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Inactivation of *Bacillus stearothermophilus* and *Bacillus coagulans* spores as indicators of sterilization by reciprocal pressurization

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The effects of the reciprocal pressurization (six reciprocal cycles of 5 min pressurization) (RP) and the continuous pressurization (30-min pressurization) (CP) on the inactivation of spores of *Bacillus coagulans* and *Bacillus stearothermophilus* were studied. The combined parameters used were hydrostatic pressure (100, 200, 300 and 400 MPa), temperature (45, 55, 65 and 75 °C) and total pressurization period (30 min). RP was more effective than CP on inactivating bacterial spores. However, the effectiveness of RP on increasing the inactivation power was observed in hydrostatic pressure over 200 MPa.

INTRODUCTION

Dormant bacterial spores are highly resistant to many physical and chemical conditions including heat, drying, radiation and chemicals such as hydrogen peroxide (Gould 1983), and their inactivation is the main objective of food sterilization. Heat, radiation and chemical preservatives are the major means for the sterilization (Russell 1991). Of these methods, moist heat sterilization is the most commonly used (Walker & LaGrange 1991). However, the heat sterilization usually results in detrimental changes in the nutritive value, color and flavor of foods (Joslyn 1991).

On the other hand, high hydrostatic pressure can inactivate microorganisms without altering the flavor and nutrient components in foods (Cheftel 1992). Hence, major investigations are currently focussed on the potentials of hydrostatic pressure treatments as an alternative to heat treatments (Hoover et al. 1989). The effects of hydrostatic pressure treatments on the destruction of microorganisms were reported 100 years ago (Hite 1899), and the application of such technology to food processing has increased in the past decade (Hayashi 1992). In hydrostatic pressure sterilization, bacterial spores were more resistant than vegetative bacteria (Timson & Short 1965; Cheftel 1992), surviving up to 1200 MPa (Larson et al. 1918; Johnson and ZoBell 1949; Timson and Short 1965; Sale et al. 1970). Hence, it has been suggested that bacterial spores are poorly sterilized by the hydrostatic pressure treatment at room temperature (Sale et al. 1970).

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Therefore, the sterilizing effects of hydrostatic pressure on bacterial spores in a combination with heat (Gould 1973; Mallidis & Drizou 1991; Okazaki et al. 1994; Roberts & Hoover 1996), irradiation (Crawford et al. 1996), low pH (Roberts & Hoover 1996) and bacteriocins such as nisin (Roberts & Hoover 1996) have been studied.

We have been studying the impulsive force generated by quick decompression and its effect on the inactivation of bacterial spores (Hayakawa et al. 1998). From this work, we considered that the reciprocal compression and decompression of hydrostatic pressure could increase the injury and inactivation power on bacterial spores comparing to a continuous pressurization.

In the present paper, we report the effect of the reciprocal pressurization (RP) on the inactivation of bacterial spores of *Bacillus coagulans* and *B. stearothermophilus* and the comparison of its inactivation effect with continuous pressurization (CP).

The spore of *B. stearothermophilus* is the most heat-tolerant species among aerobic spore-forming bacteria. This microorganism is often used as a biological indicator to evaluate sterilization processes because of its high heat resistance (López et al. 1997; Periago et al. 1998).

MATERIALS AND METHODS

Bacterium

The bacteria used were *Bacillus coagulans* IFO12583 and *Bacillus stearothermophilus* IFO12550, obtained from the Institute for Fermentation Osaka (Osaka, Japan).

Media and culture conditions

Log-phase-cultures of *B. coagulans* and *B. stearothermophilus* grown in nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan) were transferred to soil-infusion-agar-plates (Berry & Brandshaw 1980), which consisted of nutrient agar (Eiken Chemical Co., Ltd., Tokyo, Japan) plus a soil extract. The plates were incubated at 37 °C (*B. coagulans*) and 55 °C (*B. stearothermophilus*) for 10 days.

Preparation of spore suspensions

Spores were collected by flooding the surface of the culture with sterile distilled water, and then scraping the surface with a sterile microscope glass slide. After collecting, the spores were washed three times by centrifugation at 4,000×g for 30 min, and resuspended in sterile distilled water and stored at 4 °C until use. The spore suspensions were diluted to give approximately 10^6 colony forming units (CFU) ml^{-1}.

