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Efficacy of gaseous chlorine dioxide in inactivating *Bacillus cereus* spores attached to and in a biofilm on stainless steel

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ABSTRACT

We evaluated the lethal activity of gaseous chlorine dioxide (ClO_2) against *Bacillus cereus* spores attached to and in biofilm formed on a stainless steel surface. Aqueous ClO_2 was prepared by mixing sulfuric acid (5% w/v) with sodium chlorite (10 mg/mL), and gaseous ClO_2 was produced by vaporization of aqueous ClO_2 in an air-tight container. The concentration of gaseous ClO_2 in the air within the container increased rapidly at first but gradually decreased over time. The lethality of gaseous ClO_2 against *B. cereus* spores attached to stainless steel coupons (SSCs) and in biofilm formed by the pathogen on SSCs was determined. The *B. cereus* spores attached to SSCs (5.3 ± 0.1 log CFU/coupon) were completely inactivated within 1 h at 25 °C when treated with gaseous ClO_2 (peak concentration: 115.3 ± 5.0 parts per million [ppm]). The total number of vegetative cells and spores in biofilm formed by *B. cereus* on SSCs was 5.9 ± 0.3 log CFU/coupon; the spore count was 5.3 ± 0.1 log CFU/coupon. The vegetative cells and spores in biofilm were completely inactivated within 6 h (peak concentration: 115.3 ± 5.0 ppm). Results show that *B. cereus* spores in biofilms are more resistant to gaseous ClO_2 than are attached spores not in biofilms. Gaseous ClO_2 was, nevertheless, very effective in killing *B. cereus* spores in biofilm on the surface of stainless steel. Results show promise for application of gaseous ClO_2 to enhance the microbiological safety of foods that may come in contact with stainless steel and possibly other hard surfaces on which *B. cereus* biofilms have formed.

1. Introduction

Chlorine dioxide (ClO_2) is a powerful oxidizing agent that is 2.5-fold more effective than chlorine as an antimicrobial and is less corrosive (Marriott and Gravani, 2006). It exerts bactericidal activity over a broader pH range (pH 3.0 to 8.0) compared to sodium hypochlorite, which has markedly reduced activity with an increase in pH toward neutrality (Junli et al., 1997). Moreover, ClO_2 does not produce dioxins or trihalomethanes upon reaction with ammonia (Knapp and Battisti, 2001). It can be used as a sanitizer in either aqueous or gaseous form and has been widely used to control hazardous bacteria present in water and food and on a variety of surfaces in processing environments.

Aqueous ClO_2, the most widely used form as a sanitizer, has the advantage of being easy to produce and handle compared to gaseous ClO_2. However, residual moisture on food and food-contact surfaces may promote the growth of molds after treatment with aqueous ClO_2 (Sy et al., 2005b; Trinetta et al., 2011; Wu and Kim, 2007). Compared to aqueous ClO_2, gaseous ClO_2 has some advantages as a sanitizer. The gaseous form may result in smaller amounts of ClO_2 residues on food or food-contact surfaces and, because of its superior ability to penetrate, it is more effective in killing hazardous microorganisms in biofilm formed on various types of surfaces (Han et al., 2001; Knapp and Battisti, 2001).

Bacillus cereus is a Gram-positive, facultatively anaerobic, spore-forming bacterium commonly found in foods and natural environments. It can cause foodborne diseases via the production of diarrheal and emetic toxins (Ehling-Schulz et al., 2004; Granum and Lund, 1997). A prominent feature of *B. cereus* is that the bacterium produces spores. The spores are much more resistant than vegetative cells to heat treatment and chemical sanitizers, resulting in survival for long periods of time in foods and on food-contact surfaces (Kreske et al., 2006b; Ryu and Beuchat, 2005). *B. cereus* is known to form biofilms on the surface of stainless-steel, and cells can sporulate in biofilms when exposed to an atmosphere with high relative humidity (RH) (Ryu and Beuchat, 2005). Biofilms formed by *Bacillus* species on food-contact surfaces can be a source of contamination during processing of foods. Flint et al. (1997), for example, reported that biofilm formed by *Bacillus* spp. on contact surfaces in a dairy product plant was not easily removed and could act as a source of contamination of products and other surfaces. Andersson et al. (1995) observed that *B. cereus* spores remained

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attached to equipment surfaces after pasteurization of milk and cleaning. They concluded that, if dairy products produced using such equipment are stored at inappropriate temperatures, surviving spores may germinate and spoilage may occur.

