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In vitro inactivation of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp. using slightly acidic electrolyzed water

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In the current study, the effectiveness of slightly acidic electrolyzed water (SAEW) on an *in vitro* inactivation of *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Salmonella* spp. was evaluated and compared with other sanitizers. SAEW (pH 5.6, 23 mg/l available chlorine concentration; ACC; and 940 mV oxidation reduction potential; ORP) was generated by electrolysis of dilute solution of HCl (2%) in a chamber of a non-membrane electrolytic cell. One milliliter of bacteria suspension (ca. 10–11 $\log_{10}$CFU/ml) was mixed with 9 ml of SAEW, strong acidic electrolyzed water (StAEW; ca. 50 mg/l ACC), sodium hypochlorite solution (NaOCl; ca.120 mg/l ACC) and distilled water (DW) as control and treated for 60 s. SAEW effectively reduced the population of *E. coli*, *S. aureus* and *Salmonella* spp. by 5.1, 4.8, and 5.2 $\log_{10}$CFU/ml. Although, ACC of SAEW was more than 5 times lower than that of NaOCl solution, they showed no significant bactericidal difference ($p>0.05$). However, the bactericidal effect of StAEW was significantly higher ($p<0.05$) than SAEW and NaOCl solution in all cases. When tested with each individual test solution, *E. coli*, *S. aureus* and *Salmonella* spp. reductions were not significantly different ($p>0.05$). These findings indicate that SAEW with low available chlorine concentration can equally inactivate *E. coli*, *S. aureus* and *Salmonella* spp. as NaOCl solution and therefore SAEW shows a high potential of application in agriculture and food industry as an environmentally friendly disinfection agent.

[**Key words:** Slightly acidic electrolyzed water; Food pathogens; *In vitro* inactivation]

In recent years, there has been increased concern about the safety of foods especially products that are consumed fresh or slightly cooked, thus ensuring the safety of food products is the food industry's first priority today. *Escherichia coli*, *Staphylococcus aureus, Campylobacter jejuni, Salmonella* spp. and *Listeria monocytogens* are considered as the important pathogens of major public health concern and were reported to be the common food borne pathogens that can cause illness and death (1) with outbreaks reported in Canada, USA, the UK, Japan and some other parts of the world. In Japan, the statistics by the Japanese Ministry of Health, Labor and Welfare shows that about 1,000 outbreaks of food-borne poisoning, involving 30,000 to 35,000 individuals, are reported each year and since food-borne disease is caused mostly by infection with pathogenic microorganisms, three principles have been developed to aid in the prevention of food-borne disease: prevent microbial contamination, halt microbial growth, and protect against pathogens by cleaning and disinfection (2). Therefore, developing effective method for reducing or eliminating pathogens from food and agricultural products is an important step for the hazard analysis and critical control points (HACCP) of the food industry. So far, several chemical solutions such as sodium hypochlorite, chlorine dioxide, hydrogen peroxide, organic acids and ozone have been used as sanitizers in food industry. Chlorinated water of 50–200 ppm is the most commonly and widely used sanitizer for reducing bacterial contamination on whole fruit, vegetables and fresh-cut produce on the commercial processing (3,4). However, many of chemicals evaluated have minimal disinfection effectiveness on fresh produce (5). For this reason, the use of acidic electrolyzed water with low chlorine concentration has been introduced as an alternative and a novel sanitize in agriculture and food industry. In Japan, strong acid electrolyzed water (pH 2.5 ± 0.2; 20–60 mg/l ACC) and slightly acidic electrolyzed water (pH 5.0–6.5; 10–30 mg/l ACC) have been authorized for use with food by the Japanese Ministry of Health and Welfare. So far, bactericidal efficacy of StAEW (pH 2.5 ± 0.2) on most pathogenic and non-pathogenic bacteria in vitro has been extensively reported (6–10). It has shown promise against cells suspensions of *E. coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes* (9), spoilage organisms associated with vegetables (11) and pathogens in solution (12). In contrast, a solution at slightly acidic or neutral pH generated by electrolysis was reported as an effective antimicrobial agent with low available chlorine concentration (11,13,14), and recently attention is being paid to SAEW for disinfection (15). Therefore, with a near neutral pH, SAEW application seems promising as it minimizes human health and safety issues from Cl_2 off-gassing, reduces corrosion of surfaces, and limits phototoxic side effects while maximizing the application of hypochlorous acid species (16). Despite of these advantages, an in vitro inactivation of different food pathogens using SAEW has not been intensively

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studied. Current study is among the few preliminary studies aiming at determining basic SAEW's bactericidal effectiveness data against different food spoilage and pathogenic microorganism. In the present study, the effectiveness slightly acidic electrolyzed water on *in vitro* inactivation of *E. coli, S. aureus* and *Salmonella* spp. was evaluated and compared with strong acidic electrolyzed water and sodium hypochlorite solution.

