Research Papers

Effect of pH on the Efficacy of Sodium Hypochlorite Solution as Cleaning and Bactericidal Agents

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The efficacy of sodium hypochlorite (NaOCl) solution as a cleaning and bactericidal agent against *Pseudomonas fluorescens* was studied as a function of pH. Alumina (Al_2O_3) particles on which *P. fluorescens* cells were irreversibly adhered were cleaned with NaOCl solutions of pH 5 to 12 containing free available chlorine (AC) of 120 to 1000 mg/L. The efficiency of the removal of *P. fluorescens* cells from the Al_2O_3 surfaces increased with increasing pH and the AC concentration. It was found that the efficacy of NaOCl solution as a cleaning agent depended on the concentration of the dissociated hypochlorite ion (^-OCl). On the other hand, the bactericidal activity of NaOCl solution of 2.5 mg AC/L increased with decreasing pHs from 9.3 to 5.7. The logarithmic relative reductions of viable *P. fluorescens* were proportional to the product of the AC concentration and time, and the rate of inactivation depended on the concentration of undissociated hypochlorous acid (HOCl). Our results show that the cleaning and bactericidal activities of NaOCl solution are governed by the percentage of HOCl and ^-OCl existing in the solution.

Keywords : Sodium Hypochlorite, Cleaning and Disinfection, Control of Biofilm, Bactericidal Activity, Free Available Chlorine

1. Introduction

Adhesion of bacterial cells to solid surfaces and the subsequent formation of a biofilm have been demonstrated in a wide variety of environments. The occurrence of a biofilm is often associated with numerous medical, industrial, and ecological problems. The importance of avoiding bacterial adhesion or removing biofilms formed on medical implants[1)~4)] and ureteral stents[5)], tap water distribution[6)], cooling systems[1, 7)], and milk transfer pipelines[8)] is generally recognized. A biofilm consists of both bacteria and their surrounding extracellular polymeric substances (EPS), composed of acidic polysaccharides or glycoproteins[1), 9)]. Biofilm formation is initiated when bacteria along with organic molecules, such as proteins and polysaccharides, are adhered from an aqueous phase onto a solid surface, forming a conditioning film. The conditioning film alters the physicochemical properties of the solid surface which may also induce the subsequent adhesion of bacterial cells. After adhesion, if nutrients are available, the attached bacteria grow and multiply. During this period, the bacteria produce EPS that contribute to the anchorage of the cells to the surface. As further production and accumulation of EPS occur, the bacterial cells in the biofilm are embedded in an EPS matrix and become permanently attached.

Sodium hypochlorite (NaOCl) has been used as a reliable disinfectant for more than 100 years because it fulfills many requirements of the ideal disinfectant, including a broad antimicrobial activity, rapid bactericidal action, reasonable persistence in treated potable water, and ease of handling[10)]. Most common commercial products of NaOCl are strong base solutions of pH 12.5 to 13.5 containing 5 to 12% of free available chlorine (AC). NaOCl solution is commonly used in the concentration range of 50 to 200 mg AC/L (pH 8.5 to 10) to disinfect produce surfaces and processing equipment[11)]. In a diluted NaOCl solution, over the pH range of 5 to 10, there is an equilibrium between two forms, undissociated hypochlorous acid (HOCl) and the dissociated hypochlorite ion (^-OCl):

$$HOCl \rightleftarrows {}^-OCl + H^+. \quad \cdots\cdots (1)$$

Their ratio depends on pH and temperature[12)]. It is believed that the active microbicidal species is undissociated HOCl[13), 14)]. When NaOCl solution is added to water to prepare a potent disinfecting solution, it is necessary to adjust the pH to between 5.0 and 6.5 because the concentration of HOCl is optimal. On the other hand, biofilm bacteria grown on various solid surfaces are more highly resistant to HOCl at neutral pH than are unattached bacteria[15), 16)]. Presumably, bacteria become more sensitive to NaOCl solution once many of them have been detached from the surface. Therefore, it is desirable to perform the disinfecting operation in the early stage of biofilm formation or after eliminating as much on the bacterial populations as possible. However, little is known of the mode of action of NaOCl solution in removing bacterial cells from solid surfaces.

