Effects of Carbonation with Heating on Germination of *Bacillus subtilis* Spores

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Received June 1, 2011; Accepted July 30, 2011

The effects of carbonation with heating (CH) on germination of *Bacillus subtilis* spores were investigated. Treatment conditions for CH and heat treatment alone were set to obtain an approximate 1 log reduction in viable count. Pre-treatment of spores with CH at 80 °C and 5 MPa for 30 min significantly decreased their heat resistance to a subsequent heating process at 90 °C for 30 min, as compared with pre-treatment by heat alone at 90 °C for 30 min. Treatment with CH also decreased refractility and enhanced DAPI staining when compared with heat treatment alone, thus suggesting that CH effectively initiates and stimulates germination of *B. subtilis* spores.

Keywords: carbonation, heat, *Bacillus* spores, germination

Introduction

Carbonation treatment is carried out by solubilizing carbon dioxide (CO₂) into liquid foods under pressurization. Dissolved CO₂ is thought to penetrate the cells, and the generated H₂CO₃ dissociates into HCO₃⁻ and H⁺, leading to acidification of the cytoplasm (Dixon and Kell, 1989; Dil low et al., 1999; Spilimbergo et al., 2002). Acidification of cytoplasm has been shown to induce injury and affect the viability of vegetative microbial cells (Kim et al., 2008; Wu et al., 2007). Although numerous reports have demonstrated that carbonation at moderate temperature (20–40 °C) can inactivate microorganisms in their vegetative form, this is not sufficient for substantial reductions in viable spore counts (Garcia-Gonzalez et al., 2007). Enomoto et al. (1997) also reported that carbonation at temperatures above 50 °C significantly inactivated bacterial spores. The elucidation of the mechanisms for this enhanced inactivation of spores under heating may lead to more effective use of carbonation in the control of bacterial spores.

It is known that germination of *Bacillus* spores is accompanied by a decrease in heat resistance, a decrease in optical density (Hachisuka, 1998), and an increase in permeability to dyes such as DAPI (4',6'-diamino-2-phenylindole) and acridine orange (Ragkousi et al., 2002). Furukawa et al. (2004) reported that carbonation at moderate temperatures (20–40 °C) induced decreases in the heat resistance of *Bacillus* spores and turbidity of the suspension. However, it remains uncertain whether the inactivation of spores by carbonation with heating is mediated through their germination.

In the present study, we investigated whether the inactivation of *Bacillus* spores by carbonation with heating is mediated through germination.

Materials and Methods

**Bacteria and preparation of spore suspension**  *Bacillus subtilis* 168 was kindly provided by Dr. S. Ishikawa of Shinshu University. Spores were formed in G medium as described by Stewart and Halvorson (1953), and were washed in triplicate by centrifugation at 7,000 × g at 4 °C for 10 min in 0.85% sodium chloride (Nacalai Tesque, Inc., Kyoto, Japan) solution. The spore suspension was heated at 80 °C for 30 min in order to inactivate vegetative cells. A spore suspension of about 10⁹ CFU/mL was then lyophilized and stored at −20 °C until use.

**Carbonation with heating (CH), and heat treatments** A schematic diagram of the apparatus for CH is shown in Fig. 1. Spore suspension (1 mL) in a test tube was immersed in water at the desired temperature in a CO₂-dissolving vessel. CO₂ gas was introduced into the vessel at the desired pressure and
was dissolved into the spore suspension with stirring. After standing for a specified period, CO₂ gas in the vessel was released via a pressure control valve. The come-up-time to the desired temperature and pressure was about 1 min, and this was not included in the treatment time. For heat treatment, the spore suspension in the test tube was heated for 30 min in a water bath. The come-up-time to the desired temperature was about 1 min, and this was not included in the treatment time. The spore suspensions subjected to CH and heat were immediately cooled by immersion into ice-cooled water.

This study was a pre-study to establish the point of equivalence in the inactivation of spores by CH treatment versus heat treatment alone. This study used a range of temperatures (50–90°C), pressures (1–5 MPa) and times (10–50 min). A second study was then conducted to compare the germination of spores between CH at 80°C and 5 MPa for 30 min and heat treatment at 90°C for 30 min.

**Viable spore count** After CH or heat treatment, appropriate serial dilutions were prepared in 0.85% sodium chloride solution, and 0.1 mL of the suspension was plated onto nutrient agar (Eiken Chemical Co., Ltd., Tokyo, Japan). The number of viable spores was enumerated as CFU after incubation at 30°C for 24 h.

