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Comparison of disinfection effect of pressurized gases of CO₂, N₂O, and N₂ on Escherichia coli

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ABSTRACT

Based on the production of gas bubbles with the support of a liquid film-forming apparatus, a device inducing contact between gas and water was used to inactivate pathogens for water disinfection. In this study, the inactivation effect of CO₂ against Escherichia coli was investigated and compared with the effects of N₂O and N₂ under the same pressure (0.3–0.9 MPa), initial concentration, and temperature. The optimum conditions were found to be 0.7 MPa and an exposure time of 25 min. Under identical treatment conditions, a greater than 5.0-log reduction in E. coli was achieved by CO₂, while 3.3 log and 2.4 log reductions were observed when N₂O and N₂ were used, respectively. Observation under scanning electron microscopy and measurement of bacterial cell substances by UV-absorbance revealed greater cell rupture of E. coli following treatment with CO₂ than when treatment was conducted using N₂O, N₂ and untreated water. The physical effects of the pump, acidified characteristics and the release of intracellular substances caused by CO₂ were bactericidal mechanisms of this process. Overall, the results of this study indicate that CO₂ has the disinfection potential without undesired by-product forming.

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1. Introduction

Various studies have been conducted to investigate the use of different disinfectants for inactivation of pathogens in wastewater and water treatment. For nearly a century, chlorine has played a major role in standards for water disinfection in Europe, the United States and other countries around the world. The economic and effective characteristics of chlorination disinfection make it a better choice for treatment than other disinfectants. Unfortunately, during chlorination, the chlorine combines with organic matter to generate carcinogenic by-products. Accordingly, the risks posed to human health by the use of chlorinated drinking water are uncertain at this time. Alternative techniques to improve by-products releasing from the reaction of residual chlorines and ozone with organic materials during conventional disinfection have also been investigated (Richardson, 2011; Steve, 2009). UV disinfection requires a preventive maintenance program and ozone treatment generates undesired disinfection by-products (DBPs) (Guus et al., 2007;
Singer, 1993), while the membrane filtration process does not produce DBPs, but is a complicated disinfection process and quite expensive (EPA, 2001a). Recently, solar disinfection (Mistear and Gill, 2012; Lee et al., 2009), ultrasound (Ayvazidiz et al., 2011) and hydrodynamic cavitation (Mezule et al., 2010) have been reported as potential treatment technologies; however, further studies of alternative disinfectants or disinfection methods are needed. The latest technologies for water disinfection should enhance the advantages of conventional methods and eradicate their shortcomings. This study attempted to accomplish this by transferring the antiseptic properties of carbon dioxide used in food preservation to wastewater and water disinfection.

High pressure CO₂ (4.0–50.0 MPa) has been found to effectively inactivate many types of pathogens (Ballesta et al., 1996; Dillow et al., 1999; Erkmen, 2001; Haas et al., 1989; Kamihira et al., 1987). This method is dependent on contact between a liquid-film and the air (Imai et al., 2008), and involves the application of carbon dioxide to inactivate pathogens as a new disinfectant producing no by-products. More than 100 published journals in the area of food preservation have reported that high pressure CO₂ caused efficient bacterial effects, but an inactivation mechanism has not clearly been understood (Zhang et al., 2006). Thus, few studies have been conducted to investigate the use of pressurized CO₂ to enhance antimicrobial treatment of wastewater and water. Kobayashi et al. (2007, 2009) conducted one of the first studies to investigate the use of CO₂ microbubbles to inactivate Escherichia coli and coliforms within 13.3 min, but a supercritical pressure of 10 MPa and high temperature range (35–55 °C) were needed to achieve the effective results. On the other hand, E. coli disruption by the combination of high temperature (55 °C) with low pressure carbon dioxide at 1 MPa was reported by Klangpetch et al. (2011). However, these studies did not alleviate the need for conventional heat pasteurization. Enomoto et al. (1997) concluded that the depressurization rate at less than 4 MPa led to no mechanical cell rupture, while the only 0.3 MPa was found to cause cell death (Cheng et al., 2011). Therefore, these findings did not reveal whether the chemical nature of CO₂ or depressurization was related to the death of the E. coli cells. Low pH caused by CO₂ dissolution is believed to have a bactericidal effect (García-González et al., 2007), and acidified CO₂ has been found to more easily penetrate cell walls and the intracellular environment of bacteria to inhibit microbial growth (Haas et al., 1989; Hong and Pyun, 2001; Spillimbergo et al., 2002).

