranes of live bacteria. The colorless TTC is a hydrogen acceptor and is transformed into a red insoluble product TF (2,3,5-triphenylformazan) after being hydrogenated in the bacteria. The absorbance of TF at 485 nm was used to determine the TTC-dehydrogenase relative activity.

Two-milliliter of Tris–HCl buffer (1.5 mol/L, pH 8.8) and 2 mL TTC-glucose solution (0.4% TTC solution and 0.1 mol/L glucose were mixed in 1:1 ratio) were added to 6 mL treated and control samples. After shaking, the mixture was incubated at 37 °C for 2 h to promote the chromogenic reaction. Next, 5 mL methylbenzene was added and the mixture was extracted for 1 h at room temperature. The absorbance of the organic phase was measured at 485 nm with