The present study was initiated to examine the effect of pH on the efficiencies of cleaning and disinfection by NaOCl solution. *Pseudomonas fluores-*

cens was chosen as the test microorganism because it is widely found on food and vegetable surfaces[17)~19)] and produces EPS[19), 20)]. Alumina (Al_2O_3) particles were used as the model solid hard surface. We report here that the efficacy of NaOCl solution in removing *P. fluorescens* cells from Al_2O_3 surfaces depends on the ^-OCl concentration, whereas HOCl is the only potent bactericidal agent.

2. Experimental

2. 1 Bacterial strain and growth conditions

P. fluorescens NBRC 14160 was obtained from the National Institute of Technology and Evaluation (NITE), Biological Resource Center (Kisarazu, Chiba). *P. fluorescens* was grown on Luria-Bertani (LB ; Difco Laboratories, Detroit, MI) (pH 7) medium throughout the study. A stock culture was prepared by suspending the cells, collected from an LB agar plate, in liquid LB medium containing 10% glycerol. The stock culture was stored at −80℃. A working culture was prepared by inoculating 40 μL of the stock culture into 100 mL of liquid LB medium in a 200-mL flask. The culture was incubated at 30℃ for 24 h with continuous shaking (60 oscillations per minute). The cells were harvested by centrifugation (15,000× g for 10 min) and washed twice in 0.85% (w/v) NaCl solution (saline solution, pH 6). The washed cells were suspended in 20 mL of the saline solution, and this suspension (stock cell suspension) was used for adhesion and disinfection experiments.

P. fluorescens counts were quantified by direct spreading of 0.1 mL-portions of serial decimal dilutions of the cell suspension on LB agar plates[21)]. The plates were incubated at 30℃ for 48 h, and the colonies formed were counted. The viable cell count was represented as colony forming units per milliliter (CFU/mL). The number of *P. fluorescens* in the stock cell suspension was 2×10^9 CFU/mL.

2. 2 Materials and chemicals

Granular α-Al_2O_3 particles, with a mean diameter of 15.5 μm and a specific surface area of 0.37 m^2/g, were used[22)]. NaOCl (Lot DWF 2416) containing 60,000 mg AC/L was purchased from Wako Pure Chemical Ind., Ltd. (Osaka). The AC concentration was measured using the *N, N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method[21)]. All other chemicals were of analytical grade and were purchased from commercial sources

2. 3 Adhesion conditions

To prepare *P. fluorescens* cells-attached Al_2O_3 particles, a 5-ml aliquot of the stock cell suspension (pH 6) was dispensed into a 25-mL glass vial containing 2 g of Al_2O_3 particles, and then the vial was sealed with a butyl rubber stopper. The vial was placed on its side in a water bath at 30℃ and reciprocally shaken (140 oscillations per min) for 2 h. After being shaken, the particles with attached cells were collected by centrifugation (2,300× g for 10 min) and washed twice in 25 mL of saline solution by centrifugation (2,300× g for 10 min). After being washed, the mass of the cells remaining on the particles (i.e., irreversibly adhered cells) were determined using a total organic carbon (TOC) analyzer equipped with a solid sample module[23)].

2. 4 Cleaning conditions

The reagent NaOCl was diluted with a deionized water to 120, 500, and 1,000 mg AC/L because AC concentrations of 100 to 1,000 mg/L are generally used to clean food-processing equipment. The pH values (5 to 12) of diluted NaOCl solutions were adjusted by drop-wise addition of HCl or NaOH (0.1 M solutions) under conditions of constant agitation.

Batchwise cleaning was conducted at 40℃ by introducing 5 ml of the dilute HCl or NaOH solutions (pH 4 to 13) or the NaOCl solution into a 25-ml glass vial containing a 2-g portion of the washed Al_2O_3 particles with attached cells (initial attached cell mass:Γ_0). The vial was then laid on its side in a water bath and reciprocally shaken (140 oscillations per min) for 2 h as described above. After conducting the cleaning, the particles were washed twice in 5 mL of saline solution by centrifugation (2,300× g for 10 min). The mass of cells remaining on the particles after cleaning (Γ_r) was measured with the TOC analyzer described above. The removal efficiency was determined as the percentage of the mass of cells removed ($\Gamma_0-\Gamma_r$) from the Γ_0.