MATERIALS AND METHODS

Bacterial cultures and preparation of inocula The bacteria used in this study were *E. coli* (NBRC3301), *Salmonella* spp. (NBRC13245) and *S. aureus* (NBRC12732). Liquid-dried (L-dried) cultures of *E. coli*, *S. aureus* and *Salmonella spp* were obtained from NITE Biological Resource Center (NBRC), revived soon after arrival according to L-dried culture reactivation procedures provided by manufacturer. Briefly, the L-dried culture ampoule was carefully and aseptically snap opened, immediately 0.2 ml of prepared L-dried specimen re-hydration fluid 702 (Wako Pure Chemicals Ind., Ltd., Osaka, Japan) was added using a sterile pasteur pipette and left for few minutes. Re-hydrated cultures of different bacteria were gently mixed, plated on L-dried specimen growth media 802 (Wako Pure Chemicals Ind., Ltd., Osaka, Japan) and incubated at 37 ± 2 °C for 24 ± 2 h. The viable cell count of each culture was verified by serial dilution and pour plate count methodology using standard method agar (NISSUI Pharmaceutical Co., Ltd, JAPAN). The colonies from plated pure culture were propagated once after every 4 d using a 4 by 4 looping out method on solidified standard method agar for preservation.

Original *E. coli*, *S. aureus* and *Salmonella* spp. suspensions were prepared by transferring several colonies to a 10 ml of 0.1% peptone water using a sterile inoculation loop, vortexed using a thermal mixer (TM-100, Tokyo Thermonics Co. Ltd, JAPAN) and transferred to a 50 ml beaker that was filled up to a final volume of 50 ml by sterile 0.1% peptone water. The prepared original bacteria suspension was continuously stirred using a magnetic stirrer (REXIM RS-6DR, AS-ONE Corporation, Osaka JAPAN) at 500 rpm to maintain the uniform distribution. The actual bacteria cell concentration of prepared original bacteria suspension was determined by plating 1 ml of portions of appropriately diluted cultures on standard method agar plates and incubating plates at 37 ± 2 °C for 24 ± 2 h. The original bacteria suspension contained bacteria concentration of between 10 and 11 $\log_{10}$CFU/ml.

Preparation of treatment solutions Slightly acidic electrolyzed water was produced by electrolysis of a mixture of aqueous dilute solution of HCl (2%) and tap water using Apia60 generator (Apia60, HOKUTY Co., Kanagawa, JAPAN) at 5.0 V, 3.0 A and produced at a rate of 1.0 l/min. SAEW generator basically consists of an electrolytic cell with anode and cathode electrodes and no separating membrane between them. The schematic representation of SAEW generating equipment and resulting compounds during electrolysis are shown in Fig. 1A. More schematic elaboration of mixing the two solutions and the electrolysis of resulting solution in producing SAEW is shown in Fig. 2. Strong acidic electrolyzed water was generated by electrolysis of 0.15% sodium chloride (NaCl) solution using a ROX-20TA generator (base model ROX-20TA, Hoshizaki Electric Co. Ltd., Japan) at 15.0 V, 14.5 A and at a rate of 1.5 l/min. The generator was left to run for 15 min before collecting water for the treatment. The StAEW generator consists of an electrolytic cell where the anode and cathode electrodes are separated by a diaphragm or membrane (Fig. 1B). With this type of apparatus, both StAEW and Strong alkaline electrolyzed water are generated simultaneously. From the anode side of the generator, StAEW was produced and was collected to be used in this experiment. The cathode side produced strong alkaline electrolyzed water that was however, not collected. Sodium hypochlorite solution was prepared by diluting 10% sodium hypochlorite solution using distilled water to obtain a final sodium hypochlorite (NaOCl) solution (Wako Pure Chemicals Ind., Ltd., Osaka, Japan). Sterilized distilled water (DW) was used as control for this experiment

Analytical measurements The ORR, pH and ACC of treatment solutions were measured in duplicate immediately after preparation and before each bactericidal experiment. The pH was measured with a pH meter (HM-14P, TOA electronics Ltd., Tokyo, Japan) using a pH combination electrode (GST-2419C) and ORP was measured with ORP meter (RM-12P, TOA Electronics Ltd., Tokyo, Japan) using an ORP electrode (PST-2019C). The pH meter was calibrated using commercial standard buffers pH 4.01 and 6.86 (Nacalai Tesque, Inc., Kyoto, Japan). Available chlorine concentration of treatment solutions were determined by spectrophotometric method using a spectrophotometer (DR/4000 V, HACH Co., Loveland, U.S.A). The detection limit is 0.2 mg/l Cl_2. Therefore samples were first diluted to desired lower levels of ACC using deionized water prior to measurement.

