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Ultraviolet Spectrophotometric Characterization and Bactericidal Properties of Electrolyzed Oxidizing Water as Influenced by Amperage and pH

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ABSTRACT

To identify the primary component responsible in electrolyzed oxidizing (EO) water for inactivation, this study determined the concentrations of hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) and related those concentrations to the microbicidal activity of the water. The ultraviolet absorption spectra were used to determine the concentrations of HOCl and OCl^- in EO water and the chemical equilibrium of these species with change in pH and amperage. EO water generated at higher amperage contained a higher chlorine concentration. The maximum concentration of HOCl was observed around pH 4 where the maximum log reduction (2.3 $\log_{10}$ CFU/ml) of *Bacillus cereus* F4431/73 vegetative cells also occurred. The high correlation ($r = 0.95$) between HOCl concentrations and bactericidal effectiveness of EO water supports HOCl's role as the primary inactivation agent. Caution should be taken with standard titrimetric methods for measurement of chlorine as they cannot differentiate the levels of HOCl present in EO water of varying pHs.

Different analytical methods have been used for chlorine measurement such as titrimetric, spectrophotometric (colorimetric), and amperometric methods *(1).* Selection of methods depends on the pH value of samples, interest in type of chlorine, and determination range of chlorine. Chlorine can be present in available chlorine and nonavailable chlorine forms. Available chlorine (residual chlorine) can be further divided into free chlorine and combined chlorine *(16).* Both of these types of available chlorine can be used for microbial inactivation, while nonavailable chlorine (chloride) has very little disinfection power *(16).*

The iodometric method is a widely used titrimetric procedure for determining residual chlorine. The suitable pH for titration is 3 to 4, because the reaction between free iodine and sodium thiosulfate is not stoichiometric at neutral and higher pHs *(1, 10).* The DPD-FEAS (*N,N*-diethyl-*p*-phenylenediamine-ferrous ethylenediammonium sulfate) method is another titrimetric procedure for determining free available chlorine. This titration method has a determination range from 0 to 3 ppm free chlorine while working under neutral pH conditions (pH 6.2 to 6.5).

Ultraviolet (UV) absorption spectroscopy is another method for chlorine determination. The principle of UV absorption is based on the hypochlorous acid (HOCl) component having maximum absorption at a wavelength of 234 nm with molar absorptivity of 100 cm^{-1} M^{-1} while hypochlorite ion (OCl^-) has a maximum absorption at wavelength of 292 nm with molar absorptivity 350 cm^{-1} M^{-1} *(5, 8).* The approximate concentration of HOCl and OCl^- can be calculated using Beer's law equation $A = \epsilon bc$ *(9),* where A is absorbance, ϵ is molar absorptivity (M^{-1} cm^{-1}), b is path length through solution (cm), and c is concentration of HOCl or OCl^- (M).

Electrolyzed oxidizing (EO) water has been reported to have a strong bactericidal effect on most pathogenic bacteria *(2, 3, 5).* Generated on-site, EO water is formed by electrolyzing a dilute salt (NaCl) solution that is subsequently separated into an acidic fraction and a basic fraction. EO water obtained from the anode has a pH of approximately 2.6, an oxidation-reduction potential of approximately 1,100 mV, and a chlorine concentration in the range of 10 to 100 ppm. This acidic fraction has been shown to inactivate most pathogenic bacteria *(2, 6, 12),* however, Kim et al. *(7)* reported that *Bacillus cereus* vegetative cells are more resistant to the EO water treatment than *Escherichia coli* O157:H7 and *Listeria monocytogenes.*

Many applied microbiological studies have been done with EO water, but the microbicidal activity of EO water has not been fully studied *(4, 13).* One factor that has recently been reported to play a primary role in the disinfection capability of EO water is its oxidation-reduction potential *(6).* Relative concentrations of chlorine species (aqueous molecular chlorine, [Cl_2], HOCl, OCl^-) may also factor into the bactericidal potency of EO water.

The objective of this study was to characterize spectrophotometrically the free chlorine species in EO water as a function of amperage and pH and relate their concentrations to EO's microbicidal activity.

