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Inactivation of spores of three *Bacillus* strains by low hydrostatic pressure (LHP) treatment

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The effect of low hydrostatic pressure (30, 50 and 100 MPa) (LHP) treatment on the inactivation of *B. subtilis*, *B. licheniformis* and *B. coagulans* spores in combination with heat treatment at 75 °C was investigated.

The inactivation of spores was in proportion to the increase of treatment pressure at 75 °C. *B. licheniformis* spores were more resistant than those of the other two strains to LHP treatment combined with heat treatment at 75 °C. The hydrostatic pressure above 50 MPa and the treatment time at least 700 min were necessary to inactivate *B. licheniformis* spores by five log-cycles.

INTRODUCTION

The effects of hydrostatic pressure treatments on the destruction of microorganisms were reported 100 years ago (Hite, 1899), the application of such technology to food preservation has increased over the past decade (Hayashi, 1992). From many studies, it was indicated that the hydrostatic pressure can inactivate microorganisms without altering the flavor and nutrient components of foods (Cheftel, 1992).

In hydrostatic pressure sterilization, bacterial spores were more resistant than vegetative cells (Cheftel, 1992; Timson and Short, 1965), surviving up to 1200 MPa (Johnson and ZoBell, 1949; Larson et al., 1918; Sale et al., 1970; Timson & Short, 1965). Hence, it has been suggested that bacterial spores are poorly sterilized by hydrostatic pressure treatment at room temperature (Sonoike, 1997).

Because of this, the sterilizing effect of hydrostatic pressure on bacterial spores in combination with heat (Gould, 1973; Mallidis and Drizou, 1991; Okazaki et al., 1994; Roberts and Hoover, 1996), irradiation (Crawford et al., 1996), low pH (Roberts and Hoover, 1996) and bacteriocins such as nisin (Roberts and Hoover, 1996) has been studied. Especially, combination with heat was effective means of accelerating the

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inactivation rates of bacterial spores.

High temperature is effective to inactivate microorganisms, but it degrades the quality of foods (Joslyn 1991). In general, many food products are cooked at below 100 °C; thus, we examined the sterilization of bacterial spores by hydrostatic pressure treatment combined with heating below 100 °C.

In previous paper, we reported the effectiveness of low hydrostatic pressure (LHP: 10–100 MPa) treatment combined with the heat treatment (55–95 °C) on the inactivation of heat-tolerant spores of *B. stearothermophilus* IFO12550 (Furukawa & Hayakawa 2000; Furukawa & Hayakawa 2001).

B. stearothermophilus spores were inactivated effectively at 95 °C by LHP treatment (Furukawa & Hayakawa 2000; Furukawa & Hayakawa 2001), and *B. subtilis* (D95 °C= 2.31 min), *B. licheniformis* (D95 °C=2.80 min) and *B. coagulans* (D95 °C=240 min) spores were more heat sensitive than *B. stearothermophilus* (D95 °C=2260 min). Then, this time, we report the effect of LHP treatment on the inactivation of *B. subtilis*, *B. licheniformis* and *B. coagulans* spores in combination with heat treatment at 75 °C.

MATERIALS AND METHODS

Bacteria

The bacteria used were *Bacillus subtilis* IFO13722, *Bacillus licheniformis* IFO12200 and *Bacillus coagulans* IFO12583, obtained from the Institute for Fermentation Osaka (Osaka, Japan).

Media and culture conditions

Log-phase-cultures of *B. subtilis*, *B. licheniformis* and *B. coagulans* 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 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 0.1 s. The temperature of the pressure cell was regulated by a thermocontrolled water bath (Haake GH, Germany). Several combinations of hydrostatic pressure (30, 50 and 100 MPa),

temperature (75 °C) and total holding period (60 to 720 min) were used in this study.

Measurement of survivors

The number of survivors was determined by the colony count method using a nutrient agar. The plates were incubated at 37 °C for 24 h and then enumerated.

Statistical analysis

All experiments were done in triplicate. The data presented are the means of three replicate experiments.

RESULTS AND DISCUSSION

The effect of pressure on the inactivation of *B. subtilis* spores was investigated (Fig. 1). The inactivation of spores increased in proportion to the increase of treatment pressure at 75 °C. Spores were sterilized in 720 min treatment at 50 MPa, and in 180 min treatment at 100 MPa.

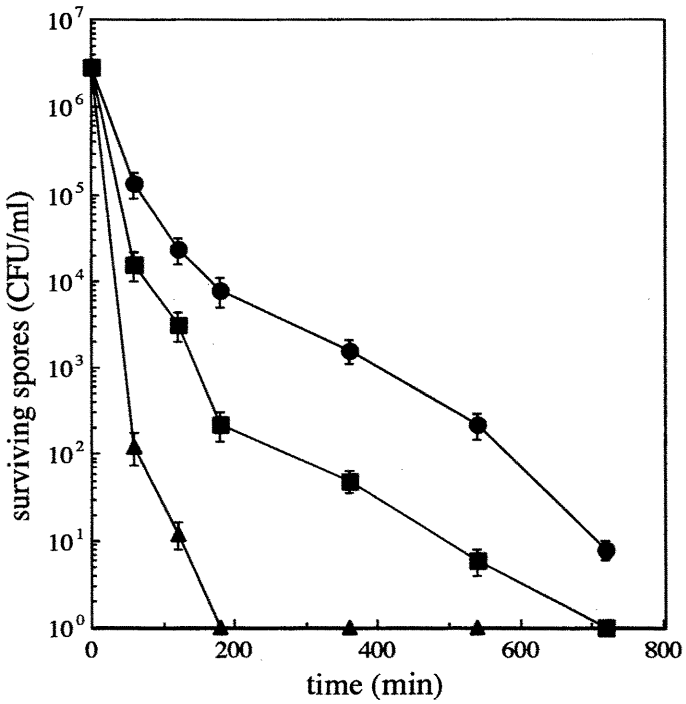

Fig. 1. Survivor curves of *B. subtilis* spores in distilled water pressurized at 75 °C. The treatment pressures used were at 30 (●), 50 (■) and 100 (▲) MPa.

