

APPLYING MEMBRANE-LESS ELECTROLYZED WATER FOR INACTIVATING PATHOGENIC MICROORGANISMS

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Abstract. The membrane-less electrolyzed water (MLEW) has been reported to possess antimicrobial effects against a variety of microorganisms and is considered as an alternative of traditional disinfectants. In the study, the inactivating efficiency was evaluated by using MLEW with free available chlorine (FAC) at concentrations of 50 and 100 mg/L against food-borne related microorganisms, including *Enterohaemorrhagic Escherichia coli* (EHC), *Salmonella spp.* and *Staphylococcus aureus* individually. D-value (decimal reduction time) was applied for evaluating the antimicrobial ability of MLEW. D-values of EHC, *Salmonella*, and *S. aureus* were about 120 sec, 5 sec and 5 sec, respectively. Our study demonstrated that MLEW is very effective in reducing the food-borne microbial contamination.

Keywords: *antimicrobial, free available chlorine, food-borne, disinfectants, microbial contamination*

Introduction

Bacteria induced food-borne illness has had serious impacts on public health. People infected with these food-borne bacteria can suffer from gastroenteritis, including inflammation of gut, diarrhea, abdominal pains and cramps, fever, bloody stool, nausea and vomiting. According to the previous study, the top five food categories linked to food-borne illness in the USA from 1990 to 2003 were seafood, dairy products, eggs, beef, and poultry products (CSPI, 2006). Recently, fresh-cut fruit, vegetables, prewashed and pre-cut salads have become popular eating choices. These food categories are generally eaten without processing. Along with this trend and changes in people's eating habits, the study found that microbial infections associated with fresh-cut fruits and vegetable increased (Qadri et al., 2015; Sureshkumar et al., 2016; Mahfuza et al., 2016).

Investigation of fresh-cut fruit and vegetables have indicated that these producing processes was unable to remove bacteria and also food-borne pathogens such as *Salmonella*, *L. monocytogenes*, *Staphylococcus aureus* and *E. coli* O157:H7 (Upadhyay et al., 2016; Sanchís et al., 2016). *Enterohaemorrhagic Escherichia coli* (EHEC) and *salmonella* have been reported as the causal factor of food-borne illness outbreaks (Switaj et al., 2015; Ebel et al., 2016). *Salmonella* and EHC were estimated to be the leading cause and should be responsible for 30% of food-borne illness hospitalization in United States individually (CDC, 2013; Jadeja and Hung, 2014). *Staphylococcus aureus* is major pathogen in the food industry and listed among the top 5 pathogens causing food-borne illness (Sun et al., 2012; Giaouris et al., 2015).

Therefore, reducing the contamination caused by these pathogenic bacteria is important for protecting consumers from adverse health exposure in food industry. Many

previous studies have shown that chlorine-related antimicrobial agent is helpful to reduce the bacterial contamination during food-production process (Rahman et al., 2012; Olaimat and Holley, 2012; Waters and Hung, 2014; Petri et al., 2015).

Electrolyzed water is generated from electrolysis of saline solution in a container within anodic and cathodic electrodes with or without ion-selective permeating membrane. The electrolyzed water contains free available chlorine (FAC) compounds such as hypochlorous acid (HOCl), chlorine gas (Cl₂) and hypochlorite ion (OCl⁻) yielding its antimicrobial activity. The distribution of fractions of FAC compounds in electrolyzed water is dependent on pH values and affects biochemical characteristics. Acidic electrolyzed water (AEW) is generated in cathode compartment of electrolysis cell within membrane. It has a strong antimicrobial activity on various microorganisms because of its low pH (2-4) and higher proportion of chlorine gas compared to hypochlorous acid. These characteristics of AEW help destroy cell membrane and disrupt metabolism of microorganisms rapidly. AEW has been widely evaluated for antimicrobial application in recent decades.

Neutral electrolyzed water, has near neutral pH value (6-8), giving similar antimicrobial mechanism but result in less metal surface corrosion or skin irritation as AEW (Huang et al., 2008; Cui et al., 2009). This work chose the membrane-less electrolyzed water (MLEW) as the antimicrobial agent. The MLEW is one of the neutral electrolyzed water. Moreover, the membrane-less electrolysis container is more productive, stable, convenient and economic because of expendable ion-selective membrane is not utilized during electrolysis (Chuang et al., 2013). MLEW becomes more popular in recent years because of above advantages but revealed less information as the alternative of conventional chlorine-related antimicrobial agents.

