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Synergistic disinfection and removal of biofilms by a sequential two-step treatment with ozone followed by hydrogen peroxide

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ABSTRACT

Synergistic disinfection and removal of biofilms by ozone (O_3) water in combination with hydrogen peroxide (H_2O_2) solution was studied by determining disinfection rates and observing changes of the biofilm structure *in situ* by confocal laser scanning microscopy (CLSM) using an established biofilm of *Pseudomonas fluorescence*. The sequential treatment with O_3, 1.0–1.7 mg/L, followed by H_2O_2, 0.8–1.1%, showed synergistic disinfection effects, while the reversed treatment, first H_2O_2 followed by O_3, showed only an additive effect. The decrease of synergistic disinfection effect by addition of methanol (CH_3OH), a scavenger of hydroxyl radical (•OH), into the H_2O_2 solution suggested generation of hydroxyl radicals on or in the biofilm by the sequential treatment with O_3 followed by H_2O_2. The primary treatment with O_3 increased disinfection rates of H_2O_2 in the secondary treatment, and the increase of O_3 concentration enhanced the rates. The cold temperature of O_3 water (14 °C and 8 °C) increased the synergistic effect, suggesting the increase of O_3 adsorption and hydroxyl radical generation in the biofilm. CLSM observation showed that the sequential treatment, first with O_3 followed by H_2O_2, loosened the cell connections and thinned the extracellular polysaccharides (EPS) in the biofilm. The hydroxyl radical generation in the biofilm may affect the EPS and biofilm structure and may induce effective disinfection with H_2O_2. This sequential treatment method may suggest a new practical procedure for disinfection and removal of biofilms by inorganic oxidants such as O_3 and H_2O_2.

1. Introduction

The formation of bacterial biofilms and their removal by disinfection has become an important subject in water quality management of swimming pools, food processing lines, industrial water systems, etc. Since microorganisms in a biofilm are protected by matrices of extracellular polymeric substances and are more tolerant to antibiotics and biocides than planktons, the importance of tests using a biofilm system has been pointed out for evaluation of disinfection efficacy as a biofilm disinfectant. Among the oxidants used for disinfection, O_3 has been given attention because of its excellent oxidation power. In the previous study (Tachikawa et al., 2009), we evaluated the disinfection efficacy of O_3 water on biofilms established from ubiquitous bacteria, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. The results indicated O_3 water to be effective for biofilm disinfection; however, it required much

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higher concentrations of O_3 than those for planktonic cells (EPA, 1999; Viera et al., 1999) and showed tail-off disinfection curves suggesting the formation of physical hindrance to O_3 penetration in biofilms. In order to use O_3 water for disinfection of biofilms, means to raise its efficiency are anticipated.

In the field of water treatment, the advanced oxidation process (AOP) has been utilized to increase O_3 decomposition by adding H_2O_2 to generate a highly reactive hydroxyl radical (•OH) (EPA, 1999). Though radicals show synergistic oxidation effects on organic solutes in water, it is known that the microbicidal activity of the radicals is greatly affected by the O_3 dose, H_2O_2/O_3 ratio, contact time, source water quality and type of microorganisms tested (Wolfe et al., 1989; Labatiuk et al., 1994). The very short life time of free radicals made it difficult to apply the AOP system directly to biofilm disinfection and removal. However, it may be considered that, if free radical generation occurs on or in biofilms, AOP may give effects on the structure of biofilms and on its disinfection efficacy. Therefore, the sequential use of O_3 and H_2O_2 for disinfection and removal of biofilms was studied by determination of disinfection rates and by observation of structural changes of biofilms by confocal laser scanning microscopy (CLSM) using an established biofilm of *P. fluorescens* (Tachikawa et al., 2005).

2. Materials and methods

2.1. Bacterial strains and biofilm preparation

P. fluorescens (JCM no. 2779) was obtained from the Japan Collection of Microorganisms (JCM) in Riken Bioresource Center, Tsukuba, Japan. Biofilms of *P. fluorescens* were grown on clean and sterile small glass slides (14 × 26 mm) according to our established method as described elsewhere (Tachikawa et al., 2005). The glass slides (20 plates) were placed in a glass culture dish (i.d. 145 mm) holding 150 mL of growth medium containing 1% of glucose and phosphate and a small amount of minerals (LeChevallier et al., 1988). The dish was inoculated with a culture of *P. fluorescens*, and incubated at 28 °C with continuous slow stirring with magnetic stirrers for 2 nights. The number of viable cells in the biofilm formed on the glass slide was determined by colony counting on tryptone glucose yeast agar (Standard Methods, 1998a) following ultrasonic dispersion and serial dilution. In the present study, the mean cell density of the biofilm was $1.1 \pm 0.5 \times 10^9$ cfu/slide.

