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https://doi.org/10.5109/24402

出版情報：九州大学大学院農学研究院紀要. 45 (2), pp.519-523, 2001-02-28. Kyushu University
バージョン：
権利関係：

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J. Fac. Agr., Kyushu Univ., **45** (2), 519–523 (2001)

Some aspects of the germination of *B. stearothermophilus* IFO12550 spores by hydrostatic pressure below 40 MPa

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(*Received October 26, 2000 and accepted November 10, 2000*)

The effect of temperature in hydrostatic pressure treatment on the germination of *B. stearothermophilus* spores was investigated. There were optimum temperatures in the germination ratio in the treatment at 10, 20, 30 and 40 MPa for 720 min. There was also an optimum temperature on the germination of *B. stearothermophilus* spores by chemical germinants at 0.1 MPa (under atmospheric pressure). Therefore, it was considered that the cause of optimum temperature of inactivation was the optimum temperature of germination enzymes under hydrostatic pressure below 40 MPa.

INTRODUCTION

Bacterial spores were much more resistant than vegetative cells in a hydrostatic pressure sterilization (Timson & Short 1965; Cheftel 1992), surviving up to 1200MPa (Johnson & ZoBell 1949; Larson et al. 1918; Sale et al. 1970; Timson & Short 1965). Therefore, the hydrostatic pressure treatments combined with heat were attempted to inactivate bacterial spores (Gould, 1973; Mallidis & Drizou 1991; Okazaki et al. 1994; Roberts & Hoover 1996).

We indicated that extraordinary heat resistant spores of *B. stearothermophilus* were effectively inactivated by the hydrostatic pressure below 100 MPa in a combination with heat (Furukawa & Hayakawa 2000). Further investigation showed that there was an optimum temperature in the inactivation of the spores at the pressure below 40 MPa (Furukawa & Hayakawa 2001). Therefore, it was found that the combination with heat was not always effective in the inactivation of the spores by hydrostatic pressure treatments. Presence of the optimum temperature evidently contradicted the established knowledge in the conventional heat sterilization (Joslyn 1991).

This time we investigate the optimum temperature in the germination of *Bacillus stearothermophilus* IFO 12550 spores by hydrostatic pressure treatment.

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MATERIALS AND METHODS

Bacterium

The bacterium used was *Bacillus stearothermophilus* IFO12550, obtained from the Institute for Fermentation Osaka (Osaka, Japan).

Media and culture conditions

Log–phase cultures of *B. stearothermophilus* IFO12550 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 55 °C for 10 days.

Preparation of spore suspension

Spores were collected by flooding the surface of the culture with sterile distilled water, and then scraped 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 in sterile distilled water, and resuspended in a 0.067 M phosphate buffer (pH 7.0) 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 1 and 10 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 (10–40MPa), temperature (55–95 °C) and total holding time (720 min) were used in this study.

Measurement of ungerminated spores

After the pressure treatments, samples (1.5 ml) were submerged in a water bath equilibrated at 100 °C for 30 min to inactivate germinated spores (Gould & Sale 1970). Surviving heat resistant spores (ungerminated spores) were then enumerated by the viable colony count method using nutrient agar. The plates were incubated at 55 °C for 24 h, then colonies were enumerated. The viability of ungerminated spores of *B. stearothermophilus* was unaffected by treatment at 100 °C for 300 min.

Germination by chemical germinants

Germination of *B. stearothermophilus* was initiated by the addition of chemical agents (KCl, L–alanin, D–glucose and D–fructose) at 35, 45, 55, 65 and 75 °C (Hachisuka 1988). The concentrations of these chemical agents were 0.1 M (KCl), 0.1 M (L–alanin), 0.1 M (D–glucose) and 0.1 M (D–fructose).

Measurement of germination by chemical germinants

Germination was measured as the reduction in optical density (OD) (expressed as a percentage of the initial OD) at 650 nm (Hachisuka et al. 1955).

Statistical analysis

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

RESULTS AND DISCUSSION

Figure 1 shows the effect of temperature in hydrostatic pressure treatment on the germination of *B. stearothermophilus* spores. The spores were treated at 10, 20, 30 and 40 MPa for 720 min. Germination was observed at every treatment and was accelerated with the increase in the treatment pressure. There were optimum temperatures in the germination ratio, and these values corresponded to the optimum temperatures of

