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Inactivation of *Bacillus subtilis* spores by high pressure CO_2 with high temperature

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ABSTRACT

The objective of this study was to investigate the inactivation of the *Bacillus subtilis* spores by high pressure CO_2 combined with high temperature (HPCD + HT) and to analyze the clumping effect of the spores on their HPCD + HT resistance. The spores of *B. subtilis* were subjected to heat at 0.1 MPa and HPCD at 6.5–25 MPa, and 82 °C, 86 °C, and 91 °C for 0–120 min. The spores were effectively inactivated by HPCD + HT, but a protective effect on the spores was also found, which was closely correlated to the pressure, temperature and time. The spores treated by HPCD + HT at 6.5 and 10 MPa exhibited a two-stage inactivation curve of shoulder and log-linear regions whereas the spores at 15–25 MPa exhibited a three-stage inactivation curve of shoulder, log-linear and tailing regions, and these curves were well fitted to the Geeraerd model. Approximately 90% of pyridine-2,6-dicarboxylic acid (DPA) was released after HPCD + HT and the 90% DPA release time depend on the pressure and temperature. Moreover, the spore clumping in suspensions was examined by dynamic light scattering. The particle size of the spore suspensions increased with the increase of pressure, temperature and time, indicating the spore clumping. 0.1% Tween 80 as a surfactant inhibited the spore clumping and increased the inactivation ratio of the spores by HPCD + HT. These results indicated that the spore clumping enhanced the spores' resistance to HPCD + HT and induced a protective effect.

1. Introduction

Spores of *Bacillus* and *Clostridium* species formed in sporulation are metabolically dormant and extremely resistant to a variety of stress factors, including heat, desiccation, chemicals and radiation because of their unique structures (Setlow, 1995, 2006). These spores in food are common agents that cause spoilage, foodborne illnesses, and detrimental changes to the organoleptic quality (Brown, 2000). The extreme resistance of bacterial spores to physical and chemical treatments makes them a significant problem in the food industry.

High pressure carbon dioxide (HPCD) was firstly shown to inactivate *Escherichia coli* cells in the 1950s by Fraser (1951). During the recent two decades, HPCD has been proposed as an alternative non-thermal pasteurization technique for foods (Spilimbergo et al., 2002). Besides the environmentally benign nature of the HPCD process (CO_2 is nontoxic), the CO_2 pressures applied for preservation purposes are much lower (generally lower than 30 MPa) compared to the high pressures (100–600 MPa) employed in high pressure processing (Garcia-Gonzalez et al., 2007), which makes it easier to manage pressure in HPCD technique. The bactericidal effects of HPCD have been reviewed by Damar and Balaban (2006), Garcia-Gonzalez et al. (2007), Perrut (2012), Spilimbergo and Bertucco (2003), and Zhang et al. (2006b). Previous studies indicated that the vegetative forms of pathogenic and spoilage bacteria, yeasts, and molds were completely inactivated by HPCD at pressures less than 30 MPa and at 20 to 40 °C. However, the use of HPCD at moderate temperatures (20–40 °C) is often insufficient to obtain a substantial reduction in bacterial spore counts due to their more complex structure as compared to vegetative cells. Limited studies indicated that bacterial spores were inactivated by HPCD with high temperature ≥ 60 °C (HPCD + HT), and the inactivation ratio was increased with the increase of temperature, pressure and time (Bae et al., 2009; Ballestra and Cuq, 1998; Enomoto et al., 1997; Hate et al., 1996; Spilimbergo et al., 2002; Watanabe et al., 2003). The inactivation kinetics of spores by HPCD + HT was described by first-order models (Enomoto et al., 1997; Hata et al., 1996; Qiu et al., 2009; Watanabe et al., 2003; White et al., 2006) and two-fraction models (Ballestra and Cuq, 1998). Different models were observed probably because of the incomprehensive HPCD conditions and insufficient experimental data numbers in these studies (Garcia-Gonzalez et al., 2007). Inactivation of spores by extensive HPCD + HT conditions and the inactivation kinetics were necessary to be studied. Meanwhile, as DPA (pyridine-2,6-dicarboxylic acid) was a major chemical component in the inner core of bacterial spores (~10% of spore dry weight) (Setlow, 2003) and its

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release was closely correlated to the damage of spores' inner membrane (Reineke et al., 2013; Zhang et al., 2006a; Zhang et al., 2007), DPA release was necessary to be studied to further understand the inactivation mechanism of bacterial spores.

