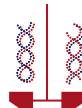

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Increase in hydrophobicity of *Bacillus subtilis* spores by heat, hydrostatic pressure, and pressurized carbon dioxide treatments

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The effects of heat treatment (HT), hydrostatic pressure treatment (HPT), and pressurized carbon dioxide treatment (CT) on surface hydrophobicity of *B. subtilis* 168 spores were investigated. The spore surface hydrophobicity was measured by determining the ratio of hydrophobic spores (RHS) that were partitioned into the *n*-hexadecane phase from the aqueous spore suspension. The RHS after HT generally increased in a temperature-dependent manner and reached approximately 10% at temperatures above 60°C. The effects of pressurization by HPT and accompanying temperature on increased RHS were complex. The highest RHS after HPT was approximately 17%. Following CT, RHS reached approximately 80% at 5 MPa at 80°C for 30 min. An increased treatment temperature enhanced RHS by CT. The increase in RHS by CT led to the formation of spore clumps and adhesion of spores to hydrophobic surfaces. Acidification of spore suspension to pH 3.2, expected pH during CT, by HCl also increased the adhesion of spores at the similar degree with CT. The spore surface zeta potential distribution was not changed by CT. Furthermore, spores with increased RHS after CT had germination-like phenomena including loss of their refractility and enhanced staining by 4′,6-diamidino-2-phenylindole. Physiological germination that was induced by the addition of L-alanine also increased the RHS. From these results, it is clear that CT under heating considerably increases RHS. CT under heating considerably increases RHS. This increase in RHS may be due to acidification or germination-like phenomena during CT.

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[**Key words:** Hydrophobicity; Adhesion; Acidification; Carbon dioxide; Germination]

It has been reported that surface hydrophobicity of bacterial spores varies among *Bacillus* and *Clostridium* strains (1). Spores of some *Bacillus* species with strong hydrophobic properties were found to have a higher ability to attach to hydrophobic surfaces (2). When some *Bacillus* spores were adhered to stainless steel, their heat resistance considerably increased (3). Additionally, it was reported that heat treatment increased the hydrophobicity of spores and caused formation of spore clumps (4–6). The formation of spore clumps resulted in decreased inactivation effects of heat treatment (4). Therefore, a change in surface hydrophobicity of spores occurring during food pasteurization process is a considerable factor in ensuring control of bacterial spores in processed food. However, alterations in hydrophobicity due to processing have been poorly studied. Therefore, the mechanism of increasing surface hydrophobicity of bacterial spores remains unknown.

Pressurized carbon dioxide treatment (CT) is performed by solubilizing carbon dioxide (CO_2) into liquid foods under pressurization. Dissolved CO_2 generates H_2CO_3, which dissociates into HCO_3^- and H^+, leading to the acidification of liquid foods. CT at temperatures above 50°C significantly inactivates bacterial spores (7). The inactivation effect of hydrostatic pressure treatment (HPT) against microorganisms was reported more than 100 years ago (8). Over the past 30 years, considerable research efforts have been directed toward the practical applications of HPT. Through these studies, it has been revealed that HPT is an advantageous alternative to heat treatment because it does not lead to deterioration of the flavor and nutrient content of foods (9). CT and HPT are promising alternatives for the pasteurization of food.

The aims of this study are to compare the effects of HT, HPT, and CT on surface hydrophobicity of *Bacillus subtilis* 168 spores. A marked increase in hydrophobicity was observed after CT; thus, the mechanism behind the increase in surface hydrophobicity of *Bacillus subtilis* spores by CT was elucidated.

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MATERIALS AND METHODS

Preparation of spore suspension *Bacillus subtilis* 168 was kindly provided by Dr. S. Ishikawa of Shinshu University. Spores of *B. subtilis* were formed on nutrient agar (Becton, Dickinson, and Company, NJ, USA) by incubation at 30°C for approximately 10 days. Spores were also formed in G medium as described by Stewart and Halvorson (10). Spore formation (>90%) was confirmed using a phase contrast microscope (BX50; Olympus Co., Tokyo, Japan). The spores formed were washed in triplicate by centrifugation at 8000 ×*g* at 4°C for 10 min in 0.85% sodium chloride (Nacalai Tesque, Inc., Kyoto, Japan) solution to give a spore suspension with high purity. The spore suspension was lyophilized and stored at −20°C until use.