Pressure treatment

Spore suspensions were sealed in 1.5 ml portions in sterile screw-capped plastic tubes (1.5 ml capacity; Greiner Labortechnik Co., Ltd., Germany), and these tubes were pressurized. The equipment used was a prototype pressurization apparatus (Hayakawa et al. 1994). The time needed to achieve the treatment pressure was between 10 and 30 s, depending on the required pressure. The decompression time was less than 1 s. The temperature of the pressure cell was regulated by a thermocontrolled water bath (Haake GH, Germany). Several combinations of hydrostatic pressure (100, 200, 300 and

400 MPa), temperature (45, 55, 65 and 75 °C), total holding period (30 min) and reciprocal time (1 and 6) were used in this study.

Measurement of survivors

The number of survivors was determined by the viable count method using a nutrient agar. The plates were incubated at 37 °C (*B. coagulans*) and 55 °C (*B. stearothermophilus*) for 24 h and then enumerated.

Statistical analysis

All experiments were done in triplicate. The data presented are the means of three replicate experiments. Significant differences between the RP and the CP treatment were determined by Student's t test ($P<0.05$).

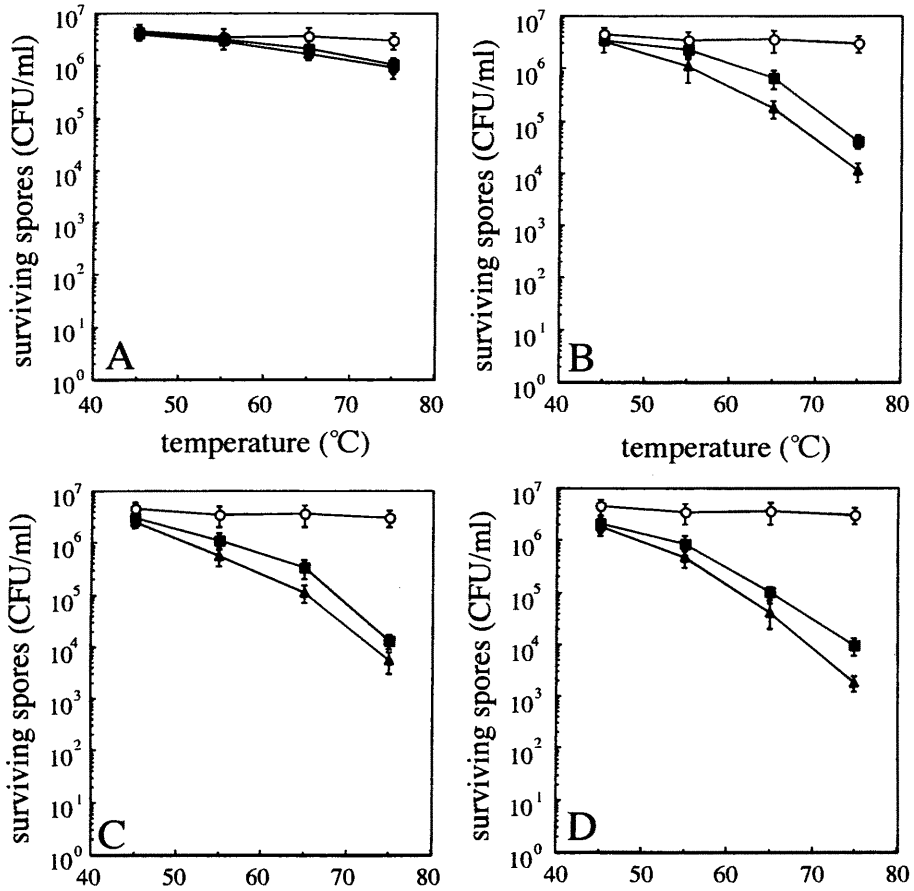

Fig. 1. Effect of the treatment temperature on the inactivation of *Bacillus coagulans* IFO12583 spores by the CP (■) and the RP treatment (▲) at (A) 100, (B) 200, (C) 300 and (D) 400 MPa or 30 min in distilled water. The symbol, ○, shows the control experiment (0.1 MPa).