2. 5 Disinfection conditions

To determine the time-dependent bactericidal action of NaOCl solution, a low AC concentration of 2.5 mg AC/L was used. Disinfection of *P. fluorescens* by NaOCl was conducted at 40℃ in 0.1 mM 2-morpholino- ethanesulfonate (MES) buffer at pH 5.7, 0.1 mM phosphate buffer at pH 7.6, and 0.1 mM *N*-cyclohexyl-2-aminoethanesufonate (CHES) buffer at pH 9.3. A 0.25-mL aliquot of the stock cell suspension was added to a 15-mL sterile polypropylene (PP) tube containing 4.75 mL of each autoclaved buffer solution (the initial number of cells: 1.0×10^8 CFU/mL). A freshly prepared NaOCl solution (1,000 mg AC/L) was added to the cell suspension at final concentrations of 2.5 mg AC/L in the tube, which was then gently shaken at 40℃. During the course of the NaOCl treatment, samples (0.01 mL) were withdrawn at appropriate intervals and immediately transferred to PP tubes containing 0.99 mL of 0.1 mM phosphate buffer (pH 7.2). Serial dilutions were prepared, 0.1 mL-portions of which were spread on LB agar plates as described above. After incubation at 30℃ for 48 h, the colonies formed were counted to determine the viable cell number.

The Chick-Watson law[24), 25)] was used to determine the rate of inactivation of *P. fluorescens*, by assuming that the fitting parameter for non-first-order (n) is equal to 1:

$$\log(N/N_0)=-kCT \quad \cdots\cdots(2)$$

where N_0 is the initial number of the cells, N is the number of the vital cells at the contact time T, C is the AC concentration (C_{AC}) or HOCl concentration (C_{HOCl}), and k is the inactivation rate constant of the

cells.

3. Results

3. 1 Adhesion of *P. fluorescens* to Al_2O_3 surfaces

When *P. fluorescens* cells were brought into contact with Al_2O_3 particles at pH 6 and 30℃, the cells adhered to Al_2O_3 particles spontaneously. After being rinsed, Al_2O_3 particles with irreversibly attached cells were obtained. The initial mass of the attached cells (Γ_0) was 0.117±0.011 mg TOC/m² (n=9), which corresponded to approximately 1×10^8 CFU/m².

3. 2 Removal of P. fluorescens

Figure 1 shows the removal efficiency of *P. fluorescens* cells from the Al_2O_3 surfaces at different pH values in the presence and absence of NaOCl during batch cleaning. In the cleaning with dilute HCl or NaOH solutions alone, the removal efficiency of the cells was less than 7% in the pH range of 4 to 10, whereas it increased markedly at pHs higher than 11 and reached approximately 96% at pH 13. It was indicated that the removal of *P. fluorescens* cells occurred in an $^-$OH-dependent manner. In the presence of NaOCl, the removal efficiency was improved markedly at all the pH values examined. The efficacy of NaOCl became greater with increasing pH and it depended on the AC concentration. At pHs less than 6, samll amounts of the cells was removed even in the presence of NaOCl at 1000 mg AC/L.

These results could be accounted for by assuming that $^-$OCl has a stronger cleaning power than HOCl. To clarify the cleaning action of $^-$OCl, we put aside the data obtained at pHs higher than 11, where the cleaning action of $^-$OH alone was pronounced, and plotted the data in the pH range of 5 to 10 against the $^-$OCl concentration (**Fig. 2**). The concentration of dissociated $^-$OCl was calculated using an acid dissociation constant (pK_a) of 7.43 at 40℃, obtained by extrapolation of the data reported by Morris[12]. It was noted that the data were situated at points capable of being depicted asymptotically by one curve as a function of the $^-$OCl concentration. The removal efficiency increased from 15 to 86% with an increase in $^-$OCl concentrations from 2 to 1000 mg AC/L. This finding clearly indicates that the efficacy of NaOCl solution in removing *P. fluorescens* cells depends on the $^-$OCl concentration.