Treatment with heat, hydrostatic pressure, and pressurized carbon dioxide The stored spores were suspended in ice-cold sterile distilled water to give an optical density (OD_{600}) of approximately 1.0 by using a spectrophotometer (UV-1700; Shimadzu, Kyoto, Japan). For heat treatment (HT), the spore suspension (2 mL) in test tubes (18 × 130 mm) was incubated at 20°C, 40°C, 60°C, and 80°C for 30 min in a water bath (Lauda, Lauda-Königshofen, Germany) under atmospheric pressure. For hydrostatic pressure treatment (HPT), the spore suspension was sealed in sterile screw-cap plastic tubes (capacity, 5 mL; Greiner Labortechnik Co. Ltd., Frickenhausen, Germany) that were pressurized using a prototype pressurization apparatus (11). The time needed to achieve the treatment pressure at 200 MPa was approximately 60 s, and the decompression time was approximately 10 s. The temperature of the pressure cell was regulated using a temperature-controlled water bath. Combinations of hydrostatic pressure (50, 200, and 400 MPa) and temperature (20°C, 40°C, 60°C, and 80°C) were used in this study along with a total holding period of 30 min. For pressurized carbon dioxide treatment (CT), the spore suspension (2 mL) in test tubes (18 × 130 mm) was immersed in water at the desired temperatures (20°C, 40°C, 60°C, and 80°C) in a CO_2-dissolving vessel (ϕ 95 mm × 140 mm). CO_2 gas was introduced into the vessel at 5-MPa pressure and dissolved into the spore suspension. The vessel was allowed to stand for 30 min; thereafter, CO_2 gas in the vessel was released using a pressure control valve to return the vessel to atmospheric pressure. The apparatus used for CT was the same as that reported in a previous study (12). As a control experiment, a spore suspension was kept on ice for 30 min.

Measurement of spore surface hydrophobicity The partition of spores between *n*-hexadecane and the aqueous phase was measured in order to evaluate hydrophobicity of the spore surface by using the BATH assay method (1). After each treatment, the OD_{600} of the spore suspension was measured (A_0). The spore suspension (1.5 mL) was added to 0.5 mL of *n*-hexadecane (Nacalai Tesque, Inc.), and the mixture was vortexed (Vortex-Genie 2, Scientific Industry Inc., Bohemia, NY, USA) at the maximum speed for 1 min in a test tube (18 × 150 mm). The mixture was allowed to stand for 15 min at 25°C. The aqueous phase was carefully recovered with a Pasteur pipette, and its OD_{600} was measured with a spectrophotometer (A_f). The hydrophobicity of the spore surface was expressed as the ratio of hydrophobic spores (RHS), which was calculated using the following equation: $RHS (\%) = 100 (A_0 - A_f)/A_0$.

Spore clumps The spore suspension subjected to CT was observed using a phase contrast microscope (BX50; Olympus Co.). The size distribution of spore clumps in the spore suspension was analyzed using a laser diffraction particle size analyzer (SALD-200V ER; Shimadzu).

Adhesion of spores to hydrophobic beads and polypropylene tube Hydrophobic beads used were the packing material of the Phenomenex Jupiter C4 column for reversed-phase chromatography (5-μm silica gel spherical beads modified with normal butyl groups with a micropore diameter of 300 Å; Phenomenex, CA, USA). The beads (45 mg) were added to 2 mL of spore suspension before or after CT. The spore suspensions were vortexed (Vortex-Genie 2) at the maximum speed for 1 min in a test tube (18 × 150 mm). The beads were allowed to stand at 4°C until they sank to the bottom of the tube, after which the supernatant was carefully recovered with a Pasteur pipette. The number of spores in the supernatant was determined by the plated count method using nutrient agar. In addition, degree of adhesion to hydrophobic surface after HT, CT, or HCl treatment was also evaluated. HCl treatment was performed by standing untreated spore suspension at room temperature for 30 min after adding 1 N HCl solution to give pH 3.2. One milliliter of the spore suspensions after HT, CT, or HCl treatment was added to polypropylene tubes (capacity, 5 mL; Watson, Co. Ltd., Tokyo, Japan); these tubes were centrifuged at 10,000 ×*g*, 4°C for 3 min for binding of the spores with increased hydrophobicity to the inner surface of the tube. Next, the tubes were vortexed (Vortex-Genie 2, Scientific Industries, Inc.) at the maximum magnitude for 10 s to re-suspend unbounded spores into the supernatant. The number of spores in the suspension before centrifugation and after vortex mixing was determined by the plate count method using nutrient agar. The plates were incubated at 30°C for 16 h, and the colonies were then enumerated. The ratio of the decrease in spore count upon hydrophobic bead or centrifugation treatment was expressed as $\log (N/N_0)$, where N_0 is the spore count of the control or after each treatment, and N is the spore count of the supernatant after the addition of hydrophobic beads or centrifugation.