Neutral electrolyzed water has been extensively used for inactivating food-borne bacteria (Zhang et al., 2016; Machado et al., 2016). However, the MLEW has not yet been applied to inactivate food-borne bacteria. Thus, this study aims to evaluate antimicrobial activity of MLEW against the selected food-borne illness bacterial microorganism including ECHC, *Salmonella spp.* and *S. aureus* strains. In addition, two commercially available chlorine-related antimicrobial agents including bleach and chlorine dioxide (ClO₂), usually used in the food-processing factory, were applied to inactivate the selected bacterial strains for comparison in our study.

Materials and Methods

Preparation of Membrane-less electrolyzed water (MLEW)

The MLEW used in this study was generated by hand-made membrane-less electrolyzing device (Chuang et al., 2013). The schematic diagram of electrolyzing device is shown in *Figure 1*. The device consists of 850 mL cylinder polycarbonate (PP) container (height: 15 cm; diameter: 10.5 cm) filled with saturated NaCl solution (6.15 M). Two Pt/Ti base electrodes module (10 × 2 cm²) was installed inside the PP container as cathode and anode with the gap of 0.8 cm between electrodes, giving 12 A electrical current inputs. The FAC concentration of the MLEW solution was measured with N, N-dimethyl-p-phenylenediamine (DPD) colorimetric method, using portable spectrometer (DR 2800, HACH, Loveland, CO, USA). The pH value of the MLEW solution was measured using pH meter (CyberScan pH 510, Eutech Inc., Singapore).

With 30 minutes of electrolyzing process, FAC concentration of NaCl solution would rise up to over 10,000 mg/L. This 850 mL solution with high FAC concentration was

subsequently diluted with deionized water (Milli-Q, Millipore, Billerica, MA, USA) to FAC 50 and 100 mg/L as the ready-to-use antimicrobial agent (pH 7.0 ~ 7.2) for further inactivating experiments. In the United States, chlorine-related wash at highest FAC 200 mg/L is allowed to be used sanitizing treatment by the food-production industry (Regulation 21CFR178.1010). Therefore, we applied MLEW with FAC 50 and 100 mg/L in the study for simulate the field application in the factory. Bleaching liquid (NaOCl, Regular-Bleach, Colorox, CA., USA) and chlorine dioxide liquid (ClO₂, CLEAN GOOD, Daisyko Trading Co. Ltd.) were diluted to FAC 50 and 100 mg/L (the same concentrations with MLEW, measured with DPD method, DR 2800 HACH, Loveland, CO, USA) to inactivate the selected food-borne bacterial strain as the reference antimicrobial agents.

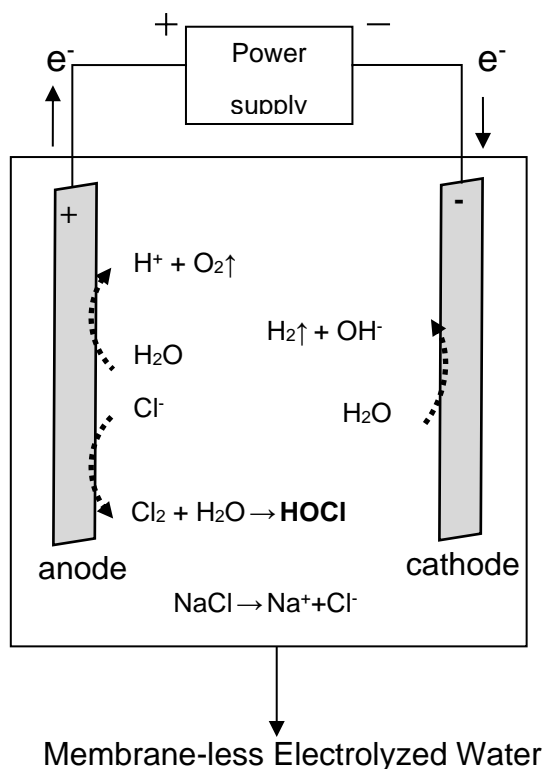


Figure 1. The schematic diagram of hand-made membrane-less electrolyzing device

Preparation of test bacterial strains

The bacterial strains used in the study including ECHC, *Salmonella spp.* and *S. aureus* were obtained from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The preparation and culture of the testing microorganisms based on the previous research (Abadias et al., 2008). ECHC (BCRC 14824), *Salmonella enterica* (BCRC12947) and *S. aureus* (BCRC 10451) pure culture collections were transferred and grown on tryptic soy agar (TSA, BD, N.J., USA) incubating at 37 ± 1°C for 48 hours. Single bacterial colony was isolated with streaking loop and transferred to tryptic soy broth (TSB, BD, N.J., USA) and incubating at 37 ± 1°C for 24 hours in order to enrichment. The enriched TSB that respectively contained ECHC, *Salmonella spp.* and *S. aureus* cells were then poured individually into sterile tubes that were then centrifuged