***In vitro* inactivation of *E. coli*, *S. aureus* and *Salmonella* spp** An *in vitro* inactivation of *E. coli*, *S. aureus* and *Salmonella spp* in suspension was designed as illustrated in Fig. 3. For each bacterium, suspensions of about 10–11 $\log_{10}$CFU/ml were prepared as described above. One milliliter of each bacterial suspension was separately added to 9 mL of SAEW, StAEW, NaOCl and sterile DW continuously hand-shaken for 1 min to mix the resultant suspension and enable the bacteria inactivation treatment (17,18) at ambient temperature of 20 ± 2 °C. To assess the effectiveness of SAEW at varying treatment time for each bacterium, the inactivation treatment was carried out for 1 min, 2 min and 3 min at ambient temperature of 20 ± 2 °C. At the end of each treatment time, inactivation experiments were stopped by transferring 5 ml of each treated sample to a sterile tube containing 5 ml (equal volume) of neutralizing 0.5% sodium thiosulphate ($Na_2S_2O_3$) solution (17–22) and the tubes were vortexed using a thermal mixer (TM-100, Thermonics C0.Ltd, Tokyo, JAPAN). After neutralization, treated and control samples were then serially diluted in sterile 0.1% peptone water. Following serial dilution, 1 ml of each sample was pour plated on standards method agar in duplicate plates and incubated at 37 ± 2 °C for 24 ± 2 h prior to counting of colonies. Microbial counts were expressed as $\log_{10}$CFU/ml sample. Further the $\log_{10}$CFU/ml reduction on bacterial population was computed to represent the inactivation effectiveness of treatment solution. The effect of the neutralizing solution (0.5% sodium thiosulphate solution) was also tested on bacterial cultures to ensure that the observed bacterial reductions were solely attributed to treatment solutions and not due to sodium thiosulphate solution.

Statistical analysis The population of surviving bacteria and bacterial count log reductions ($\log_{10}$CFU/ml) were considered for further statistical analysis to assess the difference in antimicrobial effectiveness among tested solutions. Data were subjected to one way analysis of variance (ANOVA) and Tukey HSD multiple range test was used to determine the differences at $p \leq 0.05$ using SPSS 13.0 (SPSS software for Windows, release 13.0, SPSS, Inc., USA).

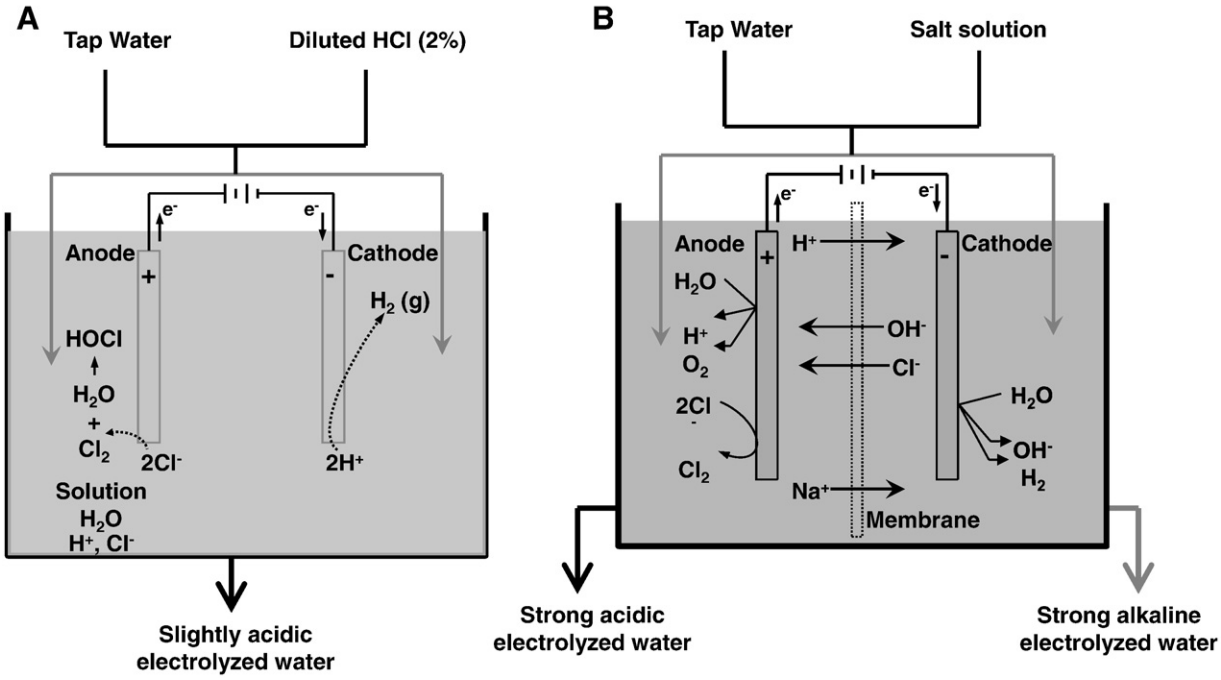

FIG. 1. Schematic diagram of electrolyzed water generators resulting compounds during electrolysis. (A) is a SAEW generator and (B) is a StAEW generator.