This study was conducted to investigate the relationship between the reduction in pH of the liquid environment induced by CO₂ applied at 0.2–1.0 MPa and pathogen inactivation. In addition, nitrogen (N₂) and nitrous oxide (N₂O) were used to provide a basis for comparison of bactericidal mechanisms involved in CO₂ disinfection. Both N₂O and CO₂ have strong solubility in water and similar characteristics; however, CO₂ leads to acidification of the solution and N₂O leads to neutralization. Moreover, N₂ has weak solubility in water. Therefore, these compounds were compared to determine whether CO₂ or N₂O led to its inactivation effects. In addition, the weak solubility of N₂was confirmed. The results of this study could facilitate the application of low pressure CO₂, an innovative bactericidal disinfectant technique, to wastewater and water disinfection.

2. Materials and methods

2.1. Microorganism preparation and enumeration

E. coli (ATCC 11303) from stock cultures (American Type Culture Collection, Manassas, VA, USA) was used as a representative pathogen in this experiment. E. coli was propagated in flasks containing 100 mL Luria-Bertani (LB) broth (Wako Chemical Co. Ltd., Osaka, Japan) at 37 °C with continuous shaking for 16–18 h at 150 rpm. The cell concentrations were then determined by plating aliquots of the culture onto LB agar (Wako) and incubating the samples at 37 °C overnight. The number of colony-forming units (CFU) was subsequently counted on plates that contained 25–300 CFUs/plate. The initial enumeration was approximately 10⁵–10⁷ CFU mL⁻¹ and cell suspensions were maintained in 20% glycerol at −80 °C. All stock cultures were used within one month, and 1–100 mL of E. coli that had been incubated at 37 °C and 150 rpm for 12–18 h was used for each experiment.

2.2. Apparatus and procedure for inactivation

The apparatus used for the experiment was designed to provide a high contact efficacy between the treatment gas and liquid (Imai et al., 2008) (Fig. 1). A nozzle and shield were set up inside the apparatus to strongly agitate the influent water. Highly dissolved treatment gas in water obviously developed inside the device. The initial temperature of 20–22 °C was maintained throughout the experiment.

Microbial suspensions of low (100–200 μL), medium (1–5 mL) or high (50–100 mL) concentration and 7000 mL of distilled water were mixed at room temperature to give the desired concentrations (low: 10⁻¹⁰ CFU/mL, medium: 10⁻⁸–10⁻⁶ CFU/mL and high: 10⁻⁶–10⁻⁸ CFU/mL), after which these mixtures were used as water samples that have been subjected to microbial contamination. Approximately 7000 mL of this wastewater was pumped into the device. During treatment, the flow rate was 13–15 l min⁻¹ and the contact time was 25 min. At the beginning of the experiment, the treatment system, which can tolerate up to 1.0 MPa, was filled with treatment gas at 0.2–1.0 MPa. A blow down valve designed for low or rapid depressurization was used to collect the samples. Performance was judged based on the inactivation of E. coli at various pressures and concentrations. All experiments were conducted in triplicate.

2.3. Inactivation mechanism assessment

The bactericidal mechanism was judged by examining the pressurized microbubbles of N₂, N₂O and CO₂ on E. coli leading to their different inactivation effects. As shown in Table 1, the solubility and nature of N₂ are much different from those of CO₂, whereas N₂O and CO₂ have similar properties in terms of molecular weight, gas density, specific volume, critical pressure and temperature and solubility in water. N₂O and CO₂ have very similar features, with the only major difference being that a reduction in pH is caused by CO₂ (CO₂ + H₂O ⇄ H₂CO₃ ⇄ 2H⁺ + CO₃⁻) but not N₂O (N₂O + H₂O ⇄ H₂N₂O₃). UV-absorbance and scanning electron
microscopy (SEM) were used to evaluate the disinfection mechanism of CO₂.

2.3.1. Measurement of UV-absorbing substances
E. coli cells destroyed by CO₂ released various substances, including nucleic acids and proteins. Therefore, treated samples were centrifuged at 1000× g for 10 min, after which the absorbance of the supernatant at 260 nm and 280 nm was measured by spectrophotometry (Hitachi, Tokyo, Japan) to determine the levels of nucleic acids and proteins, respectively (Kim et al., 2008a).