at 2,500 rpm for 15 minutes. Following the centrifugal process, the supernatant of the TSB were removed. The resulting pellet in the tubes were re-suspended with 30 mL sterilized deionized water as testing suspension. The centrifuge and re-suspension processes were repeated twice to totally remove TSB. Culturable bacterial population of the testing suspensions were determined by tenfold serial dilution of 0.1 mL aliquot on TSA by incubation at $37 \pm 1^\circ\text{C}$ for 24 hours. The final bacterial population of the testing suspensions were adjusted to $10^6 \sim 10^7$ colony forming unit CFU/mL for subsequent inactivating experiments.

MLEW, bleach and ClO₂ inactivating test against 3 bacterial strains

The experimental system and tests were operating in Taiwan, R.O.C. Quantitative inactivating experiments adapted from Robinson et al. (2010) against 3 bacterial suspensions were performed of MLEW, NaOCl and ClO₂ in the study. 1 mL of MLEW, bleach and ClO₂ with FAC 50 and 100 mg/L were added to 99 mL of 3 bacterial suspensions respectively as inactivating treatment samples (1% v/v contacting). After time interval of 10 and 30 seconds, 0.1 mL aliquot of agents-treated bacterial suspensions were extracted for tenfold serial dilution and spread on TSA, followed with incubation at $37 \pm 1^\circ\text{C}$ for 24 hours. Each test would spread on three TSA plates. The colonies of the plates were counted and convert to original culturable population in the bacterial suspensions for measuring the survival rate at the inactivating time intervals mentioned above. Sodium thiosulfate (Na₂S₂O₃, 217263 Sigma-Aldrich, USA) was used to liberate bacterial aliquot from continuously contact of antimicrobial agents while extracted. The experimental limit of the serial dilution and culture method was 10 CFU/mL. On the other hand, as control sample, another group of 3 bacterial suspensions with neither MLEW, ClO₂ nor NaOCl were added were observed by the same aliquot, serial dilution and incubation process. The experiment was repeated triple.

Inactivating efficiency were evaluated by bacterial strains survival population to the extent of being killed in terms of corresponding decimal reduction times (D-value) of antimicrobial agents. The D-value is the time required, at a given condition, to achieve a log reduction, that is, to kill 90% of relevant microorganisms.

Statistical analysis

The inactivating efficiency of testing bacteria after MLEW, NaOCl and ClO₂ were calculated by subtracting the initial mean bacteria concentration (CFU/mL) from the bacteria concentration (CFU/mL) after each treatment. All experimental values showed the means of three different experiments with 3 replicates of the inactivating treatment per experiment. The Statistical Product and Service Solutions (SPSS version 20) program was utilized for statistical evaluation of measurement data. Significant differences between inactivating test with respect to bacterial reduction were analyzed by one-way ANOVA at a significance level of 0.05.

Results and Discussion

The inactivating efficiency of MLEW, bleach and ClO₂ against 3 bacterial strains

Table 1 shows the inactivating efficiency of MLEW, NaOCl and ClO₂ against ECHC with FAC 50 and 100 mg/L. The ECHC in the test suspension was almost totally inactivated by MLEW and bleach with FAC 50 mg/L after 10 second treatment. However,

there is still 3.2×10^6 CFU/mL of ECHC remain survival (71%) in test suspension of FAC 50 mg/L ClO₂ after 10 second treatment. It reveals that MLEW and NaOCl performed rapid and effective antimicrobial reaction against ECHC than ClO₂ in the short time contacts ($p < 0.05$). It can be expected that longer contact time between antimicrobial agent and bacterial bring better inactivating effect. In our study, 30 seconds treatment of FAC 50 mg/L MLEW and NaOCl did resulted in the same < 10 CFU/mL survival population of ECHC. But ClO₂ still could not perform acceptable inactivating efficiency and remain 60% HCHC survival rate (2.7×10^6 CFU/mL) even elongate treatment time to 30 second.

