Journal of
Food & Industrial Microbiology
ISSN: 2572-4134

Kataoka et al., J Food Ind Microbiol 2015, 1:1
DOI: 10.4172/2572-4134.1000105

Research Article Open Access

Bactericidal and Sporicidal Activities against Pathogenic Bacteria of Direct Flow Electrolyzed Water

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Abstract

The purpose of this investigation was to examine the bactericidal activity of long lifetime ozone water (LLO water) against pathogenic bacteria of medical, veterinary, and public health interest. An electrolytic system using Pt mesh electrodes combined with a conventional polymer electrolyte, the Nafion 117 membrane, produced LLO water from tap water with high efficiency. LLO water killed 7.144±0.0507 $\log_{10}$ CFU of *Escherichia coli* O157:H7, 6.979±0.045 $\log_{10}$ CFU of methicillin-resistant *Staphylococcus aureus*, 6.503±0.3681 $\log_{10}$ CFU of *Pseudomonas aeruginosa* and 6.579±0.123 $\log_{10}$ CFU of *Legionella pneumophila* within one minute. In contrast, endospores of *Bacillus anthracis* were killed within 20 min. Results of observation of the bactericidal activity of LLO by using an electron microscope, it was revealed that LLO water was shown the bactericidal action by swelling and destroying on the cell wall of the bacterial cells. Our studies demonstrated that LLO water has the potential to be widely applicable in food hygiene, medicine and veterinary medicine as a sterilizing agent. This is the first study to demonstrate the bactericidal activity and characteristics of LLO water.

Keywords: Bactericidal activity; Sporicidal activity; Long lifetime ozone water

Introduction

Functional water produced by electric systems, such as electrolyzed water, has been widely used as a safe and low cost bactericidal water compared with disinfectants. Bactericidal electrolyzed waters, which consist of acidic and alkaline water, are usually produced by addition of 0.1% NaCl. However, if strong acidic electrolyzed water or a strong alkali electrolytic water is generated, those that contain few amount of sodium hypochlorite (NaClO) as one of the disinfectant [1]. We have developed a new electrolytic water generated by the system to direct electrolysis of tap water [2]. This electrolyzed water, named long-lifetime ozone water (LLO water), has a neutral pH and contains high concentrations of ozone and hydroxyl radicals, without NaClO [2]. Hydroxyl radicals produced by the decomposition of ozone water are one of the strongest oxidizers. Ozone can be used instead of chlorine as an effective water disinfectant and is able to inactivate many human pathogenic bacteria which are *Bacillus cereus, Escherichia coli, Legionella pneumophila, Mycobacterium fortuitum, Pseudomonas fluorescens, Salmonella* Enteritidis, *S.* Typhimurium and *Staphylococcus aureus* [3]. However, some protozoa, fungi and spores require a longer time of contact with ozone than bacteria and viruses [3-6]. The LLO water is long-lifetime ozone water; therefore, it is possible to inactivate many pathogenic agents of medicine, veterinary medicine and public health concern.

In the medical science, major etiological agents of nosocomial infections include methicillin-resistant *S. aureus* (MRSA), multidrug-resistant *P. aeruginosa* (MDRP) and *L. pneumophila* [7-9]. These organisms are particularly problematic because of their ubiquitous nature, ability to survive in the hospital environment, and innate resistance to many antibiotics and antiseptics. *Bacillus anthracis* is a highly pathogenic bacterium that causes anthrax and has been associated with deliberate contamination events [10]. Other *Bacillus* spp., *B. cereus* and *B. subtilis*, are endosporeformers that are widespread in the environment. There is the possibility that long-term use of disinfectants to eliminate these causative agents of nosocomical infections can result in environmental pollution. In contrast, electrolyzed water with bactericidal activity eventually changes into water with the passage of time. Stevenson et al. reported that the effect of storage of electrolyzed oxidizing (EO) water [11]. In their results, when stored in the dark, bactericidal efficacy was retained for at least 180 hrs. Therefore, electrolyzed water can be applied to clean up the environment in the hospital as well as the environmental pollution caused by bioterrorism. The purpose of our investigation was to examine the bactericidal activity of LLO water against pathogenic bacteria in the medical, veterinary, and public health fields. We observed by optical microscope and electron microscope how LLO water was acted against bacterial cells. Moreover, the characteristics of LLO water were determined by carrying out dilutions and limited values.

