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Addition of ethanol to supercritical carbon dioxide enhances the inactivation of bacterial spores in the biofilm of *Bacillus cereus*

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ABSTRACT

Supercritical carbon dioxide (SC-CO_2) was used to inactivate *Bacillus cereus* spores inside biofilms, which were grown on stainless steel. SC-CO_2 treatment was tested using various conditions, such as pressure treatment (10–30 MPa), temperature (35–60 °C), and time (10–120 min). *B. cereus* vegetative cells in the biofilm were completely inactivated by treatment with SC-CO_2 at 10 MPa and at 35 °C for 5 min. However, SC-CO_2 alone did not inactivate spores in biofilm even after the treatment time was extended to 120 min. When ethanol was used as a cosolvent with SC-CO_2 in the SC-CO_2 treatment using only 2–10 ml of ethanol in 100 ml of SC-CO_2 vessel for 60–90 min of treatment time at 10 MPa and 60 °C, *B. cereus* spores in the biofilm were found to be completely inactivated in the colony-forming test. We also assessed the viability of SC-CO_2-treated bacterial spores and vegetative cells in the biofilm by staining with SYTO 9 and propidium iodide. The membrane integrity of the vegetative cells was completely lost, while the integrity of the membrane was still maintained in most spores. However, when SC-CO_2 along with ethanol was used, both vegetative cells and spores lost their membrane integrity, indicating that the use of ethanol as a cosolvent with SC-CO_2 is efficient in inactivating the bacterial spores in the biofilm.
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1. Introduction

Bacillus cereus is one of the most common foodborne pathogens in daily life, and it is found in various environments such as soil, water, and dust (Kramer and Gilbert, 1989). *B. cereus* forms spores that are highly resistant to physical and chemical treatments (Setlow, 2006). Conventional pasteurization techniques destroy vegetative cells but do not kill spores; hence, more vigorous sterilization techniques, such as the use of superheated steam (Head et al., 2008), hydrostatic pressure (Gao and Jiang, 2005), and sanitizers (Setlow et al., 2002), have been exploited to destroy bacterial spores. However, these methods have certain drawbacks. Superheated steam can severely damage the nutritive value, color, and taste of foods. Hydrostatic pressure requires extremely high pressures (at least 200 to 600 MPa) for inactivating the bacterial spores, thus requiring high capital and operating costs. Sanitizers may cause environmental pollution and are not suitable for foods owing to their residual toxicity.

Biofilm, which indicates the complex accumulation of microorganisms growing on a solid surface, is much more resistant to antimicrobial agents than planktonic cells (Flemming and Wingender, 2001). Currently, the use of antimicrobial agents, including sanitizers, antibiotics, and detergents, is the most common method for inactivation of biofilms (Kreske et al., 2006). Physical treatment techniques such as heat and γ-irradiation are also used (Chmielewski and Frank, 2006; Niemira, 2010). However, the inactivation of biofilm is still difficult owing to the ineffectiveness of sterilization methods and also owing to certain problems, including harmfulness of sanitizers and substances that are heat-sensitive and could be damaged during the process of sterilization.

Supercritical carbon dioxide (SC-CO_2) has been known as an alternative non-thermal sterilization method for various microorganisms, mainly vegetative cells (Kamihira et al., 1987; Kim et al., 2007a, 2007b). SC-CO_2 has a relatively low critical point (7.38 MPa and 31.1 °C), and it has several advantages as a sterilizing solvent, such as high dissolving power, high diffusivity, and low viscosity (Paulaitis et al., 1983). Only a few studies have shown the effectiveness of SC-CO_2 in inactivation of biofilms of bacteria such as *Bacillus mojavensis* (Mitchell et al., 2008) and *Pseudomonas aeruginosa* (Mun et al., 2009).

In the present study, our aim was to inactivate spores of *B. cereus* in the biofilm by using non-thermal sterilization techniques. Bacterial spores within the biofilm are highly resistant to sterilization because they may be better protected by the double barriers of the biofilm and spore coats than vegetative cells (Mitchell et al., 2008). For example, when SC-CO_2 treatment was conducted on *B. mojavensis*, the $\log_{10}$ reduction level of viable cells in biofilms was 2 logs less than that of vegetative cells (Mitchell et al., 2008). In this study, the effect of using ethanol as a cosolvent on the effectiveness of SC-CO_2 in inactivating the bacterial spores in the biofilms was investigated.

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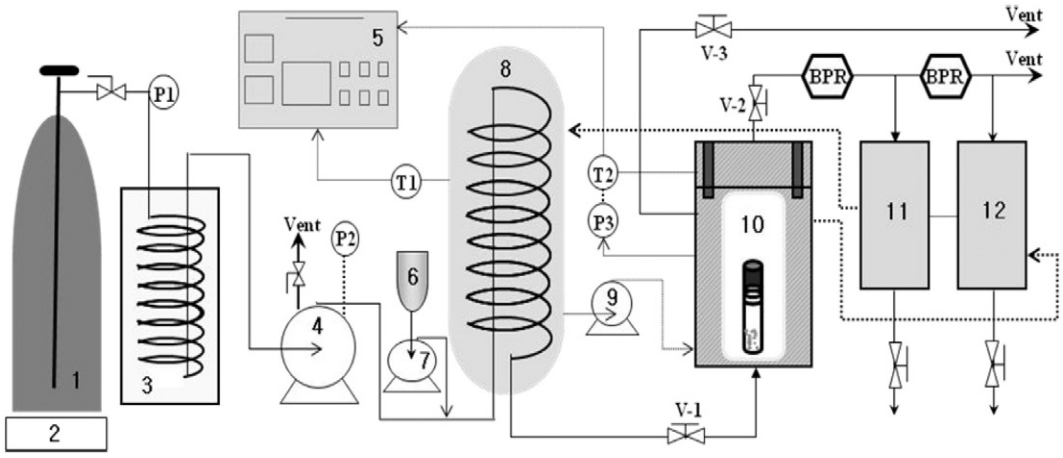

Fig. 1. Schematic diagram of supercritical CO_2 treatment system: 1, CO_2 cylinder; 2, electronic scale; 3, chiller; 4, CO_2 pump; 5, temperature controller; 6, cosolvent reservoir; 7, cosolvent pump; 8, heating bath; 9, circulation pump; 10, treatment vessel; 11, separator 1; 12, separator 2; V-1, valve 1; V-2, valve 2; BPR, back pressure regulator; dotted lines, water; solid lines, CO_2.

