DISINFECTION OF *Escherichia coli* IN SEAWATER USING PRESSURIZED CARBON DIOXIDE

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ABSTRACT

Treatment of shipping ballast water is important for controlling the dispersal of alien species into a new marine environment. This study presents the results of using pressurized gases of CO_2 (< 1.0 MPa) to inactivate *E. coli* in seawater. Bactericidal effects of the pressurized gases were observed in a liquid film-forming apparatus under varied conditions of pressure, temperature and working volume ratio (WVR). In addition, a comparison of bactericidal performance of CO_2 gas and N_2 gas at high pressure against *E. coli* in seawater was conducted, which showed CO_2 treatment was more efficient than that N_2. At 0.7 MPa, 20 °C, 10^5 CFU/mL of initial concentration, and 50 % of WVR, more than 5.0 log of *E. coli* was completely inactivated within 5 min of CO_2 treatment, while it took 25 min to achieve 5.1 log reduction with N_2 treatment. These results indicate that the use of pressurized CO_2 bubbles could be a potential technique for ballast water disinfection.

Keywords: bactericidal effect, ballast water, disinfection, *Escherichia coli*, pressurized CO_2

1. INTRODUCTION

Uptake and discharge of ballast water from vessels have been recognized as a vector for the introduction and movement of aquatic invasive species on over the world [1]. The risks of the introduction of the invasive species have been recorded in many aspects of ecology, economy and human health [1]. In response to these problems, in 2004, the International Marine Organization (IMO) established standards and procedures for the management and control of ship ballast water and sediment [2]. Following the regulatory regimes, ships are required to limit the number of viable organisms in ballast water before it can be discharged into the sea [2].

Several disinfection technologies have been applied for the treatment ballast water. Chlorination and ozonation are commonly disinfection methods because of their affective at killing microorganisms, unfortunately, it produces undesired by-product and risks to ecosystem

[3-4]. UV radiation does not have problems associated with residual toxicity, however their inactivation capability is reduced for water with high turbidity or high concentration of dissolved organic matter [4]. Currently, no single method can adequately fulfill the requirements of the D-2 ballast water performance standard [5].

Disinfection by using high pressure carbon dioxide (HPCD) is considered as a potential method for controlling harmful microorganisms. Originally, HPCD method has been widely used for food preservation and sterilization [6-7]. Recently, HPCD has been found to effectively inactivate pathogens in water and wastewater without problems of disinfection by-products [8-10]. Kobayashi et al. [8] reported that HPCD treatment of wastewater eliminated *Escherichia coli* within 13 min, but required application of supercritical CO_2 under high pressure (up to 10 MPa) and high temperature (55 °C). Vo et al. [10] showed that *E. coli* could be inactivated within 25 min by application of low pressurized CO_2 (below 1.0 MPa) at room temperature. Previous studies on HPCD treatment were conducted using distilled water or low-level saline liquids (≤ 9 ‰) as the suspension medium. The efficacy of HPCD treatment for disinfecting seawater has not yet been studied.

This research investigated the bactericidal performance of pressurized CO_2 (< 1.0 MPa) for disinfecting *E. coli* (ATCC 11303) in filtered seawater and artificial seawater (34 ‰ salinity). The effects of pressure, temperature, and WVR on the disinfection capability of pressurized CO_2 were assessed. In addition, inactivation of *E. coli* resulting from the pressurized CO_2 treatment was compared to the pressurized N_2 treatment.

2. MATERIALS AND METHODS

2.1. Microorganism preparation and enumeration

E. coli (ATCC 11303) inoculum was prepared by inoculation of 100 µL of bacterial glycerol stock from -80 °C into 100 mL of Luria-Bertani (LB) broth (Wako, Japan) containing 30 g/L sodium chloride. And then it was incubated for 18 h at 37 °C by using a reciprocal shaker rotating at 150 rpm. Cells were harvested and washed three times with 0.9 % (w/v) saline solution by centrifugation (10 min at 8000 × *g* at room temperature) in a CF15D2 centrifuge (Hitachi, Japan).The pellet was re-suspended in saline water to the initial volume 100 mL.