Material and Methods

Long lifetime ozone water and control water

Long-lifetime ozone (LLO) water produced by an electrolytic system using a felt separator was described previously [2]. This electrolytic system using platinum (Pt) mesh electrodes combined with a conventional polymer electrolyte, the Nafion 117 membrane, produced a high efficiency LLO water from tap water directly. The LLO water had a neutral pH (pH 5.5 to 7.0), contained high levels of hydroxyl radicals, and did not contain residual chlorine or NaClO. Chlorhexidine (CHG, Sumitomo Pharmaceuticals co. LTD, Osaka, Japan) at a concentration of 0.05% was used as a positive control for bactericidal activity and distilled water was used as a negative control.

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Received December 09, 2015; **Accepted** December 28, 2015; **Published** December 30, 2015

Citation: Kataoka Y, Ochi H, Sawada T, Miyakawa Y, Hatakeyama H (2015) Bactericidal and Sporicidal Activities against Pathogenic Bacteria of Direct Flow Electrolyzed Water. J Food Ind Microbiol 1: 105. doi:10.4172/2572-4134.1000105

Bacteria

Escherichia coli NIHJ and *S. aureus* 209P were used as control strains for bactericidal tests. *E. coli* O157:H7 CE194, methicillin-resistant *S. aureus* NVLU-183, *P. aeruginosa* ATCC27853, *B. anthracis* Davis, *B. cereus* ATCC14579, *B. subtilis* ATCC9372 and *L. pneumophila* ATCC27853 were used as the pathogenic bacteria. These bacterial strains were type strains, reference strains or were isolated from diseased animals or humans in our laboratory's bacterial collections.

Bacterial cultures and preparation of bacterial suspensions

E. coli, S. aureus and *P. aeruginosa* were inoculated into heart infusion broth (Becton, Dickinson and Co. USA) and cultured at 37°C for 18 hrs. *L. pneumophila* was cultured on buffered charcoal yeast extract agar (B-CYE agar; Becton, Dickinson and Co. USA) at 35°C for 48 h. After incubation, *L. pneumophila* colonies were suspended in 0.85% NaCl solution and adjusted to an optical density of 0.1 at 600 nm (OD600) using an ERMA AE200 spectrophotometer (ERMA Inc. Japan). *B. anthracis, B. cereus* and *B. subtilis* strains were cultured in trypticase soy broth (Becton, Dickinson and Co. USA) at 37°C for 18 hrs. *B. anthracis*, *B. cereus* and *B. subtilis* endospores were incubated overnight on heart infusion agar at 37°C, and further incubated for 5 days at room temperature. After incubation, *Bacillus* colonies were suspended in 0.85% NaCl solution to an OD600 of 0.1 *Bacillus* endospore solutions were treated at 62°C for 30 min to kill the vegetative cells.

Bactericidal test procedures

Bactericidal tests of LLO water were performed by using a modification of the procedure used to test bactericidal activity of disinfectants [12]. Two hundred µl of each bacterial suspension was mixed with 20 ml of LLO water, 20 ml of 0.05% chlorhexidine or 20 ml of distilled water as controls, and the mixtures were maintained for 1, 2, 3, 5 and 10 min at room temperature. One hundred µl of this mixture was taken at various time points, and 5 ml of 0.5% sodium thiosulfate stop solution was added. The treated bacterial samples were cultured on heart infusion agar at 37°C for 24 hrs, except *L. pneumophila,* which was grown on B-CYE agar at 35°C for 48 hrs. After incubation, the bactericidal activity of LLO water was determined by counting the number of bacterial colonies and comparing the CFUs with those of the samples that were treated with disinfectant and distilled water.

