STERILIZATION OF *BACILLUS CEREUS* SPORES BY A COMBINATION OF HIGH-PRESSURE TREATMENT AND SUBSEQUENT HEAT TREATMENT

Miyuki Ogino* and Tadayuki Nishiumi

Graduate School of Science and Technology, Niigata University, Niigata, 950-2181, Japan

*Corresponding author email: f14m006j@mail.cc.niigata-u.ac.jp

Abstract – We found the feasibility of sterilization by applying a pressure treatment of 200 MPa before heat treatment. The wet density of spores of *Bacillus cereus* was decreased by the pressure history a high-pressure treatment of 200 MPa was applied. When the spores were subsequently heated at around 100°C, the sterilization of spores was promoted. Furthermore, we found the turbidity of the spore suspensions greatly decreased after the pressure reduction step of the high-pressure treatment. The decrease of turbidity was also found to be highly correlated with the survival spore counts after the heat treatment. Therefore the turbidity was considered to be a sterilization indicator.

Key Words – heat-resistant spores, high-pressure treatment, sterilization

I. INTRODUCTION

Heat sterilization has been widely used as a method of sterilizing microorganisms in food. However, it is known that a deterioration of taste and flavor occurs due to excessive heating for the purpose of the sterilization of heat-resistant spores [1]. Therefore, the development of a sterilization technique using a temperature of approximately 100°C, a general cooking range, is desired. High-pressure treatment, one of the non-thermal food processing methods, is a technology which retains food quality, maintains natural freshness, and extends microbiological shelf life [2]. In meat and meat products, high pressure can contribute to improving physical and functional properties as well as prolonging shelf life [3]. It has been shown that the combination of high pressure at 300-400 MPa and 0.4 M sodium hydrogen carbonate treatment is effective to improve the quality of meat including its tenderness and color [4]. Recently, high-pressure processed foods are attracting attention in the EU and the United States as a non-thermal sterilization technique. However, heat-resistant spores have been shown to be difficult to sterilize by high-pressure treatment alone [5].

We found that a high-pressure treatment of 200 MPa reduced the heat resistance of the spores of *Bacillus coagulans*, and that the number of surviving bacteria was greatly reduced by a subsequent heat treatment at about 100°C [6]. The heat resistance of spores is known to be dependent on the water content of the spores [7]. Therefore, we presumed that the lowering of the heat resistance of spores is due to the increase in the water content of the spores, brought on by the high-pressure treatment. In this study, spores of *B. cereus*, which is a major microorganism present in agricultural products, were subjected to high-pressure treatment. Osmosis of water into spores and turbidity of the spore-suspended solutions during and after high-pressure treatment were investigated. The heat resistance of spores was also investigated.

II. MATERIALS AND METHODS

Osmosis of water into spores after high-pressure treatment

B. cereus NBRC13494 strain was used throughout this study. The spore suspension of *B. cereus* was prepared according to a previously described method [8]. Spore suspensions in 0.067 M phosphate buffer were pressurized to a hydrostatic pressure at 200 MPa at 25°C for 10 min. The compression and decompression rates

were 100 MPa/min. Wet density was measured using an universal density gradient medium, Nycodenz (Axis-Shield Diagnostics Ltd., Scotland). The density of the aqueous solutions of Nycodenz were adjusted and overlaid in a 30 mL centrifuging tube every 2 mL in a descending order of density. A 0.1 mL spore suspension was dispensed on the uppermost layer of the solution and then centrifuged at 10,000 *g* at 25°C for 30 min. Since the spores were gathered band-like in a solution of their same densities by the centrifugation, the wet density of spores could be measured.

Measurement of the turbidity of the spore suspensions during high-pressure treatment

The optical density of spore suspensions of *B. cereus* was adjusted to 0.9 at 625 nm in 0.067 M phosphate buffer (pH 7.0). The changes of turbidity during high-pressure treatment were measured using an inner-cell type high-pressure optical cell, a small high-pressure pump (Shin Corporation, Kyoto, Japan) and a spectrophotometer (HITACHI, Tokyo, Japan). The spore suspensions were pressurized at 200 MPa at 25°C for 2 min. The compression and decompression rates were 200 MPa/min. The turbidity was then measured for 60 min from the beginning of the increase in the pressure.

Measurement of heat resistance of spores by high-pressure treatment

The spore suspensions of *B. cereus* with 0.067 M phosphate buffer were pressurized at 200 MPa at 50°C for 10 min, and after a lapse time of more than 10 min, the suspensions were heated in thermal death time (TDT) tubes at 90°C, 100°C and 110°C for 1 to 30 min respectively. The number of surviving spores cultured on a standard agar medium was measured by using the pour plate method.