2.3.2. Scanning electron microscopy
Treated and untreated samples were centrifuged at 8000 rpm for 10 min, after which the supernatant was discarded and the pellet was washed with PBS buffer three times. The samples were then fixed with 2.5–3.0% glutaraldehyde (Wako) in PBS buffer (pH = 7.2) overnight at 4 °C, after which they were immersed in 1% osmium tetroxide and cacodylate buffer for 90 min at room temperature and then dehydrated at 4 °C with sequences of ethanol at 50% (twice for 10 min each), 70%, 80%, 90%, 95% and 100% (three times for 15 min each), followed by EtOH/t-butyl alcohol (v/v = 1/1 for 30 min) (Kim et al., 2008b). Finally, the samples were washed in fresh t-butyl alcohol twice for 1 h each, freeze-dried under low temperature for 3 h (VFD-21S t-BuOH free dryer), covered with gold-palladium and observed by scanning electron microscopy (SEM, Quanta™ 3D, FEI Co.).

### Table 1 – Physical and chemical characteristics of inactivation gases.

<table>
<thead>
<tr>
<th>Properties</th>
<th>CO₂</th>
<th>N₂O</th>
<th>N₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight, g/mol</td>
<td>44.01</td>
<td>44.013</td>
<td>28.013</td>
</tr>
<tr>
<td>Gas density, 1.013 bar</td>
<td>1.87</td>
<td>3.16</td>
<td>1.185</td>
</tr>
<tr>
<td>at 15 °C, kg/m³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific volume, 1.013</td>
<td>0.574</td>
<td>0.543</td>
<td>0.852</td>
</tr>
<tr>
<td>bar at 21 °C, m³/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Critical temperature, °C</td>
<td>31.1</td>
<td>36.42</td>
<td>-147.0</td>
</tr>
<tr>
<td>Critical pressure, MPa</td>
<td>7.3825</td>
<td>7.245</td>
<td>3.3999</td>
</tr>
<tr>
<td>Solubility in water, 0.1013</td>
<td>1.80</td>
<td>1.20</td>
<td>0.018</td>
</tr>
<tr>
<td>MPa, (25 °C), g/L</td>
<td>2.47×10⁻⁵</td>
<td>4.89×10⁻⁵</td>
<td>28.013</td>
</tr>
<tr>
<td>Diffusivity, cm²/s</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Cited from Murat and Giovanna (2012).

3. Results and discussion

3.1. Results

3.1.1. Inactivation effect of CO₂, N₂, and N₂O against E. coli
In this study, CO₂, N₂, and N₂O were evaluated for their inactivation performance. The inactivation efficiency of gases was compared under various pressures. As shown in Fig. 2, the bactericidal effect of CO₂ was higher than that of N₂O and N₂ at every operating pressure. When CO₂ was used, the reduction ratios of E. coli at 0.3 MPa and 0.5 MPa (Fig. 2a and b) were nearly 2.8 log within 25 min, while they were only 0.62–0.90 log and 0.85–1.44 log when N₂ and N₂O were used, respectively. When higher pressure conditions of 0.7–0.9 MPa (Fig. 2c and d) were employed, most gases showed greater E. coli inactivation. Specifically, CO₂ inactivation reached 5.2 log at 0.7 MPa and 4.7 log at 0.9 MPa. Surprisingly, N₂ induced 2.4 and
Fig. 2 – Comparison of bactericidal performance of N₂, N₂O and CO₂ against E. coli (ATCC 11303, initial concentration: 10⁵–10⁶ CFU/mL) inactivation at (a) 0.3 MPa, (b) 0.5 MPa, (c) 0.7 MPa and (d) 0.9 MPa.

2.8 log reductions in E. coli at 0.7 MPa and 0.9 MPa, respectively. N₂O, which has the same molecular weight, critical temperature and pressure, solubility in water and diffusivity as CO₂, but does not change the pH, induced an inhibition effect of 3.33 (0.7 MPa) and 3.69 log (0.9 MPa). As shown in Fig. 3, the change in the pH of water differed in response to treatment with CO₂, N₂O and N₂. Specifically, during the first minute of inactivation, the pH decreased from 8.4 to 4.9 in response to CO₂ treatment, while that of N₂O and N₂ treated water remained stable or increased slightly. These findings indicate that CO₂ may play a crucial role in attenuation of microbial growth.