**Germination** Treatment conditions used in the study of germination of spores were 80°C, 5 MPa for 30 min for CH and 90°C for 30 min for heat only.

a) **Heat resistance** Heat resistance of *B. subtilis* spores subjected to CH (80°C, 5 MPa for 30 min) or heat treatment (90°C for 30 min) was determined by investigating the viable spore count after subsequent heat treatment at 90°C for 40 min.

b) **Measurement of optical density** Optical density of spore suspensions was measured at 650 nm (OD₆₅₀) using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) after CH or heat treatment.

c) **DAPI staining** Spores were stained with DAPI (Nacalai Tesque). DAPI solution was prepared by dissolution into distilled water at a concentration of 1 mg/mL, and the stock solution was diluted 1,000-fold with methanol and used as a working solution. Spore suspension was centrifuged at 7,000 × g and 4°C for 10 min, and the resultant pellet was suspended in the DAPI working solution. After incubation at 37°C for 15 min, spores were washed three times by centrifugation in distilled water. This spore suspension was observed using a phase contrast microscope (BX50; Olympus Co., Tokyo, Japan) equipped with accessories for fluorescence observation (BH2-RFL-T3, U-ULS100HG and BX-KLA; Olympus).

**Statistical analysis** Significant differences in viable spore counts were determined by Student’s *t*-test (Microsoft Excel 2003) after *f*-test was used to ensure the equality of variance. Fisher’s least significant difference test was employed to determine statistically significant differences at OD₆₅₀.

**Results**

**Inactivation of *B. subtilis* spores by CH** Inactivation of *B. subtilis* spores by CH was investigated (Fig. 2A). The number of inactivated *B. subtilis* spores increased with treatment temperature, pressure and time. One log-order inactivation was observed at 80°C and 5 MPa for 30 min. Inactivation of *B. subtilis* spores by heat treatment is shown in Fig. 2B. A significant decrease in viable spores was observed at 85 and 90°C, and the inactivation ratio was about 1 log at 90°C. There were no significant differences in inactivation of spores between CH at 80°C, 5 MPa for 30 min and heat treatment at 90°C for 30 min. These two treatments under each set of conditions were used for subsequent studies on germination of *B. subtilis* spores.

**Germination of *B. subtilis* spores by CH**

a) **Heat resistance** Viable spores after CH were further decreased by 2.5 log after subsequent heat treatment. On the other hand, spores subjected to heat treatment (90°C for 30 min) were further inactivated by 1.5 log-order after subsequent heat treatment (90°C for 40 min). There was a significant difference (*p* < 0.01) in the additional decrease in viable spore count.

b) **Optical density** OD₆₅₀ for untreated spore suspension, 1.0, decreased to 0.22 and 0.90 after CH and heat treatment, respectively. *B. subtilis* spores subjected to CH or heat treatment were observed by phase contrast microscopy (Fig. 5, light field). Untreated spores were refractile on phase contrast microscopy, and spores treated by heat alone showed a smaller proportion of dark phase spores than spores treated by CH.
In the present study, the effects of CH on germination of *B. subtilis* spores were investigated in order to obtain evidence on the mechanism of spore inactivation under CH. In the initial stages of spore germination, loss of heat resistance is observed. Watanabe et al. (2003) showed that carbonation at ambient temperature induces loss of heat resistance in *B. coagulans* and *B. licheniformis* spores. This carbonation-mediated loss of heat resistance was also observed in *B. subtilis* spores under heating. In contrast, fewer spores showed lower heat resistance after heat treatment alone than after CH. This indicates that carbonation-mediated loss of heat resistance is induced under heating. The treatment temperature required to obtain 1 log-order inactivation of spores was 90°C by heat alone and 80°C by CH, and heat

c) DAPI staining  *B. subtilis* spores subjected to CH or heat treatment were observed by fluorescence microscopy (Fig. 5, dark field). Untreated spores were stained poorly. After CH, most spores were vibrantly stained with DAPI. After heat treatment, few spores were well stained. At the single observed cell level, dark phase (i.e., non-refractile) spores were specifically observed to stain with DAPI.

![Fig. 2](image)

**Fig. 2.** The effects of temperature, pressure and time on viable count of *B. subtilis* spores by CH (A). The effects of heat treatment on viable count of *B. subtilis* spores (B). Data represent the means of three determinations and error bars indicate the standard deviation of the mean. ‘***’ and ‘*’ indicate significant differences versus initial counts at $p < 0.01$ and 0.05, respectively.

Discussion

In the present study, the effects of CH on germination of *B. subtilis* spores were investigated in order to obtain evidence on the mechanism of spore inactivation under CH.