Moreover, Enomoto et al. (1997) reported that a pressure-dependent protective effect on *Bacillus megaterium* spores was observed at 5.8–9.7 MPa by HPCD + HT at 1.9–9.7 MPa and 60 °C for 24 h, and these authors hypothesized that the protective effect was attributed to spore clumping. This hypothesis was later confirmed by Furukawa et al. (2006), who observed spores of *Bacillus coagulans* and *Bacillus licheniformis* clumping by HPCD at 6.5 and 30 MPa and 35 °C for 0–120 min by phase contrast microscopy, and indicated that the ratio of spore clumping increased with the increase of pressure and time. However, the spores were not effectively inactivated in the study since the temperature was far less than 60 °C, therefore the contribution of the study to understand the inactivation of the bacterial spores was very limited. Moreover, phase contrast microscopy cannot reflect the spore clumping of the spore suspensions in a full-scale. Due to drawback of the studies and the method on spore clumping investigation, the spore clumping by HPCD + HT should be in-depth studied.

In this study, the inactivation of *Bacillus subtilis* spores by extensive HPCD + HT conditions was performed and the inactivation kinetics was analyzed. DPA was analyzed to further understand the inactivation mechanism of *B. subtilis* spores by HPCD + HT. Moreover, the spore clumping by HPCD + HT at these extensive conditions and its impact on spores' resistance to HPCD + HT were investigated.

2. Materials and methods

2.1. Strain and spore preparation

B. subtilis 168 was obtained from China General Microbiological Culture Collection Center (Beijing, China). Overnight cultures of *Bacillus* strains grown in nutrient broth (Beijing Aoboxing Biological Technology Co. Ltd., Beijing, China) were transferred to sporulation agar plates, nutrient agar (Beijing Aoboxing Biological Technology Co. Ltd., Beijing, China) containing 50 μg of Mn^{2+}/mL. After 1 week incubation at 37 °C, the spores were harvested in a sterile flask by flushing the surface of the culture with sterile distilled water and scrapping the surface with sterile glass microscope slide. The spores collected were washed three times by centrifugation at 7000 ×*g* and 4 °C for 15 min (CF16RXII, Hitachi, Japan), resuspended in sterile distilled water with a concentration of approximately 10^9 CFU/mL, and stored at 4 °C until they were used. All spores (>99%) used in this work were free of growing and sporulating cells, germinated spores and cell debris as determined with a phase contrast microscope (BX45-72P15, Olympus, Japan). The concentration of the spore suspension was adjusted to approximately 10^7 CFU/mL before treatments.

2.2. HPCD + HT treatments

The treatment conditions were shown in Table 1. Firstly, spore suspensions were treated by HPCD at 6.5–20 MPa, and 82–91 °C, for 60 min, and treated by heat at 0.1 MPa and 82–91 °C for 60 min. Secondly, spore suspensions were treated by HPCD at 6.5–25 MPa, and 82–91 °C for 0–120 min, and treated by heat at 0.1 MPa and 82–91 °C for 0–120 min. Thirdly, the spore suspensions with or without 0.1% (v/v) Tween 80 (polyoxyethylene sorbitan monooleate) (Xilong Chemical Co., Ltd, Guangdong, China) were treated by HPCD at 6.5–25 MPa and 91 °C for 40 min, 20 MPa and 82–91 °C for 40 min, 20 MPa and 91 °C for 0–40 min.