E. coli was enumerated using the plate count technique. Briefly, a series of ten-fold dilutions by using autoclaved artificial seawater at 34 ‰ salinity, and 100 µL of each dilution was plated on LB agar (Wako). Colony-forming units (CFU) were counted after overnight incubation at 37 °C. Each sample was analyzed in triplicate.

2.2. Seawater samples preparation

The artificial seawater was prepared by adding artificial sea salt (GEX, Osaka, Japan) to distilled water to obtain a final salinity of 34 ‰ as measured with a salinity meter (YK – 31 SA, Lutron). As for filtered natural seawater preparation, seawater was taken at Ube ports of Yamaguchi prefecture-Japan on September, 2014. Natural seawater (pH = 8.3, salinity 33 ‰) was filtered through a glass fiber filter (GA-100, Advantec, Toyo), followed by a membrane filter 0.45 µm (Millipore, Ireland). For all experiments, the *E. coli* preparation was diluted in the artificial/filtered seawater to obtain a bacterial concentration of 10^5 CFU/mL, which was used as the initial concentration for all experiments. The pH and temperature of the samples were measured by a pH meter (D-51 Horiba).

2.3. Experiment setup

The treatment system is a stainless steel pressurized chamber with an internal volume of 10 L, which was designed to include a small nozzle and a shield to enable vigorous agitation of the influent for creating bubbles (Fig. 1). To investigate the effect of pressure and temperature on the bacteria in the water, 7 L of seawater was pumped in one shot into the device as the influent. After that, the water sample was pumped and circulated inside the system with a flow rate of 14 L/min (hydraulic retention time, HRT = 0.5 min). The sensitivity of the bacteria to pressurized CO_2 treatment was determined using varying pressures (0.3 - 0.9 MPa), and temperatures (11 - 28 °C) applied for 25 min. To examine the effects of WVR, which is defined as the ratio between the sample volume and the apparatus volume, different sample volumes were used to vary the WVR (50 % to 80 %). During the treatment period, cool water was in contact with the outer wall of the device to maintain the initial temperature of the sample at ± 1.0 °C. The treated water was then collected from a bottom valve of the device. Each experiment was conducted in triplicate.

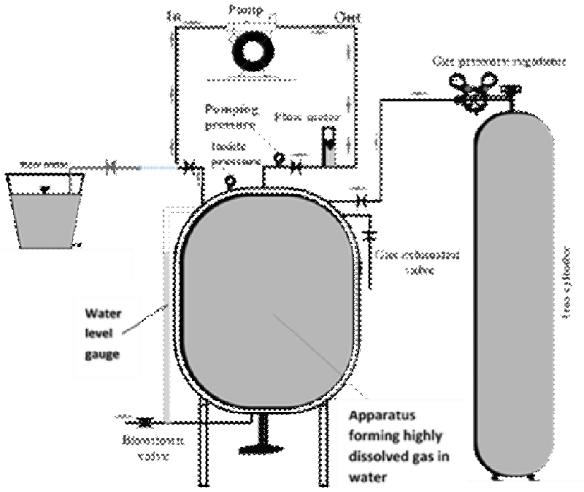

Figure 1. Experimental setup.

3. RESULTS AND DISCUSSION

3.1. Effect of pressure

The effect of using various pressure conditions (range: 0.3 - 0.9 MPa) for inactivating *E. coli* by pressurized CO_2 (concentration: 5.12 - 15.38 g/L, respectively) in comparison with pressurized N_2 (concentration: 0.06 - 0.18 g/L, respectively) is shown in Fig. 2. Overall, the bactericidal activity of both CO_2 and N_2 on *E. coli* increased with increasing pressure, and the disinfection efficiency of CO_2 was always higher than that of N_2.