3.1.2. Inactivation effect of CO₂ at various pressures against E. coli
Owing to the superior bactericidal performance of CO₂, the effects of CO₂ at pressures of 0.2 MPa–1 MPa were investigated. Fig. 4 shows the reduction of E. coli after 25 min, a common period used for water disinfection, for example with chlorine (EPA, 2001b). In contrast to tests conducted at low pressures (0.2–0.6 MPa), which showed a maximum decrease in E. coli of 3.2 log at 0.6 MPa and a minimum decrease of 2.5 log

Fig. 3 – pH change in water in response to CO₂, N₂ and N₂O at 0.7 MPa.
Fig. 4 — Inactivation effect of CO₂ at various pressures against E. coli (ATCC 11303 — initial concentration: 10⁵–10⁶ CFU/mL).

at 0.4 MPa, those at high pressures (0.7–1.0 MPa) showed decreases of 4.2 to >5.2 log. The greatest decrease in E. coli was observed at 0.7 MPa; therefore, subsequent experiments were conducted using 0.7 MPa.

3.1.3. Bactericidal effect of CO₂ against E. coli at 0.7 MPa and UV-absorbance of E. coli cell supernatant
As shown in Fig. 5, the inhibition of E. coli reached 4.7–5.2 log after 25 min at 0.7 MPa. We previously found 20 min to be the most effective period for inactivation (Cheng et al., 2011); however, in the present study, the reduction of E. coli at 20 min was not steady; therefore, 25-min was used for subsequent experiments. The decrease in pH caused by CO₂ (Fig. 3) was considered to be a reason for cell death (Spilimbergo et al., 2002). To confirm that the cells had been lysed, the levels of nucleic acids and proteins were measured based on the absorbance of samples at 260 nm and 280 nm, respectively. As shown in Fig. 5, within the first 10 min, the absorbance increased only slightly, indicating a low inactivation effect. However, the absorbance peaked at 25 min, corresponding to the maximum inactivation.

3.1.4. Inactivation performance of CO₂ at 0.7 MPa against E. coli in samples with different initial concentrations
Fig. 6 shows the inactivation of E. coli under different initial concentrations at 0.7 MPa. When the initial concentration was low (10⁵–10⁶ CFU/mL), no surviving cells were detected after 20 min, whereas samples with moderate initial concentrations showed a decrease of 5.2 log within 25 min. When high initial concentrations (10⁷–10⁸ CFU/mL) were used, the rate of cell reduction only reached 4.5 log after 25 min, but this rate grew steadily for 10 min to over 6.5 log, indicating an approximately 1.0-log reduction/5 min. In general, the bactericidal effectiveness was best at medium concentration.

3.1.5. SEM observation
Cell modifications were observed by SEM analysis of cells treated with N₂, N₂O and CO₂ for 25 min. As shown in Fig. 7a, the E. coli initially appeared healthy. After treatment at 0.7 MPa with pressurized N₂, no or only a few cells appeared

Fig. 5 — Inactivation effect of CO₂ against E. coli (ATCC 11303 — initial concentration: 10⁵–10⁶ CFU/mL) at 0.7 MPa and UV-absorbance of E. coli cell supernatant over time. Light absorbance (OD: optical density) at 260 nm for nucleic acids and 280 nm for proteins.
3.2. Discussion

Overall, the results of this study indicate that the CO$_2$ inactivation mechanism was as follows. The production of gas microbubbles and high pressure enables CO$_2$ to easily penetrate the cell membrane and change the physiological features of E. coli. When non-polar CO$_2$ molecules enter the cell, they impact the structure of the cell wall (Ishchmidt et al., 1995). Moreover, too much dissolved CO$_2$ continuously pumped into a layer of phospholipids may disrupt and change the stability of lipid chains. The accumulation of CO$_2$ also leads to a rapid decrease in intracellular pH (Spillmberg et al., 2005). The buffering capacity of bacteria is limited and increases the proton pumping system (Hutkins and Nannen, 1993), which leads to restraint of the cellular metabolism and important enzymes (Hong and Pyun, 2001; Hutkins and Nannen, 1993; Spillmberger et al., 2002).

Many studies of food preservation and water disinfection have shown that CO$_2$ has the potential to inhibit pathogens (Ishikawa et al., 1995; Haas et al., 1989; Kamihira et al., 1987; Kobayashi et al., 2007, 2009; Enomoto et al., 1997). However, the importance of cell rupture and the physiological mechanism behind such inhibition have been extensively debated. In this study, the bacterial inhibition by CO$_2$ was investigated by comparison with the effects of N$_2$ and N$_2$O treatment. The differences in the following parameters between N$_2$ and CO$_2$ (T$_c$ = $-147^\circ$C, P$_c$ = 3.39 MPa) versus T$_c$ (CO$_2$) = 36.5 $^\circ$C, P$_c$

broken (Fig. 7b). Conversely, cells treated with N$_2$O had rough surfaces and many had broken cell walls (Fig. 7c). No cells could be identified after treatment with CO$_2$, indicating that they had all been lysed (Fig. 7d).