Several attempts have been made to use gaseous ClO_2 to remove hazardous and spoilage microorganisms on foods and food-contact surfaces. Minimally processed fruits and vegetables have been a principal target in food research. Gaseous ClO_2 has been shown to be antimicrobial against *Salmonella enterica* inoculated onto the skin of Roma tomatoes (Trinetta et al., 2010), blueberries, strawberries, and raspberries (Sy et al., 2005a), and lettuce leaves (Mahmoud and Linton, 2008). Treatment of fresh-cut produce with gaseous ClO_2 has been shown to reduce *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Sy et al., 2005b). The effectiveness of gaseous ClO_2 in preventing growth of spoilage microorganisms on minimally processed carrots, lettuce, and cabbage has been studied although treatment of gaseous ClO_2 showed only limited effects in shelf life extension on those produces (Gómez-López et al., 2007, 2008). For the sanitization of food-contact surfaces, Han et al. (1999) reported that gaseous ClO_2 was lethal against spoilage microorganisms on aseptic juice storage tanks. Trinetta et al. (2012) observed that gaseous ClO_2 effectively reduced the number of *L. monocytogenes* on equipment used to process ready-to-eat products. However, we are not aware of studies reporting the lethality of gaseous ClO_2 against *B. cereus* spores attached to or in biofilms on stainless steel surfaces.

In the present study, we evaluated the efficacy of gaseous ClO_2 in killing *B. cereus* spores on stainless steel coupon (SSC) surfaces. We used an air-tight container to retain gaseous ClO_2 and treat *B. cereus* spores attached to and in biofilms on SSCs.

2. Materials and methods

2.1. Bacterial strains and preparation of B. cereus spores

Five strains of *B. cereus* were used: strain ATCC 21366 (isolated from soil), C1 (isolated from pasta), F4616A/90 (isolated from pasteurized milk), F4810/72 (isolated from cooked rice), and 038-2 (isolated from infant formula). Cryopreserved cells of these five strains were separately activated in 10 mL of tryptic soy broth (TSB; BBL/Difco, Sparks, MD, USA) incubated at 30 °C for 24 h. Activated cultures of each strain were transferred into 10 mL of TSB using loop inocula (ca. 10 μL) three times at 24-h intervals before using as inocula for each experiment.

Suspensions of *B. cereus* spores were prepared using methods described by Kreske et al. (2006a), with minor modifications. To trigger spore formation, each culture (0.25 mL) was spread-plated on four tryptic soy agar (TSA; BBL/Difco) plates and incubated at 30 °C for 72 h. Cultures were confirmed by microscopic observation to consist of more than 90% spores. Vegetative cells and spores in lawns formed on several plates were gently detached using a sterile microspatula and placed in 50-mL conical tubes (SPL Life Sciences, Pocheon, Republic of Korea) containing 30 mL of sterile distilled water. The tubes were vortexed at maximum speed for 1 min and centrifuged (800 ×*g* for 10 min at 22 ± 2 °C). The supernatants were decanted and spores in the pellets were resuspended in 30 mL of sterile distilled water. The pellets were washed two additional times, resuspended in 30 mL of sterile distilled water, vortexed at maximum speed for 1 min, and stored at 4 °C until used.

2.2. Preparation of stainless-steel coupons

Stainless steel coupons (SSCs; type 304, 5 cm by 2 cm, no. 4 finish) were immersed in 300 mL of 15% (v/v) phosphoric acid solution, sonicated at 70 °C for 20 min in a water bath (model JAC-1505, KODO Technical Research Co. Ltd., Hwaseong, Republic of Korea), and rinsed with sterile distilled water. The SSCs were then immersed in 300 mL of 15% (v/v) alkaline detergent solution (FS Pro-Chlor, Zep, Atlanta, GA, USA), sonicated at 70 °C for 20 min, and rinsed with distilled water. The SSCs were placed in a 500-mL beaker and dry sterilized in an autoclave.