2.2. Reagents

Hydrogen peroxide (30%) and methanol (CH_3OH, special grade) were obtained from Wako Pure Chemical Industry, Osaka, Japan. Fluorescence dyes, Alexa Fluor® 633 conjugate concanavalin A (AlexaFluor® 633-ConA) and *LIVE/DEAD BacLight*™, were obtained from Invitrogen™ Corp. CA. Other reagents used were all of the reagent grade.

2.3. Water

Milli Q® ultrapure water was used for preparation and dilution of the reagent solutions for the determination of ozone and H_2O_2 concentrations. For preparation of O_3 water (0.45–1.72 mg/L), tap water distributed by the Funabashi municipal water supply (pH 7.4) was dechlorinated by passing it through an activated carbon column and then led to an O_3 water generator (AOD-TH, Ai Electronic Ind. Co. Ltd., Japan). An O_3 water generator was available for continuous supply of O_3 water at a constant concentration. Since O_3 water was too unstable to maintain a constant concentration during treatment, we used a flow-through system for O_3 treatment as we used in the previous study (Tachikawa et al., 2009). Hydrogen peroxide solutions (0.92 mg/L and 0.13–1.1%) and a CH_3OH solution (0.3 M, 0.96%) were prepared by dilution with sterilized Milli Q water in order to avoid the effects of chlorine and other solute in water. The pH of 1% H_2O_2 solution was 5.4.

Concentrations of residual O_3 in the test water were determined by the indigo colorimetric method (Standard Methods, 1998b). Since the concentrations of O_3 in the water were higher than 0.3 mg/L, an accurate volume (10–20 mL) of fresh O_3 water was added into a volumetric flask containing indigo reagent II and the resulting mixture was diluted to 100 mL with Milli Q water. The optical absorbance of the solution was measured at 600 nm in 1-cm cells, and the concentration of O_3 was calculated according to the equation described below,

$$\text{mg } O_3/\text{L} = (100 \times \Delta A)/(0.42 \times b \times V)$$

where ΔA = difference in absorbance between the sample and blank, b = path length of cell in cm (1 cm), and V = volume of the sample in mL.

H_2O_2 in the test water was determined by a modified iodometric method for chlorine (Standard Methods, 1998c). One to 10 mL of test water was added into the flask with a ground stopper containing 10 mL of Milli Q water, 5 mL of 10% H_2SO_4 and 5 mL of 10% KI solution. The mixed solution was kept standing for 20 min away from direct sunlight. After a dilution with 20 mL Milli Q water, the solution was titrated with 0.1N $Na_2S_2O_3$ solution using starch solution as an indicator until the blue color disappeared. One mL of 0.1N $Na_2S_2O_3$ solution (factor 1.000) is equivalent to 1.701 mg H_2O_2.

2.4. Disinfection of biofilms by the sequential treatment with O_3 and H_2O_2

Effects of the sequential treatment with O_3 and H_2O_2 on biofilms were evaluated as follows. The biofilms established on a glass slide were immersed in sterilized water twice to remove planktonic cells. Then the biofilms were treated with O_3 water (1.3 mg/L) in a flow system and with H_2O_2 water (1.1%) in a static system for indicated times (10 or 120 s) in the following combinations: (A), treated only with H_2O_2; (B), treated only with O_3; (C), first with H_2O_2 followed by O_3; (D), first with O_3 followed by H_2O_2. For elucidation of the mechanism of the synergistic effect, the following experiments were done: I, the biofilms were treated sequentially with O_3 (1.7 mg/L) and then with a H_2O_2 solution (0.75%) containing CH_3OH (0.96%) as a scavenger of hydroxyl radicals for indicated times; II, the biofilms were sequentially treated first with various concentrations of O_3 (0.45–1.5 mg/L) and then with H_2O_2 (0.13–1.05%) for indicated times. After the treatments, the biofilms were