Table 1. Inactivating efficiency of MLEW, NaOCl and ClO₂ against ECHC

Time intervals and culturable population of ECHC							
FAC concentration (mg/L)	Control group (CFU/mL)	MLEW		NaOCl		ClO ₂	
		10 sec	30 sec	10 sec	30 sec	10 sec	30 sec
50	4.5×10^6	< 10	< 10	< 10	< 10	3.2×10^6	2.7×10^6
100	4.5×10^6	< 10	< 10	< 10	< 10	2.6×10^6	< 10

While treating with FAC 100 mg/L MLEW and bleach, ECHC were also mostly totally inactivated after 10 second contacting. The survival rate of ECHC to FAC 100 mg/L ClO₂ is 58% (2.6×10^6 mg/L) and higher than MLEW and NaOCl ($p < 0.05$). In the other way, giving longer treatment time to FAC 100 mg/L ClO₂ cause significant improvement on inactivation. < 10 CFU/mL of ECHC survival was achieved when 30 seconds treatment applied with FAC 100 mg/L ClO₂. Our experimental resulted that ClO₂ does not pose comparable inactivating efficiency to MLEW and bleach in short time contact against ECHC. ClO₂ need longer contact time to present performance the same as MLEW and bleach when treating HCHC.

Table 2 shows the inactivating efficiency of MLEW, bleach and ClO₂ against *Salmonella spp.* with FAC 50 and 100 mg/L. The *Salmonella spp.* in the test suspension was almost totally inactivated by MLEW and ClO₂ with FAC 50 mg/L after 10 second treatment. However, there is still 9.0×10^6 CFU/mL of *Salmonella spp.* remain survived (18%) in test suspension of FAC 50 mg/L NaOCl after 10 second treatment. MLEW and ClO₂ performed rapid and effective antimicrobial reaction against *Salmonella spp.* than NaOCl in the short time contacts ($p < 0.05$). After longer 30 seconds treatment, FAC 100 mg/L of three chlorine-related antimicrobial agents resulted in < 10 CFU/mL survival population of *Salmonella spp.*

Table 2. Inactivating efficiency of MLEW, bleach and ClO₂ against *Salmonella spp.*

Time intervals and culturable population of <i>Salmonella spp.</i>							
FAC concentration (mg/L)	Control group (CFU/mL)	MLEW		NaOCl		ClO ₂	
		10 sec	30 sec	10 sec	30 sec	10 sec	30 sec
50	5.0×10^7	< 10	< 10	9.0×10^6	< 10	< 10	< 10
100	4.6×10^6	< 10	< 10	< 10	< 10	< 10	< 10

As to FAC 100 mg/L three chlorine-related antimicrobial agents, all *Salmomnella spp.* survival populations were reduced to <10 CFU/mL condition. MLEW, NaOCl and ClO₂ can present comparable inactivation efficiency once enough contact time was set. The result in here indicates MLEW and ClO₂ are better effective antimicrobial agents against *Salmomnella spp.* while rapid treatment is needed.

Table 3 shows the inactivating efficiency of MLEW, NaOCl and ClO₂ against *S. aureus* with FAC 50 and 100 mg/L. Facing to *S. aureus*, FAC 50 mg/L NaOCl and ClO₂ did not give satisfied inactivation efficiency when treating with 10 second. 71% and 46% of *S. aureus* were still culturable after the 10 second treatment (4.0×10^7 and 2.6×10^7 CFU/ mL survived for NaOCl and ClO₂ treated samples). On the contrary, the MLEW performed <10 CFU/mL survival rate at the same 10 second contacting. MLEW performed rapid and effective antimicrobial reaction against *S. aureus* than NaOCl and ClO₂ in the short time contacts ($p < 0.05$).

Table 3. Inactivating efficiency of MLEW, NaOCl and ClO₂ against *S. aureus*

Time intervals and culturable population of <i>S. aureus</i>							
FAC concentration (mg/L)	Control group (CFU/mL)	MLEW		NaOCl		ClO ₂	
		10 sec	30 sec	10 sec	30 sec	10 sec	30 sec
50	5.6×10^7	< 10	< 10	4.0×10^7	1.7×10^7	2.6×10^7	< 10
100	5.6×10^7	< 10	< 10	3.2×10^7	1.0×10^7	1.4×10^7	< 10

As to longer 30 second contacting samples, only FAC 50 mg/ L NaOCl did not reach significant inactivating efficiency (30% were still survival). Both MLEW and ClO₂ give performed <10 CFU/mL survival rate in the same time of contacting. For FAC 100 mg/L antimicrobial agents experiments against *S. aureus*, NaOCl did not present significant inactivating efficiency no matter 10 second or 30 second treatment (survival rate were 57% and 17%). ClO₂ bought out satisfied inactivating efficiency after 30 second contact rather than 10 second (25% survived). MLEW presented < 10 CFU/mL survival rate against *S. aureus* both 10 second and 30 second treatment in our experiment. Therefore, ClO₂ and MLEW can be selected to inactive *S. aureus* contamination if longer contact time available.