RESULTS AND DISCUSSION

The number of spores inactivated by RP was more than that of spores by CP at pressure ranging from 100 to 400 MPa. There was no significant difference ($P<0.05$) between the RP and the CP treatment on the inactivation of *B. coagulans* spores at 100 MPa (Fig. 1A), but there was significant difference ($P<0.05$) at 200 MPa except for 45 °C and 55 °C (Fig. 1B), at 300 MPa except on for 45 °C and 55 °C (Fig. 1C), and at 400 MPa except for 45 °C and 55 °C (Fig. 1D). There was no significant difference ($P<0.05$) between the RP and the CP treatment on the inactivation of *B. stearothermophilus*

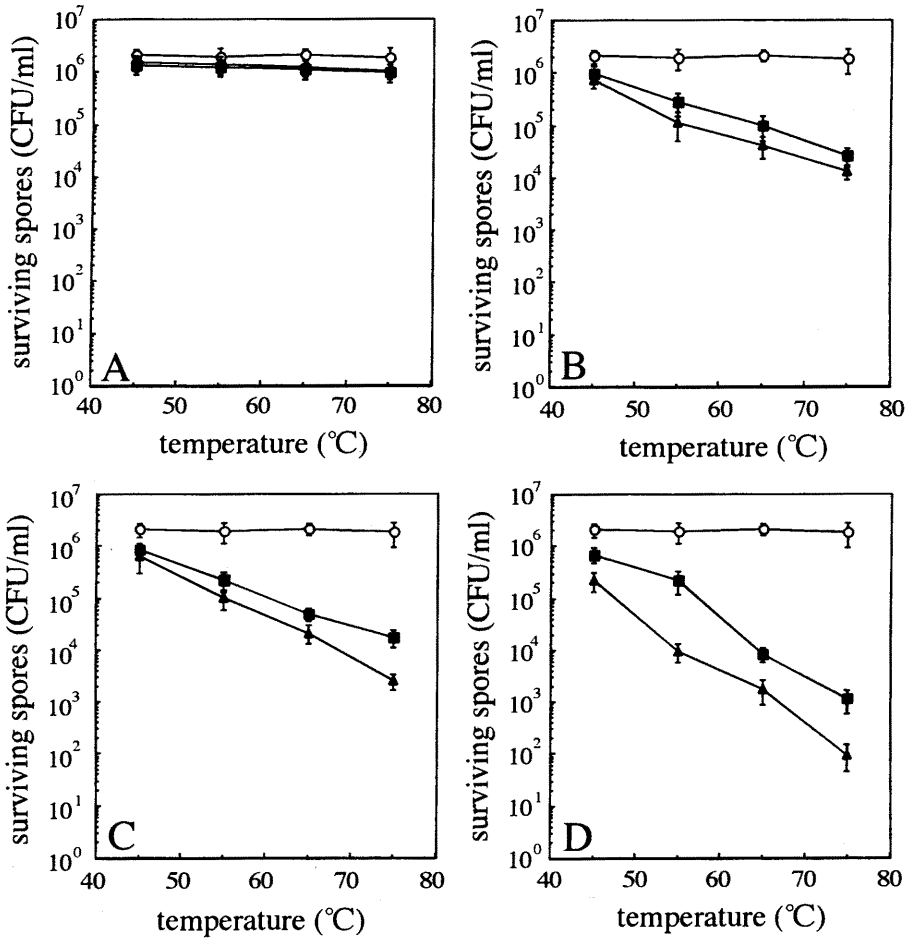

Fig. 2. Effect of the treatment temperature on the inactivation of *Bacillus stearothermophilus* IFO12550 spores by the CP (■) and the RP treatment (▲) at (A) 100, (B) 200, (C) 300 and (D) 400 MPa or 30 min in distilled water. The symbol, ○, shows the control experiment (0.1 MPa).

spores at 100 and 200 MPa (Fig. 2A and B), but there was significant difference ($P<0.05$) at 300 MPa except for 45 °C and 55 °C (Fig. 2C), and at 400 MPa (Fig. 2D). These results showed that RP was more effective in inactivating bacterial spores than CP. However, the effectiveness of RP on increasing the inactivation power was observed in hydrostatic pressure at least above 200 MPa.

With *B. stearothermophilus* spores, about 4–log–cycles inactivation was observed at 75 °C, 400 MPa (Fig. 2D). However, with *B. coagulans* spores, about only 3–log–cycles inactivation was observed at 75 °C, 400 MPa (Fig. 1D). These results showed that *B. coagulans* spores were more resistant to the CP and RP treatment, although *B. coagulans* spores were more heat sensitive than *B. stearothermophilus* spores (Shibasaki 1998). *B. coagulans* spores could be inactivated easily than *B. stearothermophilus* spores in combination with more intensive heat treatment.

The RP treatment was able to decrease the processing temperature and pressure needed to inactivate bacterial spores. Lowering the processing temperature can decrease heat damages of processed foods. From the viewpoint of reducing the cost of high pressure equipment (Kanda et al. 1992), decreasing the processing pressure is the most important point in the application of hydrostatic pressure treatment to food sterilization.

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