Microscopic and electron microscopic observation of LLO water-treated bacteria

S. aureus NVLU-183 strain was used in this study. *S. aureus* NVLU-183 was cultured in trypticase soy broth at 37°C for 18 hrs, and 200 l of each bacterial culture was mixed with 20 ml of LLO water or 20 ml of distilled water. Distilled water was used as a negative control. After incubating at 20°C for 1 second, the reaction was stopped by adding 0.5% sodium thiosulfate solution. Cells were fixed for 24 hrs with 0.2% formaldehyde in normal saline (0.85% NaCl solution). The fixed bacterial cells were washed twice by PBS and then stained with TI blue solution (Nisshin EM, Tokyo, Japan) for 2 min at room temperature according to the manufacturer's instructions on a nano-percolator filter (JEOL Ltd. Tokyo, Japan). The sample on the filter was mounted directly to the specimen holder and examined using a Miniscope® TM-3000 scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan). *B. cereus* ATCC14579 strain was cultured in trypticase soy broth at 37°C for 18 hrs, and 200 µl of the bacterial culture was mixed with 20 ml of LLO water or 20 ml of distilled water. After incubating at 20°C for 10 sec, the reaction was stopped by adding 0.5% sodium thiosulfate solution. Bacterial pellets were fixed with 2.5% glutaraldehyde in phosphate-buffered saline. The samples were post fixed with 1% osmium tetroxide (OsO4), and dehydrated using ethanol. Subsequently, the pellets were embedded in Epon 812 (TAAB Laboratories, England), ultra-thinly sectioned, and stained with uranyl acetate and lead citrate. The samples were observed with a transmission electron microscope (JEM-1011; JEOL Ltd. Tokyo, Japan).

Determination of the limits of bactericidal activity of LLO water

To determine whether LLO water was active with higher concentrations of cells, how long the LLO water retained bactericidal activity, bactericidal tests were carried out with more concentrated cell suspensions, with LLO water that had been left at room temperature. Suspensions of *E. coli* and *S. aureus* overnight cultures were adjusted to approximately 10^7 to 10^8 CFU/ml. Bacterial solutions were diluted from 2 to 8-fold with 0.85% NaCl. Bctericidal test was performed as described above and distilled water was used as the negative control. LLO water was produced by the electrolytic system and left at room temperature for 3, 6 and 24 hrs. The bactericidal activity of the LLO water was examined to determine whether it retained antibacterial capability using *E. coli*.

Statistical analysis

The bactericidal and sporicidal activities of LLO water were determined with 3 replications. Statistical significance was determined by chi-square analysis, and $P<0.05$ was considered significant.

Results and Discussion

Bactericidal activity of LLO water

Results of studies examining the bactericidal activity of LLO water are shown in Table 1. In the case of 7.059 ± 0.0567 $\log_{10}$ CFU *E. coli* NIHJ strain, 20 ml of LLO water killed to below the detection limit within one min same as 0.05% CHG. In contrast, distilled water is the negative control; the same number of inoculum bacterial cells has been detected. In the pathogenic bacteria, LLO water killed 7.144 ± 0.0507 $\log_{10}$ CFU of *E. coli* O157:H7, 6.979 ± 0.0450 $\log_{10}$ CFU of MRSA, 6.503 ± 0.3681 $\log_{10}$ CFU of *P. aeruginosa* and 6.579 ± 0.1230 $\log_{10}$ CFU of *L. pneumophila* within one minute. Furthermore, 6.898 ± 0.0811 $\log_{10}$ CFU of vegetative cells of *B. anthracis*, 6.0710 ± 1605 $\log_{10}$ CFU of *B. cereus* and 5.675 ± 0.1408 $\log_{10}$ CFU of *B. subtilis* were killed within one minute (Table 1). In contrast, endospores of 5.679 ± 0.1155 $\log_{10}$ CFU of *B. anthracis* were killed within 20 min (Table 2). The positive control, 0.05% chlorhexidine, also killed all tested bacteria except *B. anthracis* spores within one minute. On the other hand, use of distilled water as the negative control resulted in the detection of the same number of cells as the inoculum at each treatment time (Table 1).

Electron microscopic observation of bacterial cells treated with LLO water

Figure 1a and 1b shows electron micrographs of *S. aureus* cells after treatment with LLO water. As shown in Figure 1a (untreated) and Figure 1b (treated with LLO water), the morphological changes in the *S. aureus* cells are clearly evident. Bacterial cells of *S. aureus* treated with LLO water were expanded by 3 to 5-fold, and further cell ambient had changed from spherical form to irregular morphology. Therefore, LLO water directly damaged the bacterial cell walls.