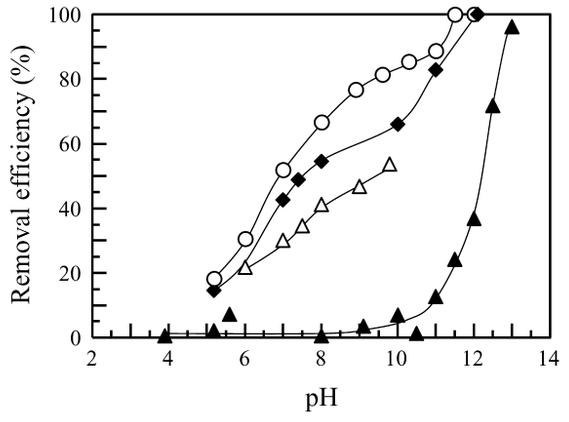

Fig. 1 Effects of pH and AC concentration on the removal efficiency of *P. fluorescens* cells from Al_2O_3 particles during batch cleaning. Cleaning was conducted in a 25-mL glass vial for 2 h at 40℃ with reciprocal shaking (140 oscillations per min). Symbols: ▲, Dilute HCl or NaOH solution alone; △, 120 mg AC/L of NaOCl solution; ◆, 500 mg AC/L of NaOCl solution; ○, 1000 mg AC/L of NaOCl solution.

3. 3 Inactivation of *P. fluorescens*

Experiments of the inactivation of *P. fluorescens* by NaOC solution were examined at pH 5.7, 7.6, and 9.3 to compare the bactericidal activities of HOCl and $^-$OCl. **Figure 3** shows the logarithmic relative reductions of viable *P. fluorescens* as a function of $C_{AC}T$ value. At pHs of 5.7 and 7.6, inactivation of *P. fluorescens* occurred according to a pseudo-first-order reaction (eq. 3) without a lag phase in which no inactivation occurred. The rate of inactivation is larger at pH 5.6 than at pH 7.6. Inactivation curve at pH 9.3 was characterized by a lag phase ($C_{AC}T<30$ mg·min/L) followed by a pseudo-first-order inactivation phase. Each solid line in **Fig. 3** was obtained from the data in the pseudo-first-order inactivation phase by using the linear regression method and eq. 3. At pH 5.7, over a 6-log-unit reduction could be achieved at a $C_{AC}T$ of 25 mg·min/L, whereas at pH 9.3, only a 0.3-log-unit reduction was achieved at a $C_{AC}T$ of 125 mg·min/L. The k values at pH 5.7, 7.6, and 9.3 were calculated to be 0.249, 0.110, and 0.00391 L/mg AC·min, respectively. The k value was 64-fold higher at pH 5.7 than at pH 9.3.

At pH 5.7, the percentage of HOCl accounts for approximately 98%, whereas it accounts for 40% at pH 7.6 and 1.3% at pH 9.3. These calculations imply that the inactivation of *P. fluorescens* by NaOCl solution depends on the HOCl concentration. Therefore, we plotted the inactivation data in **Fig. 3** against CT values based on the HOCl concentration ($C_{HOCl}T$) (**Fig. 4**). The graph demonstrated a linear correla-

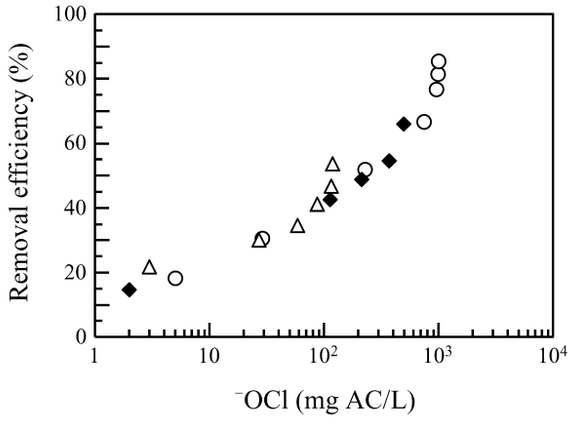

Fig. 2 Removal efficiency of *P. fluorescens* cells from Al_2O_3 particles as a function of the $^-$OCl concentration (calculated by pK_a of 7.43 at 40℃). Symbols are the same as those given in the legend for **Fig. 1**.