Zeta potential measurement Spores suspended in distilled water were subjected to CT. The zeta potential of the spores was measured with a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK).

4′,6-Diamidino-2-phenylindole staining Spores after CT were subjected to the BATH assay using *n*-pentane as the organic solvent. The spores, partitioned into aqueous and organic solvent phases, were separated and washed by centrifugation at 7000 ×*g* at 4°C for 10 min. Diamidino-2-phenylindole (DAPI) (Nacalai Tesque) solution was prepared by dissolution into distilled water at a concentration of 1 mg/mL. The stock solution was diluted 1000-fold with methanol, and this dilution was used as a working solution. The spore suspension was centrifuged at 7000 ×*g* and 4°C for 10 min, and the resultant pellet was suspended in the DAPI solution. After incubation at 37°C for 15 min, spores were washed 3 times by centrifugation in distilled water. The stained spores were observed using a phase contrast microscope (BX50; Olympus Co., Tokyo, Japan) equipped with devices for fluorescence observation (BH2-RFL-T3, U-ULS100HG, and BX-KLA; Olympus).

Germination by using L-alanine The spore suspension was incubated at 30°C in the presence of 100 mM L-alanine (Nacalai Tesque), and time-dependent changes in OD_{600} were measured using the spectrophotometer.

Statistical analysis Significant differences in RHS and in decreased ratio of spore concentration by the addition of hydrophobic beads were determined using the Student's or Welch's *t*-test (Microsoft Excel 2010) after an *f*-test used to ensure the equality of variance. Tukey' test was applied to determine significant difference ($p < 0.05$) among the decrease ratios of control, HT, CT, and HCl treatment (Ekuseru-Toukei 2012, Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Effects of HT, HPT, and CT on the RHS of *B. subtilis* Furukawa et al. (13) investigated the effects of heating time with HT at 85°C on the RHS of *Bacillus cereus*, *Bacillus coagulans*, and *Bacillus licheniformis*, and they found that the RHS increased and reached a maximum level within approximately 30 min. Therefore, in the present study, the time for HT was set at 30 min, and the effect of temperature of HT on the RHS of the spores of *B. subtilis* was investigated (Fig. 1a). RHS generally increased in a temperature-dependent manner. RHS after HT at 80°C was approximately 10%.

The effect of HPT on the RHS of *B. subtilis* is shown in Fig. 1b. The treatment time was 30 min. At 50 MPa, the RHS generally increased in a temperature-dependent manner, whereas this behavior was not observed at 200 and 400 MPa. At 20°C and 40°C, pressurization above 200 MPa increased the RHS obtained by HT alone. However, at 60°C and 80°C, the enhancing effect of pressurization was not observed. The highest RHS obtained after HPT was approximately 17%.

The effect of CT on the RHS of *B. subtilis* is shown in Fig. 1c. Treatment pressure and time was set for 5 MPa and 30 min, respectively. BATH assay was performed after vortexing the spore suspension to remove CO_2 gas. CT increased the RHS as the treatment temperature increased, and the RHS reached approximately 80% at 80°C. RHS after CT was clearly larger than that after HT and HPT.

Spore flocculation after CT and removal of spores with increased hydrophobicity We investigated whether the increase of RHS by CT results in spore flocculation by phase contrast microscopy (Fig. 2a) and size distribution analyses (Fig. 2b). CT was performed at 80°C and 5 MPa for 30 min. CT appeared to induce the formation of spore clumps as demonstrated by microscopic observation (a). For the numerical distribution analysis of spores, a slight increase in the frequency was observed at sizes larger than 2 μm after CT. In the volumetric distribution analysis of spores, a clear difference was observed between the control and CT. Upon CT, the frequency of spores with sizes of 0.8−1.2 μm considerably decreased, and that of clumped spores with sizes of 10−100 μm accounted for approximately 92% of spores. These results suggest that the increase of RHS by CT leads to the formation of a small number of large spore clumps.

The effect of the addition of hydrophobic beads on decreasing the ratio of spore count is shown in Fig. 2c. The hydrophobic beads induced no significant change in the number of control spores. In

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