Measurement of turbidity after high-pressure treatment and the number of surviving spores by heat treatment

The spore suspensions of *B. cereus* with 0.067 M phosphate buffer were pressurized at 200 MPa at 25°C for 1 min. After decompression, they were left at 25°C and the turbidity was measured at these times: immediately after decompression, 3 min after, 10 min after and 120 min after, using a spectrophotometer. Finally, the number of surviving spores was measured after a heat treatment at 100°C for 30 min.

III. RESULTS AND DISCUSSION

Osmosis of water into spores after high-pressure treatment

Compared to the wet density of untreated spores, the wet density of pressurized spores was moved to the low-density side (Table 1). Therefore, the lowering of wet density by high-pressure treatment was confirmed and the penetration of water into spores was suggested.

Table 1. Changes in wet densities of *Bacillus* spores suspended in 0.067 M phosphate buffer.

	Wet density (g/mL)
	B. cereus NBRC13494
Control (non-treated spores)	1.238 ~ 1.286
High-pressure treated spores	1.190 ~ 1.238
Vegetative cells	1.190 ~ 1.238

Wet densities were measured by density gradient centrifugation. Condition of high-pressure treatment was 200 MPa at 25℃ for 10 min.

Change in turbidity of the spore suspensions during and after high-pressure treatment

The turbidity was reduced gradually from the start of pressurization to the end of decompression, and then it fell drastically until the 10 min point (Fig. 2).

Reduction of turbidity which is one of the indexes of spore germination, is a phenomenon that appears after the lapse of some time following the start of germination [9]. However, in our previous reports, a darkening-like phenomenon had occurred in the phosphate buffer that did not contain nutritive ingredients required for germination [6,10]. In this experiment, we revealed that the turbidity is greatly reduced immediately after the end of decompression of the high-pressure treatment. From these findings, it is considered that a relation of spores and water caused by a physical phenomenon reduced the turbidity after high-pressure treatment.

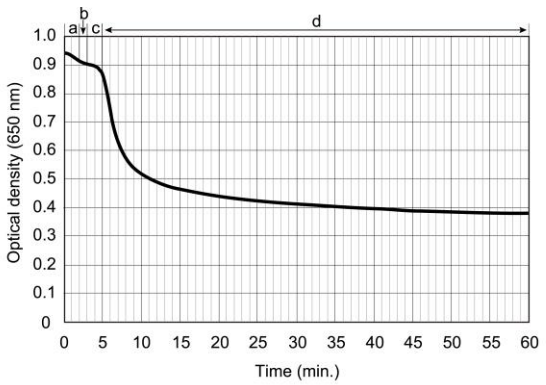

Fig. 2 Change in optical density at 650 nm of *B. cereus* NBRC13494 spores suspended in 0.067 M phosphate buffer left at 25°C after high-pressure treatment (200 MPa at 25°C for 1 min.). a) Pressure rising process (2 min.) b) Pressure holding process (1 min.) c) Pressure reducing process (2 min.) d) After pressure reduction.

The effect of high-pressure treatment on the heat resistance of spores

The counts of surviving spores from the samples subjected to heat treatment after high-pressure treatment were reduced significantly. In particular, the sample treated at 110°C fell to below the detection limit 5 min after heating (Fig. 3).

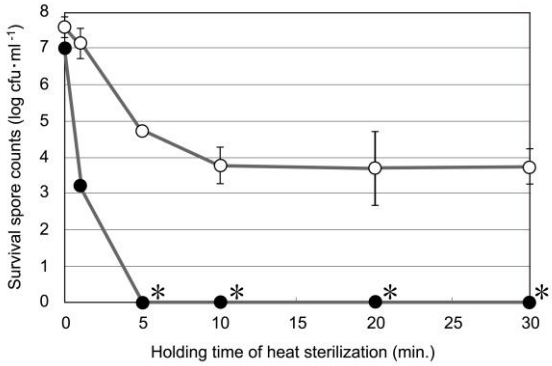

Fig. 3 Relationship between holding time of heat sterilization (110°C) and survival spore counts of *B. cereus* NBRC13494 suspended in a 0.067 M suspension of phosphate buffer (n=3). ○: Control samples only subjected to heat sterilization at 110°C
●: Samples subjected to high-pressure treatment (200 MPa at 50°C for 10min.) before heat sterilization ＊: Not detected.

Therefore, when a high-pressure treatment of 200 MPa was administered in advance, the number of surviving spores was greatly decreased by the subsequent heat treatment and the lowering of heat resistance was confirmed. As shown in Fig. 2, since the turbidity after decompression was greatly reduced, the hydrophobicity of the spores' surfaces was reduced and the water wettability was increased by high-pressure treatment. Thereby, it is presumed that water could easily flow into spores and external heat was easily transmitted through the water, thus, lowering heat resistance causing spores to be sterilized with lower temperatures.