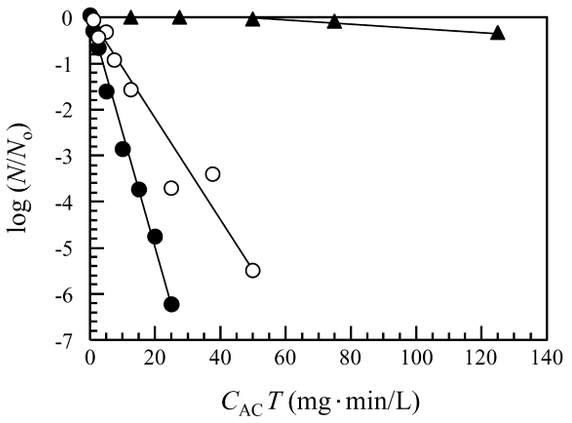

Fig. 3 Inactivation of *P. fluorescens* by NaOCl solution at different pH values. Experiments were conducted at 40℃ and initial AC concentration of 2.5 mg AC/L. Symbols : ●, pH 5.7 ; ○, pH 7.6 ; ▲, pH 9.3.

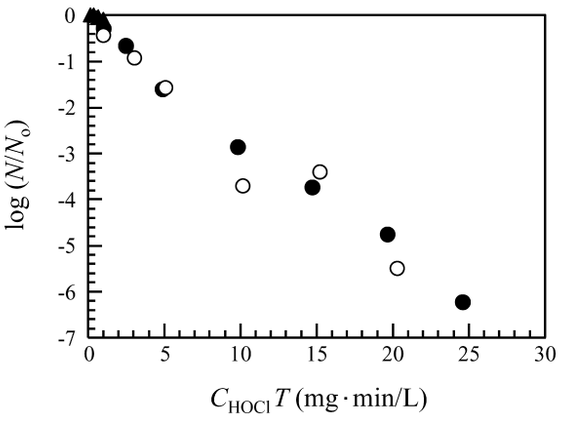

Fig. 4 Inactivation of *P. fluorescens* by NaOCl solution as a function of undissociated HOCl concentration. Symbols are the same as those given in the legend for **Fig. 3**.

tion between the logarithmic reductions and the C_{HOCl} T values. These results indicate that HOCl is the only species responsible for inactivating *P. fluorescens* under the present experimental conditions (at 40℃ and 2.5 mg AC/L).

4. Discussion

4. 1 Irreversible adhesion and susceptibility to $^{-}$OH cleaning

It is known that *P. fluorescens* produces EPS, which includes acidic polysaccharides and occurs as thin microcapsular coats[19), 20)]. EPS plays an important role in the adhesion of the cells to solid surfaces. In addition, the outer membrane proteins of *P. fluorescens* cells are also reported to be involved in their adhesion[26)]. In this study, *P. fluorescens* cells were irreversibly adhered to Al_2O_3 surfaces and could be removed only by NaOH solution of pHs higher than 11 (approximately 10^{-3}M $^{-}$OH). The relationship between the removal efficiency and pH in **Fig. 1** was similar to those obtained in caustic alkali cleaning of Al_2O_3 fouled with protein (bovine serum albumin ; BSA) and acidic polysaccharide (pectin)[27)]. BSA and pectin are believed to be irreversibly adsorbed on hydrophilic Al_2O_3 surfaces via varying numbers of carboxyl groups on the molecules through electrostatic interactions, hydrogen bonds, and possibly non-electrostatic interactions such as coordinate bonding[27)~29)]. It is conceivable that carboxyl groups on EPS and the outer membrane proteins of *P. fluorescens* cells might be involved in their irreversible adhesion. As the concentration of $^{-}$OH increases, dissociation of microbial cells and/or their surrounding EPS and Al_2O_3 surfaces is promoted by the adsorption of $^{-}$OH ions and their surfaces become more negatively charged[22), 30)], thereby creating repulsive forces between them. Attractive forces between the cells and Al_2O_3 surfaces diminish gradually, and the cells are finally detached into the NaOH solution. Thus, $^{-}$OH ions can preferentially displace the attached cells from adsorptive sites on the Al_2O_3 surfaces.