2.3. Production of gaseous ClO_2

Aqueous ClO_2 was prepared by adding sodium chlorite (10 mg/mL) to a sulfuric acid solution (5% w/v); gaseous ClO_2 was generated spontaneously. A cylindrical air-tight container (1.8 L; 142 mm diameter by 186 mm high; LOCK & LOCK Co. Ltd., Seoul, Republic of Korea) was used to collect gaseous ClO_2. The container consisted of three components structurally designed to produce gaseous ClO_2, treat SSCs on which *B. cereus* spores were attached or in biofilms, and measure the concentration of gaseous ClO_2 (Fig. 1). The reaction solution (0.33 mL) was deposited in a Petri dish placed in the ClO_2 production component and the container was sealed. The concentration of gaseous ClO_2 produced was measured using a gas detector pump and tube (model 8 H; Gastec Corporation, Tokyo, Japan). The detector tube marked the concentration of ClO_2 by color change (from white to vermillion) and the concentrations of ClO_2 were calculated by multiplying the correction factor (0.5) by the manufacturer's speculation.

2.4. Inactivation B. cereus spores attached to SSCs using gaseous ClO_2

2.4.1. Attachment of B. cereus spores to SSCs

A five-strain mixture of *B. cereus* spores (25 mL, ca. 7.6 ± 0.1 log CFU/mL) was prepared by combining 5 mL of each of the five spore suspensions in a 50-mL conical centrifuge tube. The suspension was centrifuged (2000 ×*g* for 15 min at 22 ± 2 °C), supernatants were decanted, and spores were resuspended in 25 mL of sterile distilled water. The suspension (20 mL) was diluted in sterile distilled water (980 mL) to yield a spore population of ca. 6.0 log CFU/mL. To attach spores onto SSC surfaces, the spore suspension (30 mL) and a sterile SSC were placed in a 50-mL centrifuge tube and incubated at 22 °C for 4 h. The SSCs on which spores were attached were rinsed in sterile distilled water (500 mL) by gently moving in a circular motion for 15 s using sterile forceps, dried at 22 ± 2 °C for 2 h in a laminar flow biosafety cabinet, and used in experiments.

2.4.2. Treatment of B. cereus spores attached to SSCs with gaseous ClO_2

A solution containing 5% (w/v) sulfuric acid and sodium chlorite (10 mg/mL) was prepared as described above, and 0.33 mL was deposited in a Petri dish which had been placed in the gaseous ClO_2 production compartment of the air-tight container (Fig. 1). A sterile lid with a wire-screened ventilation hole (40 mm in diameter) was placed on the top of the Petri dish. The SSCs containing attached *B. cereus* spores were placed over the hole using sterile forceps, and the container was tightly sealed. The SSCs were exposed to gaseous ClO_2 at 25 °C for 0 min, 10 min, 20 min, 30 min, 1 h, 2 h, and 6 h. At each sampling time, SSCs were transferred to centrifuge tubes containing 30 mL of 0.1% (w/v) peptone water and 3 g of glass beads (425–600 μM diameter; Sigma-Aldrich, St. Louis, MO, USA), and vortexed at maximum speed for 1 min. Undiluted suspensions (0.25 mL in quadruplicate and 0.1 mL in duplicate) and suspensions serially diluted in 0.1% (w/v) peptone water (0.1 mL in duplicate) were spread-plated on TSA. The number of colonies formed after incubation at 30 °C for 24 h was counted. The remaining portions of suspensions were poured into 250-mL bottles containing 100 mL of TSB and enriched by incubation at 30 °C for 24 h. When no colonies formed on TSA, the enriched suspension was streaked on Mannitol Egg Yolk Polymyxin agar (MYPA; Hangang, Gunpo, Republic of Korea) and TSA, followed by incubating at 30 °C for 24 h. The detection limits for *B. cereus* spores using direct plating and enrichment were 1.5 log CFU/coupon (30 CFU/coupon) and 0.0 log CFU/coupon (1 CFU/coupon), respectively.

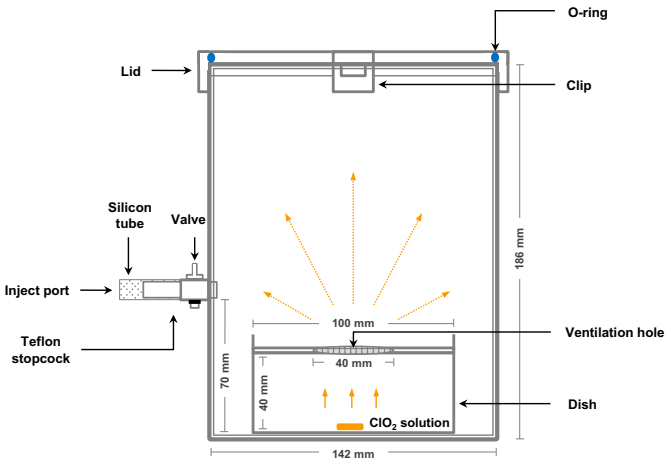

Fig. 1. Air-tight container used to collect gaseous ClO_2 produced by a mixture of sulfuric acid and sodium chlorite for use in treating stainless steel coupons on which *B. cereus* spores were attached or in biofilms.