Relationship of the changes in turbidity and the number of surviving spores after high-pressure treatment

It became clear that the elapsed time after the pressure reduction affected the survival spore counts when heat treatment was subjected subsequently (Fig. 4).

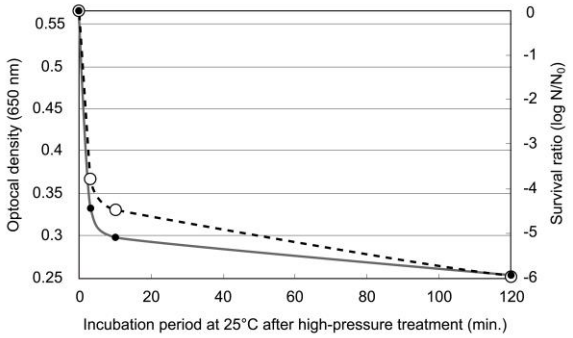

Fig. 4 Relationship of optical density at 650 nm (OD_{650}) and reducing survival spore counts of *B. cereus* NBRC13494 spores in incubation periods at 25°C after high-pressure treatment (200 MPa at 25°C for 1 min.). ●: OD_{650} after high-pressure treatment ○: Reducing survival spore counts after heat sterilization (N/N_0). N_0: Initial survival spore counts, N: Survival spore counts after heat sterilization followed high-pressure treatment.

It was observed that the reduction of turbidity after high-pressure treatment highly correlated with the number of surviving spores after heat treatment followed high-pressure treatment (r = 0.9899). From these results, it is thought to be possible to predict the effects of heat sterilization by looking at the pressure history [11]. We can predict the survival rate of spores from the turbidity and the heating temperature by using

microorganism-specific factors as parameters in advance.

IV. CONCLUSION

Until now, heat-resistant spores had been thought to be difficult to sterilize by pressure alone and by pressure plus concurrent heat. However, it has been shown that heat resistant spores can be sterilized or greatly reduced by lowering their heat resistance by subjecting pressure of about 200 MPa plus subsequent heat treatment. This suggests the possibility of sterilization or reduction of bacteria at lower temperatures than conventional heat sterilization temperatures for a number of bacterial species. Furthermore, the technique for predicting the survival spore count after heat sterilization by measuring the decrease in turbidity is considered to be an indicator for managing microorganisms according to the characteristics of each food. Therefore, this technique is effective as an application to food manufacturing.

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REFERENCES

1. Miyamoto, T. (2009). Growth characteristics and control of bacterial spore in foods. Japanese Journal of Food Microbiology 26: 92-97.
2. Yamamoto, K. (2009). Food processing by utilizing a high-pressure. Journal of Cookery Science of Japan 42: 417-423.
3. Jofré, A. & Serra, X. (2016). Processing of meat products utilizing high pressure. In V. M. Balasubramaniam, G. V. Barbosa-Cánovas, & H. L. M. Lelieveld, High pressure processing of food (pp 591-623) New York: Springer.
4. Nishiumi, T., Ohnuma, S., Watanabe, Y. & Sakata, R. (2014). Combined effects of high pressure and sodium hydrogen carbonate treatment on beef texture and color. In Proceeding 60th International Congress of Meat Science and Technology, 17-22 August 2014, Punta Del Este, Uruguay.
5. Sonoike, K. (1997). High pressure sterilization technology. Subject for the application to food. Nippon Shokuhin Kagaku Kogaku Kaishi 44: 522-530.
6. Kobayashi, A., Kawamura, M., Ohara, E., Ogino, M., Hoshino, J., Yamazaki, A. & Nishiumi, T. (2014). Application of high-pressure treatment to sterilization of foods. The Review of High Pressure Science and Technology 24: 48-51
7. Beaman, T. C., Greenamyre, J. T., Corner, T. R., Pankratz, H. S. & Gerhardt, P. (1982). Bacterial spore heat resistance correlated with water content, wet density, and protoplast/sporoplast volume ratio. Journal of Bacteriology 150: 870-877
8. Kondo, M. & Watabe, K. (1995). Spore experimental manual. In H. Shibata, T. Tsuzuki, M. Tsuboi, & K. Watabe, Preparation method of spores (pp 19-42). Tokyo: Gohodoshuppan, Co., Ltd.
9. Levinson, H. S. & Hyatt, M. T. (1966). Sequence of events during *Bacillus megaterium* spore germination. Journal of Bacteriology 91: 1811-1818
10. Ogino, M., Kobayashi, A., Kawamura, M., Ohara, E., Hoshino, J., Yamazaki, A. & Nishiumi, T. (2014). Application of high-pressure treatment to sterilization of foods. Special Issue of the Review of High Pressure Science and Technology 24: 164.
11. Yamazaki, A. (2005). Application of high pressure and its effects on rice grains and rice starch. Foods & Food Ingredients Journal of Japan 210: 29-36.