2. Materials and methods

2.1. Strain

Bacillus cereus (KCTC 1012) was obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). A stock culture was preserved in tryptic soy broth (TSB; Bacto, Detroit, MI) containing glycerol at 10% (v/v) (Junsei Chemical, Tokyo, Japan) and was stored at −80 °C until use.

2.2. Preparation of cell suspension

B. cereus from the deep-frozen cultures was thawed at 37 °C. One loop (approximately 10 μl) was streaked on brain–heart infusion agar (Difco, Detroit, MI) and incubated for 24 h at 30 °C. A single colony of *B. cereus* was transferred to 10 ml of TSB and incubated at 30 °C for 24 h. Four milliliters of cell suspension was inoculated into 200 ml of TSB and incubated for 8 h at 30 °C at 200 rpm. The harvested cells were centrifuged at 2300 ×*g* for 10 min to obtain the cell pellet. The cell pellet was washed twice using an equal volume of phosphate-buffered saline (PBS; KH_2PO_4 0.24 g/l, KCl 0.2 g/l, NaCl 8 g/l, Na_2HPO_4 1.44 g/l; pH 7.4; Sigma, St. Louis, MO) and then re-suspended in the same solution.

2.3. Formation of the biofilm

Cell suspension (30 ml) was decanted into a sterile tube, and a sterile steel plate (2 cm × 5 cm × 1 mm; stainless steel type 304, POSCO, Pohang, Korea) was then immersed in the cell suspension and incubated at 22 °C for 4 h to facilitate cell attachment. The cells attached to the steel plate were rinsed with sterile water and then transferred into a tube containing 30 ml of fresh TSB medium, and incubated at 22 °C for 48 h to induce the formation of biofilm. The steel plates containing the biofilm were then transferred into a tube containing 0.8 ml of sterile water and incubated for 72 h at 22 °C to induce sporulation of *B. cereus* in the biofilm. The concentration of initial spores in biofilm was approximately 10^4 colony forming units (CFUs)/plate. For the confocal laser scanning microscopy (CLSM) analysis, biofilms were formed on microscope glass slides using the above-mentioned method applied to the formation of biofilms on the steel plates.

2.4. Supercritical CO_2 treatment

A schematic diagram of the SC-CO_2 treatment system (Ilshin Autoclave, Daejeon, Korea) is shown in Fig. 1. *B. cereus* spores in the biofilm were treated with SC-CO_2 in a static mode by varying pressure (10–30 MPa), temperature (35 to 60 °C), and treatment time (10 to 120 min). Treatment procedures and the SC-CO_2 treatment apparatus

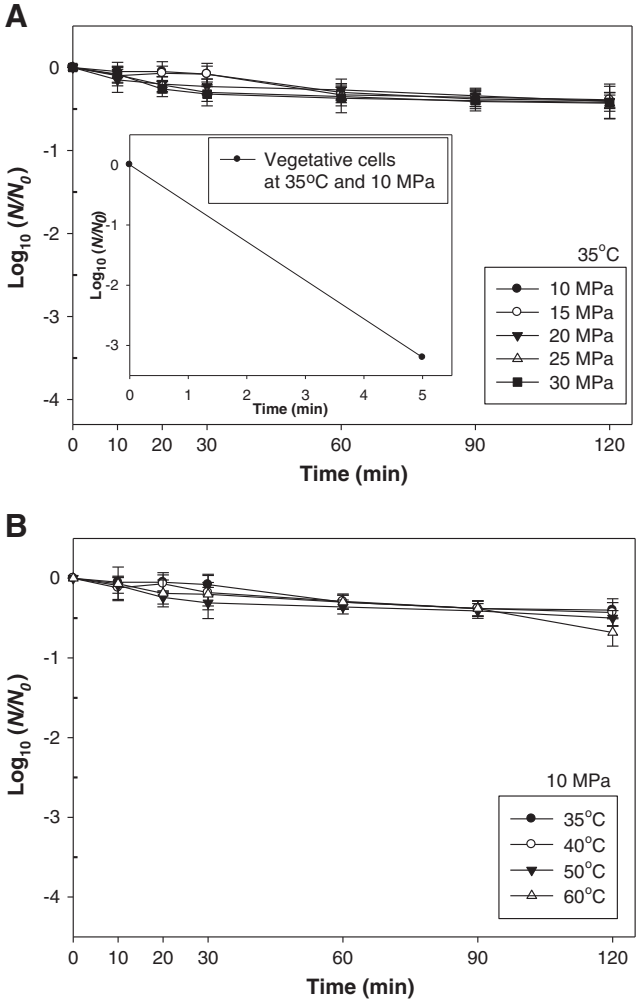

Fig. 2. Effect of pressure (A: 10–30 MPa at 35 °C) and temperature (B: 35–60 °C at 10 MPa) of supercritical CO_2 on the inactivation of *B. cereus* spores and vegetative cells (the embedded graph in A) in biofilm.

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