2.5. Inactivation of *B. cereus* spores in biofilms formed on SSCs using gaseous ClO_2

2.5.1. Biofilm formation and subsequent sporulation of *B. cereus* on SSCs

Biofilms of *B. cereus* were formed on SSCs using methods described by Ryu and Beuchat (2005). Briefly, after three consecutive transfers of five *B. cereus* strains, each culture (0.1 mL) was inoculated into TSB and incubated at 22 °C for 48 h. To prepare a five-strain *B. cereus* suspension (250 mL), cultures of each strain (50 mL) were combined and centrifuged at 2600 ×*g* for 10 min at 22 ± 2 °C. The supernatant decanted and the cells were resuspended in 250 mL of phosphate-buffered saline (PBS, pH 7.4). The suspension was combined with PBS (1750 mL) to give a population of ca. 6.0 log CFU/mL. To attach *B. cereus* cells (vegetative cells plus spores) to SSC surfaces, 30 mL of suspension and a sterile SSC were placed into a 50-mL centrifuge tube and incubated at 22 °C for 4 h. The SSCs on which *B. cereus* was attached were rinsed in 500 mL of sterile distilled water by gentle moving in a circular motion for 15 s using sterile forceps, then transferred to 50-mL centrifuge tubes containing 30 mL of TSB and incubated at 22 °C for 24 h to allow the biofilm formation.

Sporulation of *B. cereus* cells in biofilms on SSC surfaces was induced using methods described by Ryu and Beuchat (2005). Briefly, SSCs on which *B. cereus* biofilm had formed were rinsed in 500 mL of sterile distilled water and transferred to a 50-mL centrifuge tube in an atmosphere of 100% RH. To create a 100% RH, sterile distilled water (0.8 mL) had been deposited in a 50-mL centrifuge tube and incubated at 22 °C for at least 48 h before the SSCs containing biofilm were positioned above the water. Tubes were sealed and SSCs were incubated at 22 °C with 100% RH for 4 days before transferring to centrifuge tubes containing 30 mL of 0.1% peptone water and 3 g of glass beads. The SSCs and peptone water were vortexed at maximum speed for 1 min. To measure the number of total cells (vegetative cells plus spores), the suspension was serially diluted in 0.1% peptone water and spread-plated (0.1 mL in duplicate) on TSA. To determine the number of spores, suspensions (4 mL) were heated at 80 °C for 10 min, serially diluted in 0.1% peptone water, and spread plated (0.1 mL in duplicate) on TSA. Plates were incubated at 30 °C for 24 h before counting the numbers of colonies.

2.5.2. Treatment of *B. cereus* biofilms on SSCs with gaseous ClO_2

A mixed solution (0.33 mL) of 5% (w/v) sulfuric acid and 10 mg/mL sodium chlorite was placed in a Petri dish which had been placed inside the air-tight container and covered with a sterile lid containing a wire-screened ventilation hole. SSCs on which *B. cereus* biofilms had formed were placed over the hole using a sterile forceps, and the container was tightly sealed. The SSCs were exposed to gaseous ClO_2 at 25 °C for 0 min, 30 min, 1 h, 2 h, and 6 h, and populations of *B. cereus* (vegetative cells plus spores) present on SSC surfaces were determined as described above.

2.6. Statistical analysis

All experiments were performed at least three times and two SSCs were evaluated in each replication. Data were analyzed using the general linear model of a statistical software package (SAS 9.3; SAS Institute, Cary, NC, USA). The concentration of gaseous ClO_2 in the air-tight container, changes in the number of viable spores attached to SSCs by treatment time when exposed to gaseous ClO_2, and changes in the number of viable cells in biofilms formed on SSCs by treatment time when exposed to gaseous ClO_2 were analyzed by Fisher's least significant difference (LSD) test to determine if differences were significant ($P \leq 0.05$).

3. Results and discussion

3.1. Production of gaseous ClO_2

Fig. 2 shows the amount of spontaneously produced ClO_2 gas over 6 h at 25 °C in an air-tight container containing a sulfuric acid and sodium chlorite solution. The concentration of gaseous ClO_2 reached a maximum of 115.3 ± 5.0 parts per million (ppm) within 1 h and decreased gradually thereafter to 56.7 ± 5.8 ppm after 6 h.