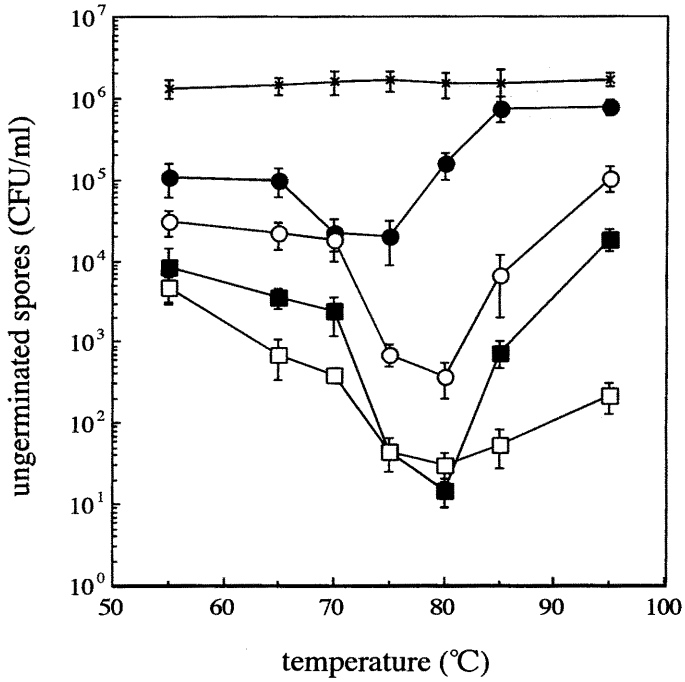

Fig. 1. Effect of temperature in hydroatatic pressure treatment on ungerminated spores of *B. stearothermophilus*. Treatment conditions: 10 (●), 20 (○), 30 (■) and 40 (□) MPa for 720 min in 0.067 M phosphate buffer (pH 7.0). The symbol, ×, shows the control experiment (0.1 MPa).

inactivation, these were investigated in previous paper (Furukawa & Hayakawa 2001). The optimum temperature was 75 °C at 10 MPa and 80 °C at 20, 30 and 40 MPa.

The germination of bacterial spore has been defined as a series of interrelated degradation events by some spore–lytic enzymes (Foster & Johnstone 1990; Moir & Smith 1990). It was also considered that the germination of bacterial spores by hydrostatic pressure was a series of reaction of the germination enzymes (Gould & Sale 1970).

There was an optimum temperature on the germination of *B. stearothermophilus* spores by chemical germinants at 0.1 MPa (under atmospheric pressure) (Fig. 2). The optimum temperature was almost 55 °C, since the germination was little observed at 45 and 65 °C. This result corresponded to the previous study (Mol 1957).

The optimum temperature of the germination by chemical germinants at 0.1 MPa could be attributed to the optimum temperature of germination enzymes. Therefore, we considered that the cause of optimum temperature of inactivation was the optimum temperature of germination enzymes under hydrostatic pressure below 40 MPa.

The optimum temperature of germination increased from 55 °C at 0.1 MPa to 75 or 80 °C in hydrostatic pressure treatment. This results suggested that the optimum temperature of germination enzymes increased under the hydrostatic pressure.

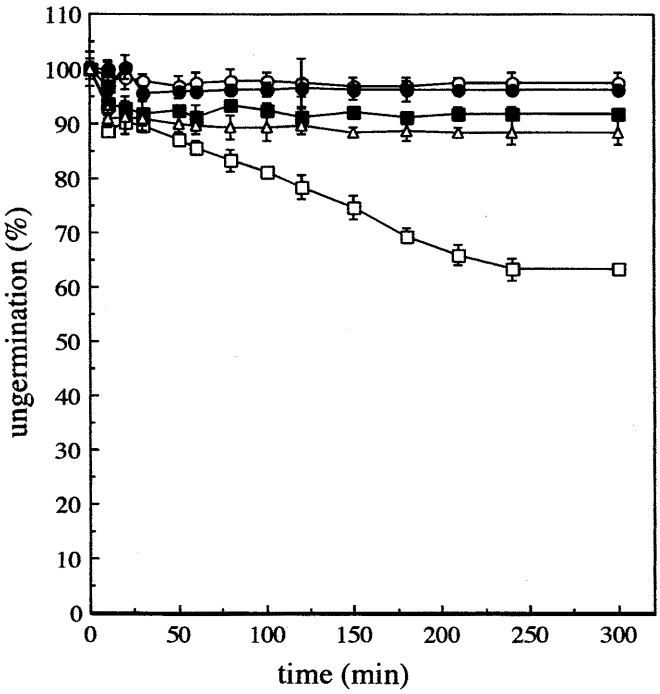

Fig. 2. Effect of temperature on the germination induced by chemical agents of *B. stearothermophilus* spores at 35 (○), 45 (●), 55 (□), 65 (■) and 75 (△) °C in 0.067 M phosphate buffer (pH 7.0).

ACKNOWLEDGMENT

A part of this research was supported by grant of Scientific Research Fund from the Ministry of Education, Science, Sports and Culture.

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