In the initial stages of spore germination, loss of heat resistance is observed. Watanabe et al. (2003) showed that carbonation at ambient temperature induces loss of heat resistance in *B. coagulans* and *B. licheniformis* spores. This carbonation-mediated loss of heat resistance was also observed in *B. subtilis* spores under heating. In contrast, fewer spores showed lower heat resistance after heat treatment alone than after CH. This indicates that carbonation-mediated loss of heat resistance is induced under heating. The treatment temperature required to obtain 1 log-order inactivation of spores was 90°C by heat alone and 80°C by CH, and heat

![Fig. 3](image)

**Fig. 3.** Viable spore count after CH or heat treatment alone, and subsequent heat treatment. White columns indicate initial spore count. Dark gray columns indicate CH with heat or heat alone. Light gray columns indicate heat treatment (90°C for 40 min) after CH with heat (80°C, 5 MPa for 30 min) or heat alone (90°C for 30 min). Data represent the means of three determinations and error bars indicate standard deviation of the mean. ‘***’ indicates a significant difference at $p < 0.01$.

![Fig. 4](image)

**Fig. 4.** Effects of CH with heat (80°C, 5 MPa for 30 min) or heat alone (90°C for 30 min) on optical density (OD$_{650}$) of spore suspension. ‘***’ and ‘*’ indicate significant differences at $p < 0.01$ and 0.05, respectively.
treatment alone at 80°C did not measurably reduce spore numbers (Fig. 2). Therefore, it is considered that *B. subtilis* spores lost their heat resistance by carbonation, and the resultant spores were inactivated by heating at 80°C during carbonation.

Following the loss of heat resistance, loss of refractility, decreased optical density and increased permeability to fluorescent stains are observed in the process of spore germination (Hachisuka, 1988). Furukawa *et al.* (2004) reported that carbonation at 6.5 MPa and 35°C decreased optical density of spore suspensions of *B. coagulans* and *B. licheniformis*. In the present study, CH clearly decreased the optical density of spore suspension and induced the loss of refractility. In addition, CH also enhanced DAPI staining of *B. subtilis* spores. However, heat treatment weakly induced these changes. These results indicate that carbonation stimulates germination under heating.

Germination of bacterial spores is a result of sequential reactions of spore-lytic germination enzymes (Foster and Johnstone, 1990). Adbelmadjid and Foster (2001) reported that optimal pH for germination was from 7 to 9, and optimal pH for germination-specific lytic enzymes was from 5 to 8. However, Spilimbergo *et al.* (2005) reported that the pH of cell suspension medium was lowered to about 3.3 at 50 bar and 333 K. At 50 bar and 313 K, the pH was calculated to be 3.2. From these data, the pH of spore suspensions is expected to be less than 5 during carbonation. In addition, Adbelmadjid and Foster (2001) demonstrated that heat treatment of spores at 90°C for 15 min led to impaired cortex hydrolysis/ modification. Therefore, it is unlikely that the carbonation-mediated germination involves the contribution of typical spore-lytic germination enzymes, as described by Furukawa *et al.* (2004).

During carbonation, protons are generated from CO2 in the spore suspension. Ions such as Ca2+, Na+, K+, Mg2+ and Mn2+ present in the spores are slowly exchanged by the generated protons, turning spores into so-called H-spores (Wuytack and Michiels, 2001). Heat resistance of H-spores is lower than that of spores without ion exchange (Igura *et al.*, 2003). It is possible that CH-mediated decreases in heat resistance are caused by the transition to H-spores. However, it is unclear whether CH turns spores into H-spores or whether CH stimulates germination in H-spores. Alternatively, the cortex layer of spores is negatively charged, and this negative charge is important for retaining heat resistance (Gould, 1975). The cortex layer is also essential for the reduction of the water content in the spore core (Setlow, 2005). Uptake of water changes spores from a phase-bright to a phase-dark state (Foster, 1994). Protons generated by CO2 may neutralize the negative charge in the cortex layer. This impaired cortex can decrease the barrier function of spores, leading to approximating germination. Further detailed investigations are necessary in order to clarify the germination mechanisms under carbonation.

In conclusion, the present study demonstrated that carbonation with heating effectively initiated and stimulated the germination of *B. subtilis* spores. Inactivation of *B. subtilis* spores by carbonation with heating was apparently induced through germination. It is likely that the spores germinated and were then inactivated by concomitant heat treatment. Optimization of conditions for germination through the combination of carbonation and heat treatment may lead to improved processing technologies for inactivation of *Bacillus* spores.

**References**