Gómez-López et al. (2009) demonstrated that the concentration of gaseous ClO_2 in an experimental system could increase, decrease, or maintain, depending on the experimental design. If gaseous ClO_2 is continuously generated, the concentration will continue to increase. If not, the concentration of gaseous ClO_2 will decrease due to reaction of the gas with organic material that may be present, spontaneous degradation, and absorption into the experimental surface. These researchers stated that if the amount of sample to be treated was very small, the concentration of gaseous ClO_2 can be maintained. Gómez-López et al. (2007) and Han et al. (2003) used systems in which the concentration of gaseous ClO_2 decreased during treatment. They evaluated the effect of gaseous ClO_2 on the shelf-life of minimally processed carrots. An air stream containing gaseous ClO_2 was injected into an experimental system containing carrots for 30 s during which the concentration of gaseous ClO_2 increased to a maximum of 1.33 mg/L after 1 min, then gradually decreased thereafter. Han et al. (2003) examined the concentration of gaseous ClO_2 in an Irvine plexiglass cylinder (10 L) containing materials with various types of

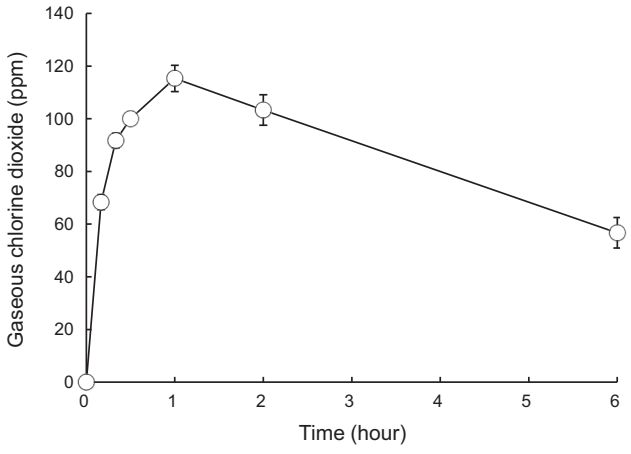

Fig. 2. Production of gaseous ClO_2 from a sulfuric acid and sodium chlorite solution (sulfuric acid [5% w/v] plus sodium chlorite 10 mg/mL) in an air-tight container at 25 °C. Bars indicate standard deviations.

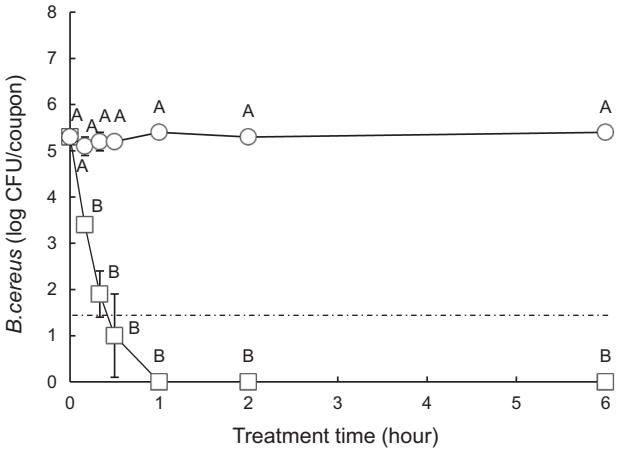

Fig. 3. Number of *Bacillus cereus* (a five-strain mixture) spores on SSCs treated with gaseous ClO_2 produced by a sulfuric acid and sodium chlorite solution (sulfuric acid [5% w/v] plus sodium chlorite 10 mg/mL) at 25 °C for up to 6 h. Key: Control (○), gaseous ClO_2 treatment (□). At a given exposure time, values not annotated with the same letter are significantly different ($P \leq 0.05$). The detection limit for direct plating was 1.5 log CFU/coupon (the horizontal dotted line). The detection limit upon enrichment was 0.0 log CFU/coupon. To plot the numbers of *B. cereus* cells on the SSCs, the number of cells was considered as 1.5 log CFU/coupon when cells were not detected on agar plates but detected after enrichment. If cells were not detected after enrichment, it was considered as 0.0 log CFU/coupon. Bars indicate standard deviations.

surfaces. They injected gaseous ClO_2 into the cylinder and measured concentrations at 0, 6, and 12 h. The initial concentration of gaseous ClO_2 (30 mg/L) decreased by 80% (to 6 mg/L) at 6 h and by more than 90% (to 2 mg/L) at 12 h. This showed that the decrease was caused by rapid gas decomposition and absorption by or reaction with experimental materials.