The effect of pressure on the inactivation of *B. licheniformis* spore was investigated (Fig. 2). The inactivation of spores increased in proportion to the treatment pressure at 75 °C. Spores, however, were not sterilized even in 720 min treatment at 100 MPa.

The effect of pressure on the inactivation of *B. coagulans* spores was shown in Fig. 3. The inactivation of spores increased in proportion to the treatment pressure at 75 °C, and spores were sterilized in 720 min treatment at 50 MPa, and in 540 min treatment at 100 MPa.

From these results, *B. licheniformis* spores were more resistant than the those of other two strains to LHP treatment combined with heat treatment at 75 °C. In the sterilization process for manufacturing commercial foods, the sterilization time is calculated on the basis of the 5×D (Decimal reduction time) concept (Lund 1977). Therefore, the hydrostatic pressure above 50 MPa and the treatment time of approximately 100 to 500 min were necessary to inactivate *B. lsubtilis* spores by five log–cycles. On *B. licheniformis* spores, the hydrostatic pressure of 100 MPa and the treatment time at least 700 min were necessary to inactivate them by five log–cycles. On *B. coagulans* spores, the hydrostatic pressure above 50 MPa and the treatment time of

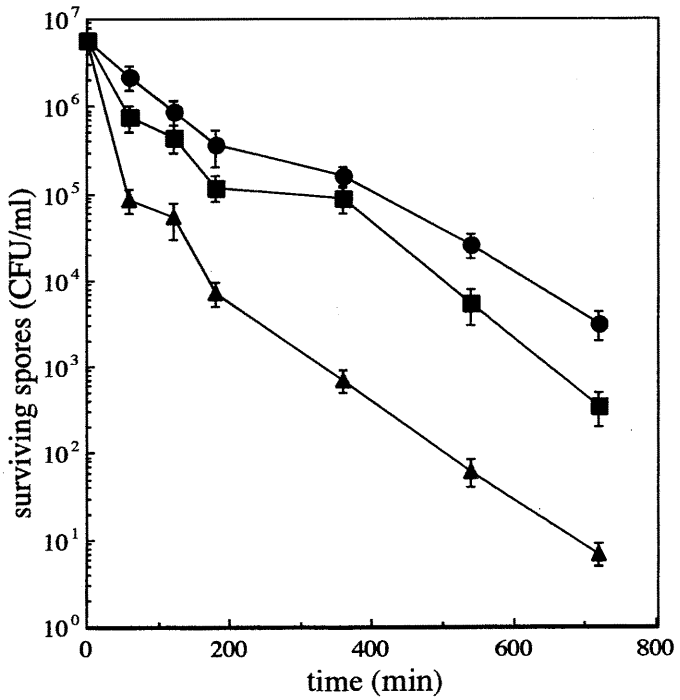

Fig. 2. Survivor curves of *B. licheniformis* spores in distilled water pressurized at 75 °C. The treatment pressures used were at 30 (●), 50 (■) and 100 (▲) MPa.

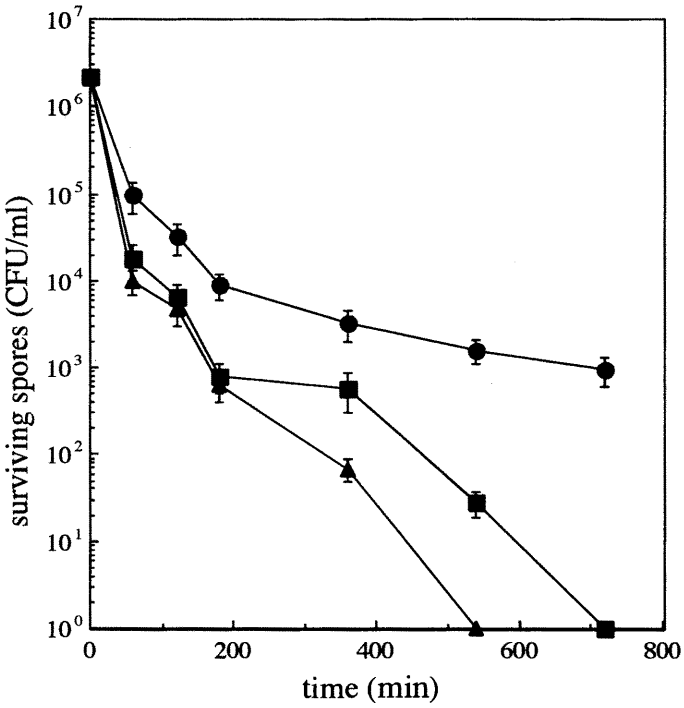

Fig. 3. Survivor curves of *B. coagulans* spores in distilled water pressurized at 75 °C. The treatment pressures used were at 30 (●), 50 (■) and 100 (▲) MPa.

approximately 500 to 600 min were necessary to inactivate them by five log-cycles.

Spores of *B. licheniformis* (D95 °C=2.80 min) were more sensitive to heat treatment but more resistant to LHP treatment than *B. coagulans* (D95 °C=240 min).

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