The bactericidal efficiency of pressurized CO_2 treatment was not difference between filtered seawater and artificial seawater, of which increasing pressure resulted in shorter treatment time to inactivate *E. coli* (Fig. 2). Here, a treatment application period of 25 min was required to reduce the *E. coli* load by approximately 5.0 log with pressure application of 0.3 MPa. Pressure application of 0.5 MPa resulted in reduction of treatment period to 20 min. The treatment period was further reduced to 10 min with pressure application of 0.7 - 0.9 MPa. However, the increase pressure from 0.7 MPa to 0.9 MPa did not result in significant increase in rate of bacterial inactivation in both filtered seawater and artificial seawater. The data indicated that the optimal pressure for this device may be in range of 0.7 – 0.9 MPa. Therefore, an operating pressure of 0.7 MPa was used for subsequent experiments.

In addition, CO_2 reduced pH of both filtered seawater and artificial seawater to around 5.0 after first minute, whereas N_2 did not acidify the sample during treatment process (Fig. 2e). The

decrease in pH caused by CO_2 is considered as a vital factor to the bactericidal performance of pressurized CO_2 treatment [10, 11]. Under low pH conditions, hydrogen ions penetrate the protein coats of cells, dissolve the phospholipid bilayer and alter the physiological feature of proteins [11]. CO_2 may penetrate into the bacterial cell membrane and accumulated into its lipophilic inner layer [11, 12]. The absorbed gas lead to reduce of pH value inside the cells [7], which resulted in metabolic disorder and inactivate *E. coli* [7, 10, 11].

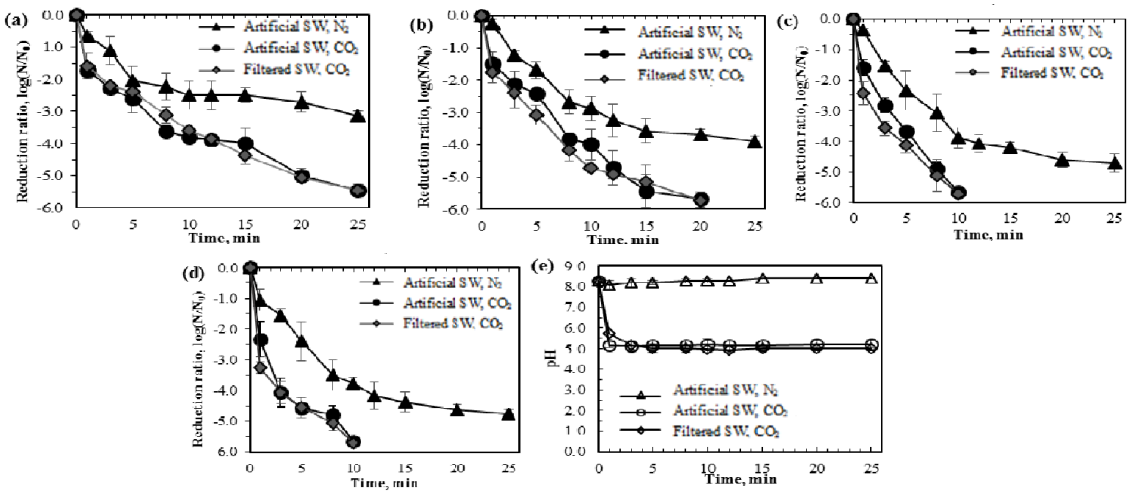

Figure 2. Effect of pressure on inactivating *E. coli* in seawater by pressurized CO_2 and pressurized N_2 on at 20 ± 1.0 °C and 70 % WVR. Inactivation effect at (a) 0.3 MPa, (b) 0.5 MPa, (c) 0.7 MPa, and (d) 0.9 MPa. (e) The influence of CO_2 and N_2 treatment at 0.7 MPa on the pH of seawater.

3.2. Effect of temperature

Inactivation effect of *E. coli* by pressurized CO_2 treatment was investigated at different initial temperatures (11 – 28 °C), at 0.7 MPa with 70% WVR for 25 min (Fig. 3). The results found that the disinfection efficiency increased significantly as the temperature increasing. As show in Fig. 3, the period required for complete inactivation of *E. coli* decreased as the temperature increased. For example, 25 min was required for reducing 5.0 log at 11 °C, whereas no *E. coli* survival after 10 min of the treatment at 20 - 28 °C.