In our study, the rate of gaseous ClO_2 production was not constant since hydrogen ions were quickly depleted upon rapid reaction between sulfuric acid and sodium chlorite. The concentration of gaseous ClO_2 increased rapidly but then decreased gradually, probably due to natural degradation of gaseous ClO_2 over time or by adsorption of gas onto the plastic surfaces of the container and the Petri dish. Kim et al. (2008) reported that aqueous ClO_2 could be continuously produced by reacting organic acids (acetic, lactic, citric, and malic acids), instead of hydrochloric acid, with sodium chlorite because the dissociation rates of organic acids are lower than that of hydrochloric acid. They demonstrated that, if aqueous ClO_2 is generated continuously, the concentration of aqueous ClO_2 can be maintained for a long period of time. If aqueous ClO_2 is produced by the reaction of an organic acid and sodium chlorite in solution, gaseous ClO_2 would be produced continuously and replenish the gas lost by natural degradation or absorption to the surfaces of the sample and container, thereby maintaining the concentration of gaseous ClO_2 in the system. Further investigations are required to develop a system that allows desired concentrations of gaseous ClO_2 to be maintained over time.

3.2. Inactivation of B. cereus spores attached to SSCs using gaseous ClO_2

Fig. 3 shows the number of viable spores attached to SSCs after treatment with gaseous ClO_2 (peak concentration: 115.3 ± 5.0 ppm) in an air-tight container at 25 °C for 0 min, 10 min, 20 min, 30 min, 1 h, 2 h, and 6 h. The initial number of *B. cereus* spores on SSC was 5.3 ± 0.1 log CFU/coupon. Without treatment with gaseous ClO_2 (control), the number of spores on SSCs remained constant over the entire 6 h. When treated with gaseous ClO_2, the number of spores decreased to below the detection limit (1.5 log CFU/coupon) within 30 min, and inactivation was complete (<0.0 log CFU/coupon) within 1 h, indicating that gaseous ClO_2 effectively killed spores attached to the SSCs.

To our knowledge, antimicrobial activity of gaseous ClO_2 against *B. cereus* spores has not been reported; however, inactivation of spores other bacterial species attached to abiotic or biological surfaces has been studied. Han et al. (2003) observed that spores of *Bacillus thuringiensis* (ca. 6 log CFU/surface) attached on paper, wool, epoxy, and plastic surfaces were inactivated within 12 h when treated with gaseous ClO_2 at concentrations of 30, 30, 25, and 20 mg/L, respectively, at 22 ± 1 °C and 85 to 92% RH. Lee et al. (2006) reported that when *Alicyclobacillus acidoterrestris* spores (ca. 5 log CFU/mL) on the surface of apples were treated with gaseous ClO_2 (peak concentration: 0.60 mg/L) for 3 h, the number was reduced by 4.5 log CFU/mL.

The modes of lethal activity of gaseous ClO_2 against bacterial spores have not been described. However, the mechanism of killing of *Bacillus subtilis* spores by aqueous ClO_2 has been described (Young and Setlow, 2003). The spore coat was found to contribute to the resistance of spores and lethality was not attributed to damage of DNA. It was suggested that one of the major mechanisms of lethal activity of ClO_2 against bacterial spores is damage of the inner membrane, resulting in a change in the permeability of the plasma membrane of the germinated spore. Spores treated with ClO_2 underwent the initial steps in spore germination but did not grow out because of membrane damage. The mechanisms of gaseous ClO_2 in killing *B. cereus* spores, especially in biofilms, should be further investigated.

3.3. Inactivation of B. cereus spores in biofilms formed on SSCs using gaseous ClO_2

In the present study, we assumed that vegetative cells and spores of *B. cereus* in biofilms would exhibit an increased resistance to gaseous ClO_2. Fig. 4 shows the populations of total cells (vegetative cells plus spores) and spores of *B. cereus* in biofilm. SSCs on which biofilm had formed were immersed in TSB at 22 °C for 1 day and sporulation was subsequently induced by exposing cells to an atmosphere of 100% RH at 22 °C for up to 4 days. Total counts and spore counts after biofilm formation were 7.1 ± 0.1 and 2.5 ± 0.1 log CFU/coupon (0.003% of total cells), respectively. After exposure of the biofilm to 100% RH, the number of spores dramatically increased to 5.3 ± 0.3 log CFU/coupon (9.4% of total cells). The increase of the number of spores is in agreement with our previous study (Ryu and Beuchat, 2005).