HPCD + HT treatment was performed with a batch of HPCD system (Liao et al., 2007). For each experiment, twenty mL of the spores suspended in sterile distilled water was transferred to a 50 mL sterile glass tube and the tube was covered with a plastic film with a 0.22 μm membrane filter in the center of aeration to prevent microbial contamination. As the pressure vessel of the HPCD system reached the experimental temperature (82 °C, 86 °C, 91 °C), the sample tubes were placed in the pressure vessel. Then, the vessel was pressurized by the plunger pump to 6.5 MPa, 10 MPa, 15 MPa, 20 MPa, and 25 MPa within 0.1 min, 0.5 min, 1 min, 2 min, and 2.5 min, respectively. After holding for required treatment time, the depressurization was performed by opening the pressure relief valve at CO_2 outlet on the pressure vessel. The depressurization time was 2.5 min, 4 min, 6.5 min, 9 min, and 11.5 min for 6.5 MPa, 10 MPa, 15 MPa, 20 MPa, and 25 MPa, respectively. After HPCD, the sample tubes were taken out from the vessel and analyzed immediately. The CO_2 purity was 99.5% in all the experiment treatments.

The inactivation of the spore suspensions by heat treatment at 82 °C, 86 °C and 91 °C was carried out at 0.1 MPa without the addition of CO_2 using a water bath. Twenty mL of the spores suspended in sterile distilled water was transferred to a 50 mL sterile glass tube, which was then immersed in a water bath equilibrated at 82 °C, 86 °C and 91 °C for 0–120 min. After treatments, the sample tubes were taken out and analyzed immediately.

Table 1
HPCD treatment conditions for the different figures.

	Temperature (°C)	Pressure (MPa)	Holding time (min)
Fig. 1	44–91	0.1–20	60
Fig. 2A	82	0.1–25	0–120
Fig. 2B	86	0.1–25	0–70
Fig. 2C	91	0.1–25	0–80
Fig. 3A	82	0.1–25	0–120
Fig. 3B	86	0.1–25	0–70
Fig. 3C	91	0.1–25	0–80
Fig. 4A	91	0.1–25	40
Fig. 4B	82–91	20	40
Fig. 4C	91	20	0–40
Fig. 5	91	20	10–40
Fig. 6A	91	0.1–25	40
Fig. 6B	82–91	20	40
Fig. 6C	91	20	0–40

2.3. Enumeration of surviving spores

The number of surviving spores was determined by the viable plate count method. Each sample was serially (1:10) diluted with sterile distilled water and pour-plated on nutrient agar (Beijing Aoboxing Biological Technology Co. Ltd., Beijing, China) in duplicate. The plates were incubated at 37 °C for 24 h. After incubation, the colonies were counted.

2.4. Measurement of DPA release

Spores' release of DPA following HPCD was determined individually for each kinetic point. DPA concentration was measured using the fluorescence method (Hindle and Hall, 1999). One mL of spore suspensions was added to 3 mL 50 μmol/L terbium (III) chloride hexahydrate (99.9%, Aladdin Industrial Corporation, Shanghai, China) at pH 5.6 by adding 1 M acetic acid (99.8%, Beijing Chemical Works, Beijing, China) (Hindle and Hall, 1999). All the samples were analyzed with a Cary Eclipse fluorescence spectrofluorometer (Cary Eclipse, Varian, USA). Steady-state emission and excitation spectra were collected with magic-angle polarization. Samples were excited at 270 nm, and emission spectra were collected at 545 nm. The total amount of DPA in each individual batch was determined after autoclaving at 121 °C for 20 min (Zhang et al., 2006a), which was used as a positive control while the one in untreated spores was used as a negative control. HPCD-induced DPA release was calculated by the equation which is as follows:

$$\mathrm{DPA}\% = \frac{F_1 - F_0}{F_2 - F_0} \quad (1)$$