MATERIALS AND METHODS

Chemical and spectral analysis of EO water. EO water was generated by a ROX-20TA generator (Hoshizaki Electric Inc., Ja-

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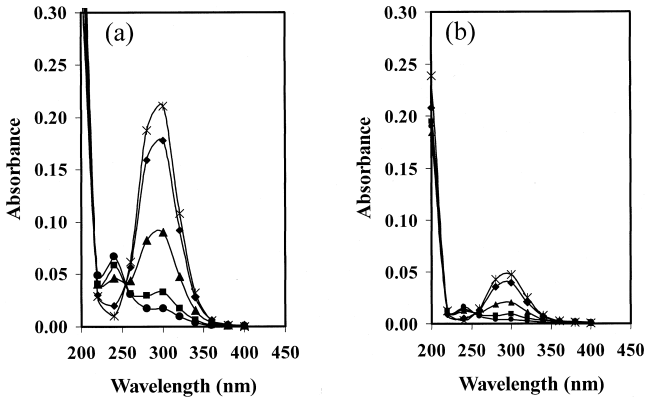

FIGURE 1. *Ultraviolet spectra of diluted acidic EO water at pH 4 (●), pH 6 (■), pH 7 (▲), pH 8 (♦), and pH 9 (*). (a) EO water generated at 14 A with 40 to 45 ppm residual chlorine. (b) EO water generated at 6 to 7 A with 10 to 12 ppm residual chlorine.*

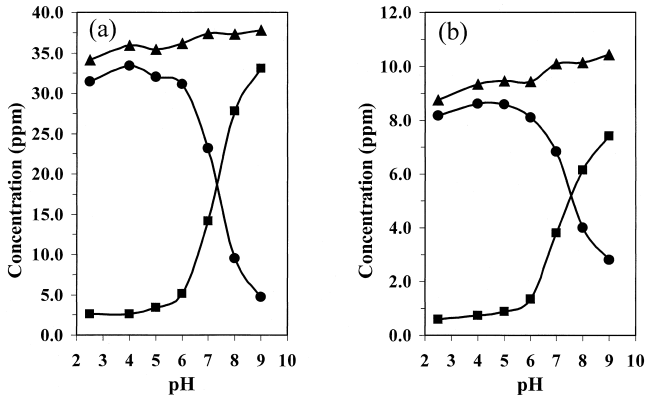

FIGURE 2. *Effect of pH on the concentrations of HOCl (●), OCl^- (■), and sum of HOCl and OCl^- (▲) in (a) EO water generated at 14 A and (b) EO water generated at 6 to 7 A.*

pan) using 6, 14, and 19 amps (A). Portions of the acidic fraction, having an initial pH of 2.5 to 2.7, were immediately adjusted to pH 4 to 9 with 1 N NaOH in amber bottles. Three methods were used to measure chlorine in EO water (iodometric, DPD-FEAS, and UV absorption methods). UV spectrophotometric scans (200 to 400 nm) were taken on these pH-adjusted fractions within 1 h using an HP 8451 A diode-array spectrophotometer (Hewlett Packard, Palo Alto, Calif.). UV spectra of EO water were measured at 25°C in 1-cm quartz cells. Deionized water was used as the reference. Absorbances at 234 nm and 292 nm were used to determine concentrations (ppm) of HOCl and OCl^-, respectively, using Beer's law equation *(5, 8).* A total chlorine test kit (Hach Co., Ames, Iowa; based on the iodometric method) was used to measure residual chlorine (free and combined chlorine), while another test kit (Hach; based on the DPD-FEAS method) was used to measure free chlorine. Both of these test kits are approved by the U.S. Environmental Protection Agency. Results for all methods were expressed in ppm. The oxidation-reduction potential and pH of EO water fractions were measured using a pH meter with pH and oxidation-reduction potential electrodes (Accumet model 15, Fisher Scientific Co., Fair Lawn, N.J.).