Fig. 5 shows populations of *B. cereus* in biofilm in which sporulation was induced followed by exposure of the biofilm to gaseous ClO_2 (peak

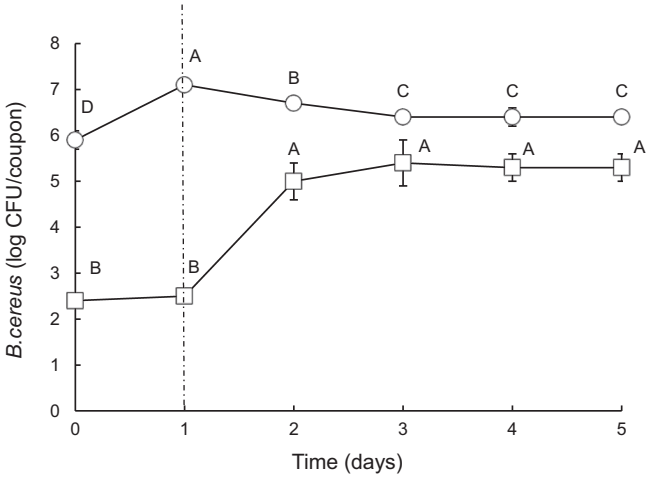

Fig. 4. Total cell counts (vegetative cells plus spores) and number of spores produced by *Bacillus cereus* (a five-strain mixture) on SSCs. Biofilms were developed on coupons immersed in TSB at 22 °C for 1 day (the vertical dotted line), then exposed to 100% RH and incubated at 22 °C for up to 4 days. Key: total cells (○), spores (□). Within each type of cell (total cells or spores), values not annotated with the same letter are significantly different ($P \leq 0.05$). Bars indicate standard deviations.

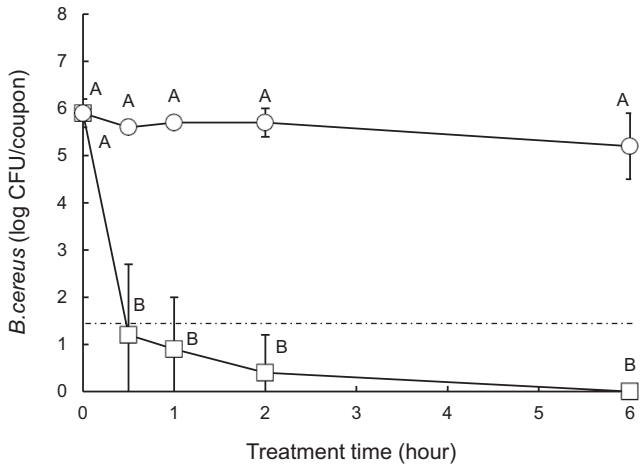

Fig. 5. Total cell numbers (vegetative cells plus spores) of *Bacillus cereus* (a five-strain mixture) surviving in biofilms on SSCs treated with gaseous ClO_2 produced by a sulfuric acid and sodium chlorite solution (sulfuric acid [5%, w/v] plus sodium chlorite 10 mg/mL) at 25 °C for up to 6 h. Key: Control (○), ClO_2 gas treatment (□). At a given treatment time, values not annotated with the same letter are significantly different ($P \leq 0.05$). The detection limit for direct plating was 1.5 log CFU/coupon (the horizontal dotted line). The detection limit upon enrichment was 0.0 log CFU/coupon. To plot the numbers of *B. cereus* cells on the SSCs, the number of cells was considered as 1.5 log CFU/coupon when cells were not detected on agar plates but detected after enrichment. If cells were not detected after enrichment, it was considered as 0.0 log CFU/coupon. Bars indicate standard deviations.