The TEM images of the *B. cereus* cells showed notable changes to their cell wall after treatment with LLO water (Figure 2a and 2b). The

Bacterial strain	Test water	The value of ORP(mV)	Inoculated cells (Log±SD)	Cells (Log±SD) remaining following reaction time (min) of :				
				1	2	3	5	10
E. coli NIHJ	LLO water	974±12.49[a]	7.059±00567	<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	364.3±4.51		<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	259.7±1.53		7.364±0.0753	7.321±0.0415	7.343±0.1247	7.365±0.0652	7.322±0.1819
E. coli O157:H7 CE194	LLO water	979±20.67	7.144±0.0507	<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	359±7.55		<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	264.3±6.51		7.206±0.1467	7.144±0.052	7.223±0.1393	7.244±0.0632	7.111±0.1307
S. aureus 209P	LLO water	969.7±9.07	6.966±0.0375	5.1±0.1384	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	355.3±13.31		<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	259±1.00		6.392±0.1229	6.417±0.1095	6.522±0.1093	6.604±0.0485	6.581±0.1985
Methicillin-resistant *S. aureus* NVLU-183	LLO water	979±23.00	6.979±0.045	<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	360±3.21		<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	269±10.15		6.662±0.0959	6.8±0.141	6.725±0.0557	6.612±0.3134	6.728±0.1131
P. aeruginosa ATCC27853	LLO water	957±9.54	6.503±0.3681	<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	346.7±11.06		<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	257.7±6.81		6.930±0.1134	6.911±0.1661	6.712±0.3403	6.968±0.0720	6.924±0.0889
L. pneumophila ATCC27853	LLO water	989.7±13.58	6.579±0.123	<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	355.3±4.51		<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	273.7±9.61		6.199±0.1780	6.073±0.1589	5.876±0.1478	6.039±0.1084	6.283±0.1149
Vegetative cells of *B. anthracis* Davis	LLO water	985.7a±14.64	6.898±0.0811	<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	352.7±11.37		2.300±0.001*	<2.301*	2.229±0.001*	<2.301*	<2.301*
	Sterile distilled water	252.3±11.59		6.631±0.2039	6.590±0.2603	6.686±0.0953	6.641±0.0596	6.564±0.1034
Vegetative cells of *B. cereus* ATCC14579	LLO water	981.7±10.4	6.071±0.1605	<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	0.05% CHG	362.7±3.06		<2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	257.3±3.06		6.539±0.1786	6.371±0.1622	6.221±0.1512	6.212±0.1171	6.375±0.1899
Vegetative cells of *B. subtilis* ATCC9372	LLO water	977.3±25.11	5.675±0.1408	2.301*	<2.301*	<2.301*	<2.301*	<2.301*
	Sterile distilled water	255.7±6.66		6.419±0.1871	6.348±0.2945	6.455±0.1639	6.212±0.1938	6.675±0.0865

*P<0.005; aValues are means±standard deviation of triplicate determinations.

Table 1: Bactericidal activities of LLO water against *Escherichia coli, Staphylococcus aureus, Bacillus anthracis, B. cereus, B. subtilis, Pseudomonas aeruginosa* and *Legionella pneumophila*.

bactericidal effect of LLO water was shown to destroy the structure of the cell wall of bacterial cells. In addition to this, the viable cell count revealed that LLO water was an effective treatment against the survival of bacterial cells.

Limits of bactericidal activity of LLO water

Although LLO water killed 6.0792 $\log_{10}$ CFU of *E. coli,* 6.3617 $\log_{10}$ CFU remained viable. Similarly, 8.1461 $\log_{10}$ CFU of *S. aureus* were viable even though LLO killed 7.5441 $\log_{10}$ CFU (Table 3).

After LLO water was left at room temperature for 3, 6, 24 hrs, the oxidation-reduction potential (ORP) value was maintained at 950 for over for six hrs, and 7.5185 $\log_{10}$ CFU of *E. coli* were killed by 6 hr old LLO water (Table 4).

Discussion

LLO water, which was produced from tap water, was shown to work as well as bactericidal disinfectants against various pathogenic bacteria found in medical and public health settings. In addition, LLO water had bactericidal activity against endospores of *B. anthracis*, which have been used as an agent of biological terrorism in recent years. Based on these findings, LLO water may be widely applicable in food hygiene, medicine and veterinary medicine as functional water. The characteristics of LLO water include a neutral pH, the same ion concentration as tap water, no sodium hypochlorite, > 0% ozone concentration, and a lifetime >150 hrs [2]. In contrast, acidic electrolyzed water has a pH of 2 to 3, and an active chlorine content of 10 to 90 ppm; basic electrolyzed water has a pH of 10 to 13 and

Test water	The value of ORP (mV)	Inoculated cells (Log±SD)	Cells (Log±SD) remaining following reaction time (min) of :				
			1	3	5	10	20
LLO water	987±13.53[a]	5.679±0.1155	3.094±0.1029*	3.314±0.0216*	2.835±0.6811*	2.602±0.301*	<2.301*
Sterile distilled water	259±7.00		5.628±0.1384	5.413±0.1756	5.502±0.1738	5.621±0.0533	5.528±0.0655
* P<0.005; [a]Values are means±standard deviation of triplicate determinations.							