Preparation of treatment solutions for the microbiological study. EO water with a pH of 2.8 and an available chlorine concentration of 10 to 12 ppm was generated at a current setting of 6 A. From this original EO water, seven different pH levels of buffered EO water were prepared by adjusting with 1 N NaOH solution and four different (1 M) chemical buffering solutions. Acetic acid was used for pH 4.0 and 5.0 adjustments, monobasic phosphate was used for pH 6.0 and 7.0 adjustments, dibasic phosphate was used for pH 8.0 adjustment, and sodium carbonate was used for pH 9.0 adjustment. Seven different controls were also prepared to match the pH of the adjusted EO water by modifying deionized water with the same chemicals and procedures described above. A 0.1-ml aliquot of each buffered EO water and control solution (i.e., buffered deionized water) was plated on tryptic soy agar (Difco, Detroit, Mich.) and incubated at 37°C for 24 h to confirm the absence of bacterial growth in the control and treatment solutions.

Preparation of inocula. *B. cereus* (F4431/73) was cultured in tryptic soy broth (pH 7.3, Difco) at 37°C. Culture of *B. cereus* was transferred three times to tryptic soy broth by loop inocula at successive 24-h intervals. The culture was then centrifuged (1,800 × *g*, 24°C) in a clinical centrifuge (model IEC 809, International Equipment Company, Waltham, Mass.) for 10 min. The cell pellet was washed twice in 5 ml of 0.85% saline solution. After washing, the pellet was resuspended in 0.85% saline solution. The initial plate count of *B. cereus* was 6.4 $\log_{10}$ CFU/ml.

Microbiological study. One milliliter of the *B. cereus* inoculum was added at 24°C to 9 ml of each of the buffered EO water fractions or the buffered deionized water fractions. After 30-s treatment, serial dilutions were made and plated on tryptic soy agar. The inocula were uniformly distributed on the agars using sterile bent glass rods. The plates were incubated at 37°C for 24 h before colonies were counted. The experiment was replicated three times. Results were expressed as log reductions by comparing the CFU/ml of control buffered solutions to the CFU/ml in EO buffered solutions.

Statistical analysis. Data were analyzed using general linear model procedures *(11).* Comparisons of means were performed using Duncan's multiple range test.

RESULTS AND DISCUSSION

Spectroscopic characterization of EO water. UV absorption spectra of EO water demonstrated two absorption peaks, one at 234 nm and one at 292 nm (Fig. 1), corresponding to the wavelength maximal previously found for HOCl and OCl^-, respectively *(5, 8).* As pH of the EO water increased, A_{292} increased while A_{234} decreased, reflecting the pH-dependent equilibrium previously described between OCl^- and HOCl in chlorine-treated water *(5).* In addition, Figure 1 illustrates that absorbances were higher in EO water generated at 14 A than for EO water generated at 6 A, indicative of higher chlorine concentrations being produced at higher amperage.

Based on the UV spectroscopic measurement method, concentrations of HOCl and OCl^- in EO water adjusted to pHs between 2.6 and 9 are shown in Figure 2. The maximum concentration of HOCl was around pH 4. At higher pHs, the concentration of HOCl decreased, reflecting its dissociation to H^+ and OCl^- *(5, 16).* The sum of HOCl and OCl^-, however, increased with increasing pH. This imbalance could be attributed to Cl_2 that would be present predominantly at low pHs, but not measurable by UV, and its subsequent conversion to HOCl with increase in pH *(5).*

TABLE 1. *Properties of EO water*[a]

Type of EO[b] water	UV[c] absorption HOCl (ppm)	UV[c] absorption OCl^- (ppm)	UV[c] absorption Sum of HOCl and OCl^- (ppm)	IODO[d] residual chlorine (ppm)	DPD free chlorine (ppm)	Initial pH	ORP[e] (mV)
19 A	61.0 A	4.7 A	66.0 A	84.5 A	67.6 A	2.50	1,119
14 A	32.0 B	2.6 B	34.0 B	43.3 B	35.7 B	2.50	1,119
6 A	8.2 C	0.6 C	8.80 C	10.8 C	8.4 C	2.70	1,024

[a] Values in a column sharing a common letter are not significantly different ($P \leq 0.05$).
[b] EO water generated at 6, 14, and 19 A.
[c] UV absorption method (measured at initial pH).
[d] Iodometric method.
[e] Oxidation-reduction potential.