concentration: 115.3 ± 5.0 ppm) in an air-tight container at 25 °C for up to 6 h. The initial number of *B. cereus* cells (vegetative cells plus spores) in biofilm was 5.9 ± 0.3 log CFU/coupon, and the initial number of spores was 5.3 ± 0.7 log CFU/coupon. When SSCs on which biofilm of *B. cereus* had formed were held at 25 °C in the absence of gaseous ClO_2 (control), the number of total cells (vegetative cells plus spores) did not change significantly for 6 h. When treated with gaseous ClO_2, vegetative cells and spores were inactivated within 6 h. The number of *B. cereus* spores attached to SSCs (5.3 ± 0.1 log CFU/coupon; Fig. 3) was not significantly different from the number of *B. cereus* spores in biofilms (5.3 ± 0.7 log CFU/coupon; Fig. 5). However, when treated with gaseous ClO_2, 6 h was required to inactivate spores in biofilms, compared to 1 h for spores simply attached to SSCs. Regardless of whether spores were in biofilms or attached on the surface of SSCs, gaseous ClO_2 was an effective sanitizer.

Other studies have shown that the lethality of gaseous ClO_2 against foodborne pathogens in biofilms on food-contact surfaces is generally good. Vaid et al. (2010) reported that *L. monocytogenes* in biofilms on SSCs was reduced by 3.21 log CFU/cm^2 when treated with 0.3 mg/L gaseous ClO_2 at 75% RH and 22 °C for 10 min. They indicated that treatment with 0.3 mg/L gaseous ClO_2 under the same conditions would result in a 5 log CFU/cm^2 reduction within 20.8 min. Trinetta et al. (2012) investigated the lethality of gaseous ClO_2 against *L. monocytogenes* in biofilms on SSCs and found that populations were reduced by ca. 3.8 ± 0.23 log CFU/cm^2 upon treatment with 2 mg/L gaseous ClO_2 for 10 min, whereas inactivation was complete after treatment for 70 min.

The antimicrobial activity of gaseous ClO_2 clearly appears to be higher than that of aqueous ClO_2 against foodborne pathogens attached to or in biofilms in food-processing environments and on food-contact surfaces. However, the application of gaseous ClO_2 to abiotic surfaces in food-processing plants is not without challenges. One of the challenges is that gaseous ClO_2 must be generated within a sealed chamber to maintain a constant gas concentration (Wu and Kim, 2007). Thus, the use of gaseous ClO_2 has been principally directed toward controlling spoilage microorganisms and foodborne pathogens present on the surfaces of fruits and vegetables in closed systems, rather than for sanitizing food-contact surfaces. One example in which gaseous ClO_2 could be used to control hazardous microorganisms in biofilm formed on food-contact surfaces is the "clean-in-place" (CIP) system commonly used in dairy manufacturing plants. The CIP system has been employed to control biofilms formed by undesirable microorganisms in dairy processing pipelines (Bremer et al., 2006; Shi and Zhu, 2009). However, it has been reported that bacteria can survive and produce biofilms on internal surfaces of pipes even after completion of a "successful" CIP procedure (Shi and Zhu, 2009). Since processing lines in the dairy industry provide a closed system during cleaning and sanitization, gaseous ClO_2 may have application for reducing *B. cereus* spores in biofilms present on pipelines as a part of the CIP procedure.

In summary, the concentration of gaseous ClO_2 produced by a sulfuric acid and sodium chlorite solution increased rapidly in an air-tight container, then gradually decreased over time. When *B. cereus* spores attached to SSC surfaces were treated with gaseous ClO_2 (peak concentration: 115.3 ± 5.0 ppm), spores were inactivated within 1 h. When *B. cereus* spores in biofilm were treated with gaseous ClO_2 (peak concentration: 115.3 ± 5.0 ppm), inactivation occurred within 6 h. These results indicate that gaseous ClO_2 is a very effective sanitizer when used to reduce *B. cereus* spores on stainless steel.

The efficacy of lethality activity of gaseous ClO_2 should be further evaluated, particularly in terms of removing undesirable microorganisms in biofilms on CIP equipment used in dairy and other food industries. A system by which a desired concentration of gaseous ClO_2 can be maintained in a sanitizing chamber should be developed; this would feature continuous production of gaseous ClO_2. The conditions, e.g., concentration of the gas, temperature, and RH, for successful treatment with gaseous ClO_2 should be optimized. Possible synergistic effects between gaseous ClO_2 and other stresses such as heat and RH in killing hazardous microorganisms on foods and food-contact surfaces should be investigated. Additionally, there is limited information for the potential hazards of gaseous ClO_2 on workers in the plant, especially when large amounts of ClO_2 are applied. Therefore, the toxicity of gaseous ClO_2 to human should be accessed and effective neutralization method should be developed.

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