Table 2: Bactericidal activity of LLO water against spores of *Bacillus anthracis* Davis.

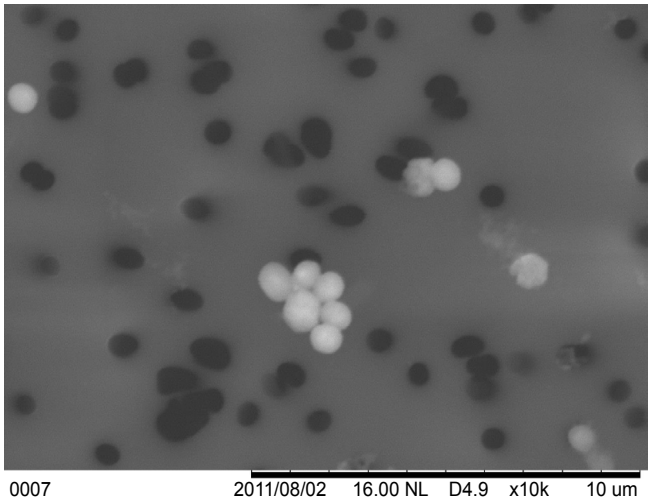

Figure 1a: A scanning electron microscopic observation with the bacteriocidal activity of LLO water (TI blue stain, *Staphylococcus aureus* ×10,000) shows *S. aureus* 209P strain as control.

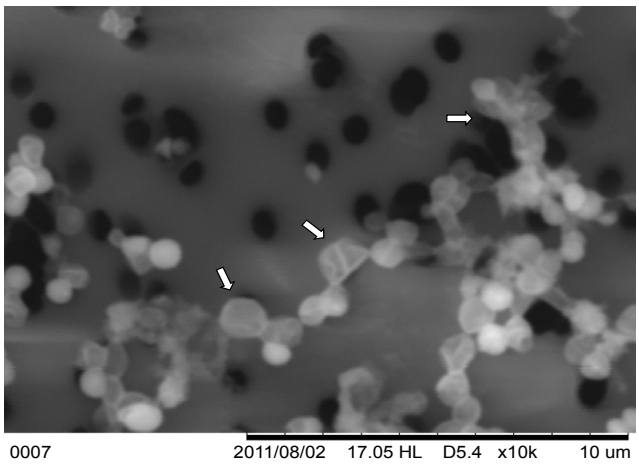

Figure 1b: A scanning electron microscopic observation with the bacteriocidal activity of LLO water (TI blue stain, *Staphylococcus aureus* ×10,000) shows *S. aureus* treated with LLO water.

an oxidation-reduction potential of -800 to -900 mV [1]. However, acidic electrolyzed water and basic electrolyzed water show a high potential for application in the agriculture and food industries as an environmentally friendly disinfection agent. Thus, LLO water is not limited to industrial applications such as agriculture and food; a wide range of applications could be expected in fields including medical and veterinary medicine, and food hygiene.

In addition to the pH of LLO water, the ORP values should also be taken into consideration. This ORP can be used as an indicator of the ability of electrolyzed water to oxidize or reduce compounds in the bacterial cell wall. The corresponding high ORP has been associated with the strong bactericidal activity of Electrolyzed water [13]. Solutions with high ORP values can induce modifications to bacterial membranes by causing changes to the cell's electron flow [14]. In this study, the ORP values of the LLO water was about +1000 mV, and had been maintained for 6 hrs. Therefore, it was revealed that the bactericidal activities of LLO water had been at least 6 hrs could be kept.