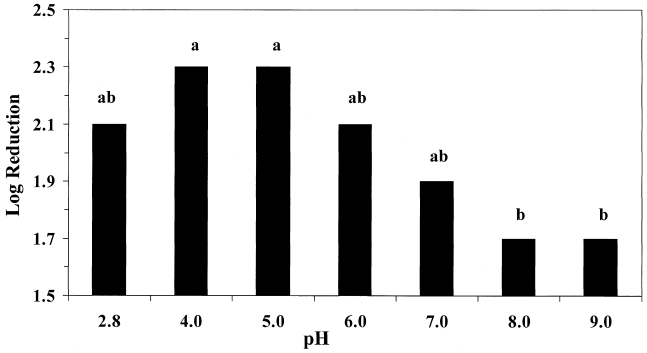

FIGURE 3. *Log reduction of* B. cereus *F4431/73 in EO water treatment (10 to 12 ppm Cl) at various pHs. Values on top of each bar sharing a common letter are not significantly different (*$P \leq 0.05$*).*

Titrimetric characterization of EO water. Titrimetric methods for determination of chlorine concentrations in EO water were also determined and are displayed in Table 1. The highest chlorine concentrations were found with the iodometric method. Differences between methods may be attributed to the chlorine constituents being measured. While DPD-FEAS measures only free chlorine (Cl_2, HOCl, and OCl^-), the iodometric method measures both free chlorine and combined chlorine (OCl^- bound to inorganic constituents). Because both of these methods require that the water samples be adjusted to specific pH ranges (pH 3 to 4 for the iodometric method and pH 6.2 to 6.5 for the DPD-FEAS method), neither is capable of differentiating the concentrations of HOCl and OCl^- present in the water. In measuring total free chlorine concentrations, however, larger concentrations were expected from the DPD-FEAS method compared to the UV spectrophotometric method, as the DPD-FEAS method measures Cl_2 in addition to HOCl and OCl^-. While these expectations were satisfied on analyses made of EO water generated at 14 and 19 A (Table 1), they were not on analyses conducted on EO water generated at 6 A. Cl_2 lost to vaporization during vigorous stirring in the DPD-FEAS method might be a larger proportion of the residual chlorine in EO water generated at small amperages than at larger amperages.

Bactericidal activity of acidic EO water. No growth occurred on the tryptic soy agar for controls and treatment solutions, indicating the absence of contamination in these solutions. More than 100-fold reduction of *B. cereus* occurred upon exposure to EO water adjusted to a pH in the range of 2 to 6, with maximum log reduction occurring around pH 4 (Fig. 4). In contrast, no significant inactivation of *B. cereus* occurred upon exposure to pH-adjusted deionized water treatments (data not shown). A highly significant correlation ($r = 0.95$) between concentration of HOCl and microbicidal activity of EO water suggested that HOCl is the primary bactericidal agent in this medium, confirming previous reports of this agent's effectiveness *(14–16).* According to White *(16),* OCl^- is about 20 times less effective than HOCl. HOCl's bactericidal effectiveness may be attributed to its neutral charge, allowing it to penetrate bacterial cell walls and interact with key metabolic systems *(16).* OCl^-, on the other hand, needs a high activation energy to penetrate the cell membrane due to its negative charge. Because HOCl will dissociate into a hydrogen ion (H^+) and a hypochlorite ion (OCl^-), disinfection efficiency of free chlorine decreases with increasing pH.

The UV spectrophotometric measurement is an attractive and simple method for chlorine determination that allows separate determination of the concentration of two free chlorine species (HOCl and OCl^-). This method does not require the addition of chemicals, thus enabling a better understanding of the properties of EO water and observance of pH effect on the equilibrium of free chlorine species. Based on the results obtained from the chemical analysis and microbiological study, it can be concluded that microbicidal activity of EO water can be explained by chemical equilibrium of Cl_2, HOCl, and OCl^-. The maximum microbicidal activity of EO water occurred around pH 4 where the concentration of HOCl is also the highest, indicating that HOCl is the primary component for inactivation. Because lower concentrations of this bactericidal agent are found in nonadjusted EO water, improvements in the effectiveness of EO water could be foreseen if adjustments in pH are made. In addition, standardized methods for chlorine measurement are incapable of differentiating concentrations of the active component and hence effectiveness of treatments.

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