4. 2 $^{-}$OCl as the active cleaning agent

The efficacy of NaOCl in the cleaning process was evaluated in the pH range of 5 to 10, where no significant amount of *P. fluorescens* cells was removed by the action of $^{-}$OH alone. It was clearly shown that $^{-}$OCl was the active agent in the removal of *P. fluorescens* cells from the Al_2O_3 surfaces, not HOCl (**Fig. 2**). The major cleaning power of $^{-}$OCl is probably attributed to its oxidizing power and possibly to preferential displacement on the Al_2O_3 surfaces. The Cl atom in $^{-}$OCl (and HOCl) behaves as Cl^{+}, a strong electrophile, and therefore preferentially attacks parts of the substrate that have high electron densities, e.g., the C=C double bond, peptide bond, amino groups, and thiol groups. As for the removal of protein, the primary function of $^{-}$OCl is to decompose the molecule into low-molecular-weight fragments, resulting from the formation of chloramines and hence of nitrogen-centered radicals[27), 31), 32)]. Although HOCl is a potent oxidant, the reactivity of the AC components (Cl_2 [aq], HOCl, and $^{-}$OCl) with BSA is minimal around pH 4.5 where HOCl exists predominantly[33)]. It is reasonable that the attack of $^{-}$OCl on proteins in EPS or the outer membrane can contribute to the detachment of *P. fluorescens* cells. On the other hand, there is little available information about the mechanism of the reaction of $^{-}$OCl with EPS composed mainly of acidic polysaccharides. Although the reactivity of $^{-}$OCl with acidic polysaccharide such as pectin is relatively low compared with that of protein[33)], the $^{-}$OCl concentration has a significant influence on the removal of pectin from Al_2O_3 surfaces[27)]. In addition, as well as $^{-}$OH, $^{-}$OCl ions can adsorb on Al_2O_3 surfaces over the pH range of 5 to 10 (authors' unpublished data). These findings suggest the possibility that $^{-}$OCl ions can displace the pectin molecules on the surface. It is presumed that in the presence of high concentrations of $^{-}$OCl up to 1000 mg AC/L, corresponding to 2.8×10^{-2}M, $^{-}$OCl ions might also

partially displace or cleave anchoring sites, e.g., carboxyl groups on the EPS of *P. fluorescens* cells from Al_2O_3 surfaces.

4. 3 HOCl as the bactericidal agent

Several authors have previously reported that the microbicidal activity of NaOCl solution became maximum in a weak acidic pH region and that HOCl was the potent bactericidal agent[13), 14), 16), 34)]. In this study, NaOCl solution exhibited a more potent bactericidal activity toward *P. fluorescens* at pH 5.7 (HOCl: 98%) than at pH 7.6 (HOCl: 40%) and 9.3 (HOCl: 1.3%) (**Fig. 3**). In addition, the data in **Fig. 4** strongly indicate that the bactericidal activity depended on the HOCl concentration. These findings also confirm the general concept that HOCl is the more active form as the bactericidal agent and $^-$OCl is entirely inactive. The bactericidal activity of HOCl is attributed to its ability to penetrate into the microbial cell across the cell wall and membrane. The penetration of HOCl is due to its electrical neutrality and to its modest molecular size comparable to that of water. On the other hand, ionized $^-$OCl is not able to penetrate the microbial cell membrane because of the existence of the lipid bilayer, a hydrophobic layer, of the plasma membrane. HOCl can attack the bacterial cell from inside the cell, thereby accelerating the inactivation rate and enhancing the bactericidal activity. Although the mechanism of the bactericidal activity of HOCl has not been fully elucidated, the primary effect of HOCl is believed to be either or both (i) the oxidation of sulfhydryl (SH) groups of essential enzymes and antioxidants and (ii) deleterious effects on DNA synthesis[35)].

5. Conclusions

The results presented here demonstrate the significance of the pH as a factor in determining the efficacy of NaOCl solution both in removing and in inactivating *P. fluorescens*. The effect of the pH on the cleaning and disinfecting efficiencies of NaOCl solution is attributed to its effect on the equilibrium between HOCl and $^-$OCl. Undissociated $^-$OCl has a far stronger cleaning power, whereas undissociated HOCl has a potent bactericidal activity. For optimum control of biofilm formation, it is necessary to strengthen the cleaning and disinfecting actions of NaOCl solution by pH adjustment.

(Received January 4, 2007 ; Accepted May 28, 2007)

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