When the effect on the bacteria was observed using electron microscopy, LLO water was found to destroy bacterial cell walls. This observation suggested that free radicals are produced from ozone, which is the main component of LLO water, and cell wall damage was caused by free radicals, resulting in cell lysis in response to the osmotic pressure. In contrast, acidic electrolyzed water produced from a dilute sodium chloride solution has a similar bactericidal effect as

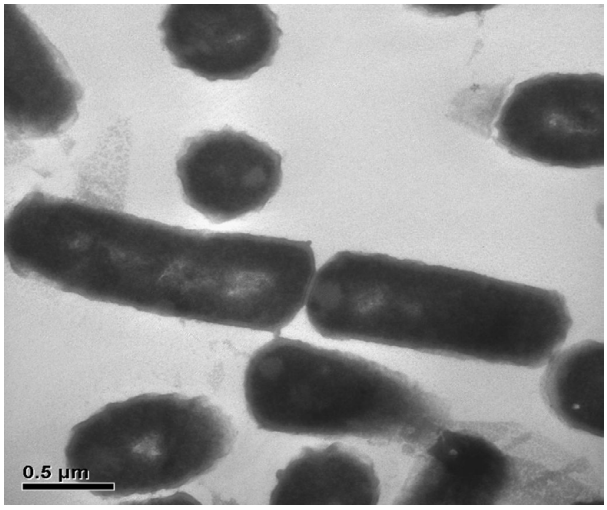

Figure 2a: A transmission electron microscopic observation with the bacteriocidal activity of LLO water (stain, *Bacillus cereus* × 10,000) shows *B. ceureus* ATCC14579 strain as control.

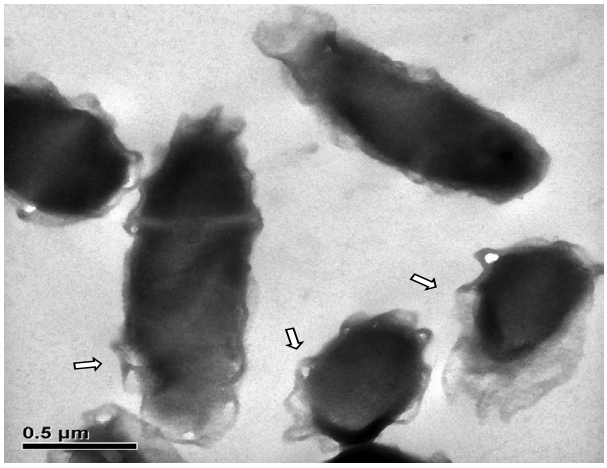

Figure 2b: A transmission electron microscopic observation with the bacteriocidal activity of LLO water (stain, *Bacillus cereus* ×10,000) shows *B. ceureus* treated with LLO water.

Bacteria	Test water	The value of ORP (mV)	Inoculated cells (Log)	Cells (Log) remaining following reaction time (min) of :		
				1	5	10
E. coli	LLO water	1007	6.3617	5.9243	5.9912	6.017
			6.0792	<2.301*	<2.301*	<2.301*
			5.8129	<2.301*	<2.301*	<2.301*
			5.5051	<2.301*	<2.301*	<2.301*
	Sterile distilled water	215	7.3617	7.2041	7.2041	7.2304
S. aureus	LLO water	1003	8.1461	7.8195	7.6434	7.5797
			7.845	6.6334	6.7242	6.716
			7.5441	<2.301*	<2.301*	<2.301*
			7.2552	<2.301*	<2.301*	<2.301*
	Sterile distilled water	216	7.1461	7.0791	6.8451	7.0414
*P<0.005						

Table 3: The limit value of bactericidal activity of LLO water against *Escherichia coli* NIHJ and *Staphylococcus aureus* 209P.

Test water	Inoculated cells (Log)	Cells (Log) remaining following LLO water incubation time (hrs) of :			
		0	3	6	24
LLO water	7.5185	<2.301*	<2.301*	<2.301*	6.7993
The value of ORP (mV)		1014	974	958	620
Sterile distilled water	7.5185	7.4314	7.4149	7.2553	7.3424
*P<0.005					

Table 4: Bactericidal activity of LLO water after collection against *Escherichia coli* NIHJ.

the disinfectant sodium hypochlorite [15]. Therefore, it is clear that the bactericidal effect of LLO water is due to the ozone and radicals generated by electrolysis of tap water.

In our current study, we have demonstrated the bactericidal and sporicidal activities of LLO water. The effect of LLO water as a disinfectant is attenuated by incorporation of organic matter, which is similar to findings with disinfectants. However, the fact that inactivated LLO water becomes mere water would allow its use in a wide range of applications without the possibility of polluting the environment. In the future, we will explore additional applications of LLO water.

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