Fig. 7 – E. coli cells under SEM observation (a) untreated (b) N$_2$ treated (25 min, 0.7 MPa), (c) N$_2$O treated (25 min, 0.7 MPa) and (d) CO$_2$ treated (0.7 MPa, 25 min).
(CO₂) = 7.24 MPa) led to various bactericidal effects. N₂ induced little or no bactericidal effect at low pressure (0.2–0.6 MPa), but did show a bactericidal effect at 0.7 MPa. Nevertheless, these effects were much lower than those induced by CO₂ under the same conditions. The solubility of N₂ may prevent it from modifying bacterial cells during treatment, which would explain the greater effect observed at higher pressures. Indeed, SEM analysis confirmed that some cells were sheared by high pressure forces, but that the shapes of E. coli were unchanged.

N₂O has a similar molecular weight, solubility in water, critical temperature, and critical pressure as CO₂. Despite these similarities, treatment with N₂O produced less effective inactivation than treatment with CO₂. N₂O did not acidify the treated water, while CO₂ reduced the pH to nearly 4 during the first minute. Nevertheless, N₂O had a greater bactericidal effect than N₂. The anesthesia and non-polar characteristics of N₂O enable it to be easily dispersed into the phospholipid layer of cell membranes with the support of high pressure (Spilimbergo et al., 2002). This may lead to dissolution of fatty sections, changes in the activity of the cells and obstruction of the bacterial growth. Indeed, E. coli cells were peeled and lysed in response to treatment with N₂O (Fig. 7c).

The reduction in pH induced by treatment with CO₂ was likely the mechanism through which CO₂ attenuated E. coli. The bacterial deaths caused by CO₂ were inhibited to the same degree. SEM images of treated cells confirmed the superior treatment performance of CO₂. The cell membranes of E. coli were severely damaged and their initial structures were unrecognizable. Analysis of the absorbance of the samples revealed that nucleic acids and proteins had been extracted from the E. coli cells. These findings are in accordance with those of previous investigations in the field of food preservation (Ertmen, 2001; Ishikawa et al., 1995; Haas et al., 1989; Kamihira et al., 1987) and were further confirmed in water disinfection by comparison with the results of the N₂ and N₂O experiments. After exposure for a sufficient time, bacterial cells were easily damaged and lysed (Fig. 7d). Although the discharge of water appeared to change the pressure of the cells, this was likely not responsible for most bacterial deaths, and rapid or slow depressurization was not the principal factor involved in the inactivation effect (Enomoto et al., 1997).

Conversely, the inactivation effect of N₂ was merely due to physical factors (pressure and pump cycling), while that of N₂O was primarily in response to a combination of physical factors, and to a lesser degree, its ability to penetrate and dissolve the cells via its chemical properties.

The optimal conditions for CO₂ treatment were found to be 0.7 MPa and 25 min at room temperature. Cheng et al. (2011) found that a 20-min period was sufficient for bacterial inactivation, but in the present study, the cells were lysed after extending treatment time to 25 min because this was determined to be the point at which CO₂ accumulation inside the cells surpassed their limitations. Residual CO₂ after treatment may diffuse to air and gradually recover neutral pH later.

• When compared with those of N₂O and N₂, the bactericidal effect of CO₂ was much greater. Additionally, operation of the apparatus at higher pressure (0.7–1.0 MPa) led to a more prominent reduction of E. coli, as compared with operation at 0.2–0.6 MPa.
• The decrease of pH in water induced by treatment with CO₂ is considered to be the most effective factor leading to its bactericidal effects.
• A pressure of 0.7 MPa, room temperature and an exposure time of 25 min were determined to be the optimum operating conditions for the treatment of artificial wastewater when E. coli were the target pathogens.

Overall, CO₂ has the potential for use as a disinfectant of wastewater and drinking water with low and medium concentrations of E. coli. Furthermore, this method does not produce disinfection by-products, resulting in reduced health risks and operation costs. Further research is needed to confirm the disinfection effect of CO₂ toward bacteriophages and to fully elucidate the role of intracellular pH.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.04.053.

REFERENCES


4. Conclusion

Using microbubbles of pressurized CO₂, N₂ and N₂O to inactivate E. coli (ATCC 11303) revealed the following:


