Sterilization of *Bacillus subtilis* in Powdered Food by Iterative Processing of Instantaneous High-Pressure

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Powdered foods, primarily made from cereal grains, carry a high risk of contamination by soil bacteria at the time of harvest. However, due to the low thermal conductivity of powdered food and the spore-forming nature of soil bacteria, thermal sterilization is challenging. Currently, iterative processing with instantaneous high pressure generated by shock waves is a promising method for destroying bacterial cells without heat. In this study, rice flour, inoculated with *Bacillus subtilis* spores, was processed iteratively using instantaneous high pressure. The processing conditions were examined based on the number of iterations. The cultivation of *B. subtilis* and the observation of the cells by scanning electron microscopy were used as the evaluation method. As a result, 99.5% inactivation of *B. subtilis* was achieved. Additionally, deformed bacterial bodies and bacterial aggregation were observed. Therefore, the iterative processing using instantaneous high pressure can ensure the safety and security of powdered food.
Keywords: Heating, Shock waves, Sterilization, High pressure (Physics), Food products, Heat, Pressure

1. INTRODUCTION

Powdered foods has a low moisture content; therefore, it is difficult for bacteria to grow in it. Furthermore, powdered foods are subjected to secondary processing. Since powdered food is typically consumed to eating after heating, the risk of food poisoning is minimized. Soil bacteria, such as those from the *Bacillus* genus, adhere to the grain, which is the raw material for powdered food, during harvesting. Moreover, these bacteria are known to form spores that have high heat resistance. This indicates that the need for sterilization of powdered food cannot be ignored.

Safety assurance in microbiology, heat sterilization is common. However, heat conductivity of powder is poor because air exists among the particles. Therefore, heat sterilization is difficult to maintain for the powder food's quality and reliability of sterilization (Isobe, 2010). On the other hand, UV irradiation sterilization is unsuitable for overlapping powder particles (Takemoto et al., 2007). Radiation sterilization is prohibited for use in food except for potatoes (Shibazaki, 1998).

In this study, we examined the inactivation of spore-forming bacteria through destruction using iterative processing of instantaneous high pressure generated by shock waves. The instantaneous high-pressure was generated by underwater shock waves created by a discharge from a high-voltage power supply. A shock wave is a high-pressure wave that transmits at a supersonic speed. The speed of sound in water is approximately 1,300 m/s. Consequently, the time required for the shock wave to transmit through a bacterial diameter of about 3 μm is instantaneous, lasting only several tens of picoseconds. Therefore, we refer to this phenomenon as "the instantaneous high pressure." The instantaneous high pressure, that is, the shock wave is transmitted through the material at a speed that exceeds the speed of sound and is divided into a penetration wave and a reflected wave at the surface of the density difference in material. The expansion wave travels at a speed below that of sound. The reflected wave on the high-density side generates pull stress due to negative pressure, which causes an exfoliation effect (Spalling destruction) at the surface of the density difference. This high-speed destruction phenomenon is termed spalling destruction. Spalling destruction also affects bacterial cells (Takemoto et al., 2007). By accumulating damage to the spores through iterative processing, a surely destructive action can be expected.

2. EXPERIMENTAL CONDITIONS

2.1 Samples

In this study, the target spore-forming bacteria for sterilization were *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 (NBRC 3134), transferred from the National Institute of Technology and Evaluation (Tokyo, Japan). The powdered food used was rice flour.

2.2 Preparation of *B. subtilis* spores

First, sterile water (3.0 mL) was added to the agar medium on which *B. subtilis* had been cultured, and this solution was transferred to a 15 mL plastic tube. The composition of the medium was: 10 g/L of polyptone, 2.0 g/L of yeast extract, 1.0 g/L of $MgSO_4 \cdot 7H_2O$, and 15 g/L of agar. Next, 5.0 mL of sterile water was added to the tube, diluting the bacterial solution 1.7 times. The mixture was then centrifuged at 3,000 rpm for 10 min, at 4°C, and the supernatant was discarded. This washing procedure was repeated twice.

Afterward, a heat treatment at 80°C for 10 min was applied to kill vegetative cells. The resulting *B. subtilis* solution, which contained only spores, was refrigerated and stored at 4°C. The conditioned state of the bacterial spore solution was confirmed by optical microscopy using the Wirtz method. The bacterial solution was applied to a glass slide, warmed for 5 min with a 5% malachite green aqueous solution, and washed with running water for 30 s. Thereafter, the cells were stained with an aqueous solution of 0.25% safranine, washed again with water, and dried. After this processing, the glass slides were observed under an optical microscope at 1,000× magnification (Figure 1). Green spores were confirmed, and no vegetative cells were observed.

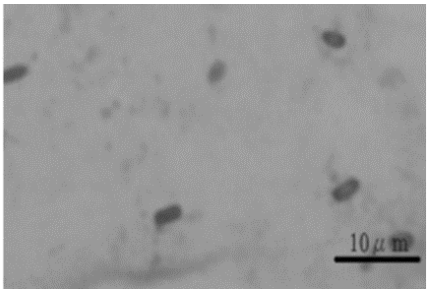

Figure 1 *B. subtilis* observed by optical microscopy using the Wirtz method.

2.3 Sterilization by instantaneous high pressure

5.0 g of rice flour was weighed into a silicone tube and 100 μl of 1.0×10^6 CFU/ml spore solution was added. The silicone tube (18 mm i.d., 22 mm o.d., 105 mm in length), with both ends sealed, was used to completely isolate the rice flour from the water and shockwave generation source. The silicone tube was set in an instantaneous high-pressure processing apparatus to process the instantaneous high pressure created by a discharge from a high-voltage power supply. Four patterns (50 times, 100 times, 150 times, and 200 times) of instantaneous high pressure were processed under the conditions shown in Table I.

Table Ⅰ The generation condition of instantaneous high pressure

Voltage	3.5 kV
Capacitance	800 μF
Energy	4.9 kJ

2.4 Evaluation of sterilization effect by culture

The sample was suspended in 200 mL of sterile water, and 100 μL of the suspension was applied to 15–20 plate mediums of the same composition as described in section 2.2. The medium was incubated at 30°C for 3 days. The number of viable bacteria was measured based on the resulting colonies, and the sterilization rate was calculated using the formula (1):

$$\text{Sterilization rate (\%)} = \left(1 - \frac{sample(CFU/g)}{control(CFU/g)}\right) \times 100 \qquad (1)$$

2.5 Evaluation of sterilization effect by scanning electron microscope

Instantaneous high pressure was applied to the spores obtained by lyophilizing the spore suspensions. The samples were immobilized with glutaraldehyde and then lyophilized again, followed by platinum evaporation through ion sputtering. The cells were observed under a scanning electron microscope (SEM; S-3600N, Hitachi High-Technologies Corporation, Tokyo, Japan)).

2.6 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) of two independent experiments. Statistical analyses were performed using one-way ANOVA followed by Tukey's test. $p < 0.05$ was considered significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan; Kanda, 2013), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander (version 1.68) designed to add statistical functions frequently used in biostatistics.

3. RESULTS AND DISCUSSION

The results showing the number of surviving spores are presented in Figure 2. While the viable cell count of the control sample was 5.4×10^4 CFU/g, the count was reduced to 1.4×10^4 CFU/g after 50 times of instantaneous high-pressure processing. After 150 times, the viable cell count further decreased to 4.6×10^3 CFU/g. A sterilization rate of 91.4% was achieved with 150 times of instantaneous high-pressure processing. Additionally, 99.4% of spores were sterilized after 200 times. This

indicates that with instantaneous high-pressure processing, the number of viable bacteria decreased to 10^{-2} or less compared to that in the control sample. Therefore, it can be concluded that iterative processing with 200 times of instantaneous high pressure can effectively sterilize powdered food.

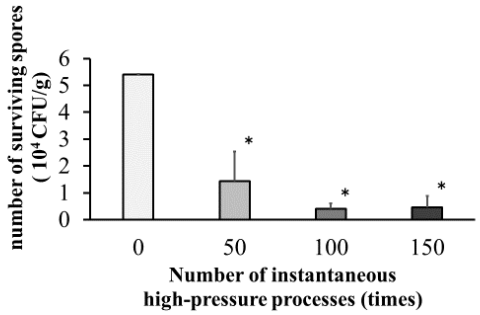

Figure 2 The number of surviving spores. Data represent the mean ± SEM of two independent experiments. $*p < 0.05$ versus control sample.

SEM images of the control and instantaneous high-pressure-processed *B. subtilis* are shown in Figure 3. Comparing the two images, many neat-shaped spores were observed in the control sample. In contrast, it was confirmed that 20–30 *B. subtilis* spore conglutinated after undergoing instantaneous high-pressure processing, and individual spores exhibited deformation.

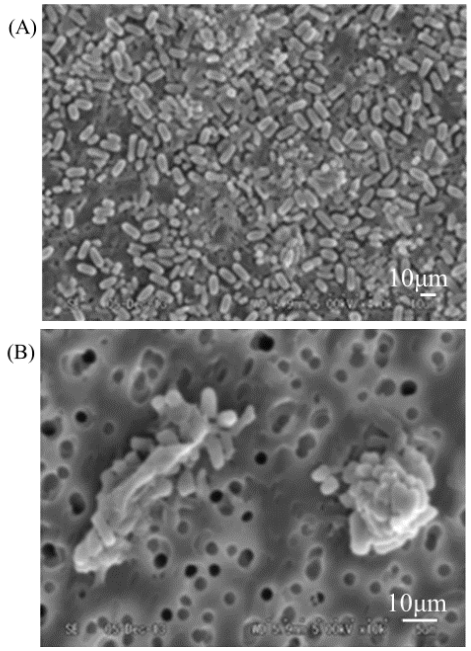

Figure 3 SEM images of *B. subtilis* spores: (A) control sample; (B) instantaneous high-pressure-processed sample.

4. CONCLUSION

This study evaluated the inactivation of *B. subtilis* spores via iterative processing using instantaneous high-pressure. By processing the instantaneous high-pressure with an energy value of 4.9 kJ 200 times, an inactivation rate of 91.4% was achieved, reducing the viable bacteria count to 10^{-2} or less.

This result demonstrates that the method is effective for spore inactivation.

Instantaneous high-pressure processing is not significantly affected by thermal metamorphism because the propagation time is extremely short—about several microseconds. This suggests that the technique is highly effective for sterilization without affecting the quality of powdered food.

Additionally, SEM observations confirmed inactivated spores, with deformation of the individual and multiple conglutinated spores observed. This is likely due to collisions between spore bodies and spalling destruction. These results suggest that *B. subtilis* spores, which are not usually inactivated by ordinary heat sterilization, can be inactivated using instantaneous high-pressure processing, owing to the physical disruption of spores.

5. ACKNOWLEDGMENTS

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