The higher temperature enhanced a better bactericidal efficiency of CO_2. Since CO_2 is both lipophilic and hydrophilic in nature, it can easily penetrate into the phospholipid bilayer of the cell membrane and accumulate there [12]. Thus, we speculate that high temperature and pressure conditions in the bubble forming apparatus may synergistically improve the diffusion of CO_2 in the seawater and may also alter the fluidity of lipids bilayer in the cell membranes to enable efficient penetration of CO_2 into the cells [12]. The results indicated that the disinfection by pressurized CO_2 could be efficiently conducted at room

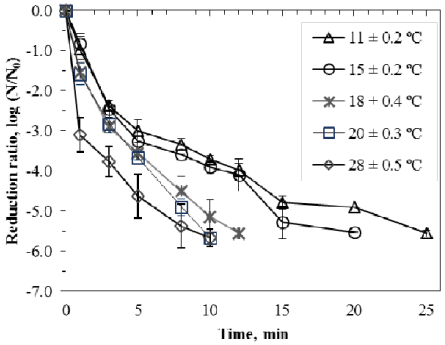

Figure 3. Inactivation effect of *E. coli* in artificial seawater by pressurized CO_2 treatment at various temperature (Δ, 11 °C; ○, 15°C; ӂ, 18 °C; □, 20°C; and ◊, 28°C) at 0.7 MPa, and 70 % WVR is shown.

temperature (20 - 28 °C).

3.3. Effect of working volume ratio

The effect of WVR was investigated using four ratios (50 %, 60 %, 70 % and 80 %) by applying pressure at 0.7 MPa, and temperature of 20 °C with a flow rate of 14 L/min for 25 min (Fig. 4). During this period, there was a slightly decrease in WVR (~ 2 %) due to withdrawal of samples. However, the WVR change was small, and it was therefore assumed that the change does not have a significant influence on the treatment process. As shown in Fig. 4, the inactivation rates significantly increased with decreasing WVR. More than 5.0 log of *E. coli* was completely inactivated within 15 min at 80 % WVR by CO_2 treatment; whereas, 5 min of treatment was required at 50 % WVR. A similar tendency was found in case of N_2 treatment, though disinfection efficiency by N_2 treatment was lower than that by CO_2 treatment. About 5.1 log reduction were achieved within 25 min at 50 % WVR in compared with 4.7 log at 70 % WVR.

Overall, using a smaller WVR results in higher inactivation rate, which is related to the influence of mass transfer of CO_2 in water [13]. In particular, low WVR may lead to a larger space for generation of CO_2 bubbles and for increasing the number of circulation cycles. Consequently, operation of the disinfection apparatus with a low WVR could enhance the solubility of CO_2 in seawater and increase the contact area between CO_2 and bacterial cells.

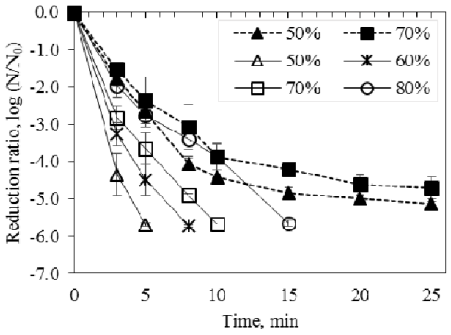

Figure 4. (a) Effect of WVR on pressurized CO_2 treatment (solid line) and pressurized N_2 treatment (dotted line) against *E. coli* in artificial seawater at 0.7 MPa, and 20 ± 0.5 °C, and 10^5 CFU/mL.

4. CONCLUSIONS

Disinfection efficiency of *E. coli* by pressurized CO_2 treatment was always higher than that by pressurized N_2 treatment. In addition, the sensitivity of *E. coli* to pressurized CO_2 treatment was not difference between filtered seawater and artificial seawater. The data indicated that the bactericidal efficiency of pressurized CO_2 on *E. coli* in seawater increased with increasing pressure and temperature, and with decreasing WVR. Treatment application at 0.7 MPa, at 20 °C and at 50 % WVR with an initial bacterial concentration of 10^5 CFU/mL resulted in completely inactivation of *E. coli* within 5 min. These results indicate that pressurized CO_2 showed a high potential apply for controlling pathogens in ballast water.

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