D-values of MLEW, bleach and ClO₂ against 3 bacterial strains

Table 4 presents D-values (decimal reduction time) of chlorine-related antimicrobial agents applied against 3 bacterial strains in the study. According our experimental results, MLEW were the most suitable antimicrobial agents against ECHC, *Salmonells spp.* and *S. aureus* strains. The inactivating efficiency of NaOCl toward to *S. aureus* is limited. ClO₂ also presented limited inactivation against ECHC.

The MLEW reveals comparable even stronger antimicrobial effects with NaOCl in our study. The same trend could also be observed in previous studies of neutral electrolyzed water (Abadias et al., 2008; Machado et al., 2016). The antimicrobial property of MLEW is contributed to multiple factors. Firstly, at the near-neutral pH chlorinated solution, the predominant FAC species is hypochlorous acid. It has stronger biocidal capacity than hypochlorite ion, which is the predominant species in high pH NaOCl liquid (Rahman et

al., 2010; Waters and Hung, 2013). Besides, the high oxidation-reduction potential (ORP) in the electrolyzed water is regarded as the primary factor yielding microbial inactivation. ORP can damage the outer and inter membrane of bacterial cell. Then, severely ORP stress can deplete the energy stores and damage the machinery that produced the energy associated with protein structure and cell function. High ORP provides MLEW additional antimicrobial factors apart from FAC species. It may explain the comparative efficacy observed in this study when FAC matched with chlorine dioxide (Thron et al., 2013; Machado et al., 2016). In Thron et al. (2013) study, the 20 minute antimicrobial treatment of electrolyzed water, bleach and ClO₂ against *S. aureus* was conducted. The results showed the order of antimicrobial efficiency was as follows: neutral electrolyzed water > ClO₂ > NaOCl. However, it should be noted there were instances in which no significant difference between agents was observed (Machado et al., 2016). It would going to the same trend if the contact time was elongated to minute level in our experiment. The MLEW did revealed comparable inactivation efficiency against the three common food-borne contagious bacterial strains to ClO₂ and bleach according to our present experimental results.

Table 4. D-values of MLEW, bleach and ClO₂ against 3 bacterial strains

D-values and FAC of chlorine-related antimicrobial agents						
Bacterial strains	MLEW		Bleach		ClO ₂	
	50 mg/L	100 mg/L	50 mg/L	100 mg/L	50 mg/L	100 mg/L
ECHC	5 sec	5 sec	5 sec	5 sec	144 sec	42 sec
<i>Salmonella spp.</i>	5 sec	5 sec	11 sec	5 sec	5 sec	5 sec
<i>S. aureus</i>	5 sec	5 sec	57 sec	40 sec	5 sec	5 sec

From the standpoint of field usage in food-processing factory and kitchen, MLEW and ClO₂ take the advantages of on-site generation. Since ClO₂ traditionally requires manual mixing of sachets whereas MLEW generation can be automated by device which is simply fabricated and economically maintained. Only water, salt and electrical power are required to generate the MLEW continuously on site. No hazardous matter storage and mixing involved in the MLEW generation (Thorn et al., 2013). Since highly microbial reactive species contained, the disadvantage of MLEW is the rapidly free chlorine loss in the open air. Greatly lost of free chlorine in the electrolyzed water is observed when settled in the open-light environment during several days, following with the significantly decreasing of biocidal efficiency (Han et al., 2018). The generation model comprehend sufficiently supply and using just-in-time is highly demands for the MLEW application in the food-production industry.

Conclusions

Our experimental result indicated that food-borne bacteria inactivation efficiency of MLEW is comparable to conventional chlorine-related agent, including bleach and ClO₂. MLEW even presented more ensuring inactivation to the target strain especially short time treatment set in our study. However, the sanitization in food-production involves

complicated processes and interfering factors, such as various deploying surface (metal plate, fabric surface or wood cutting board), organic matter reaction (blood, feces or tissue residual), co-existence of microbes (viral and bacterial strains). Therefore, further investigations of broad spectrums and various conditions should be fulfilled to clarify the s and applications of MLEW in the food-production industry.

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