Note

Effects of Solution pH and Ions on Suicidal Germination of Bacillus subtilis Spores Induced by Medium High Temperature-Medium High Hydrostatic Pressure Treatment

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Spores of Bacillus subtilis suspended in water or aqueous solution of NaCl, CaCl₂, sodium lactate, or calcium lactate at pH 4 – 7 was subjected to spore inactivation by simultaneous combination of medium high hydrostatic pressure (MHHP; 100 MPa) treatment for germination and medium high temperature (MHT; 65°C) treatment for pasteurization of germinated vegetative cells. The spores at pH 4 in NaCl solution and those at pH 5 and 6 in Na lactate solutions were less killed than in water by MHHP+MHT treatment. Spore inactivation was promoted by calcium ion in NaCl solution at pH 4 and in Na lactate solutions at pH 5 and pH 6, while it was more suppressed at pH 5 and pH 6 in Na lactate solutions than at pH 4 in NaCl solution. The spores treated by MHHP+MHT in NaCl or Na lactate solution at pH 4 were further killed by subsequent MHT treatment.

Key words : Bacillus subtilis / Spore / High hydrostatic pressure / Germination / Pasteurization.

Bacillus subtilis is often found in soil and it may contaminate agricultural produce such as grain, meat, milk, and vegetable (Andersson et al., 1995). Vegetative cells of B. subtilis can turn into spores which are highly resistant against drying, heat, bactericide, and other stresses (Setlow, 2006). In food processing, intervention technologies inactivating Bacillus spores have been demanded to ensure food safety and prolong shelf life of foods. Since most of Bacillus spores survive boiling, heat treatment at above 100°C (e.g. retort processing) must be conducted to kill Bacillus spores in food processing (Nicholson et al., 2000). However, excess heating often leads to deterioration of food quality in terms of taste, color, flavor, and nutritional value (Ross et al., 2003). Therefore, great attention has been paid to novel technologies for pasteurization of Bacillus spores at lower temperatures than 100°C toward minimizing loss in food quality.

Bacillus spores are highly resistant to heat, while their vegetative cells after germination are much less heat resistant and they can be inactivated by medium high temperature (MHT), for instance at 60°C (Desai et al., 2010). It has been indicated that medium high hydrostatic pressure (MHHP) treatment at around 100 MPa can nonthermally induce spore germination which follows the same pathway as nutritional germination (Wuytack et al., 2000; Paidhungat et al., 2002; Black et al., 2005; Reineke et al., 2012). In addition, when the spores are subjected to simultaneous combination of MHHP and MHT treatments, the MHHP treatment can induce spore germination nonthermally and the MHT treatment may simultaneously inactivate germinated spores, namely vegetative cells, thermally. This event can be referred to as suicidal germination (Yamamoto et al, 2014) since the germination can proceed even under lethal temperature conditions. The efficacy of suicidal germination by MHHP+MHT treatment is dependent on the number of spores germinated by MHHP treatment. MHHP+MHT treatment is expected to pasteurize Bacillus spores while minimizing heat-induced damages of food quality (Aoyama et al., 2006;
Islam et al., 2006; Yamamoto et al., 2014). Accordingly, suicidal germination by MHHP+MHT treatment has been studied toward establishing high quality pasteurization technologies. On the other hand, spore can be injured by various stresses such as heat, irradiation, cold storage, and chemicals (Foegeding & Bustab, 1981). It can be speculated that MHHP treatment would also induce injured spores, which could be inactivated by subsequent heat treatment.

Islam et al. (2006) reported that efficacy of MHHP+MHT (100 MPa at 65°C) to inactivate B. subtilis spores in potage or ketchup was affected by food components and that low pH in the foods suppressed the inactivation. Reduced heat resistance and germination ability of spores can be observed at ambient pressure after immersing the spores in acidic solutions, being ascribed to demineralization of dipicolinic acid (DPA) in the spore core (Nishihara et al., 1981; Inukai et al., 1984; Marquis et al., 1985; Palop et al., 1999). Solutions containing organic acids as metal chelators enhanced the demineralization of the spores, while spore germination was suppressed (Nishihara et al., 1981; Inukai et al., 1984). Therefore, it can be speculated that suicidal germination might be suppressed by demineralization which can be induced by lowering pH and/or addition of organic chelators. However, the effect of food composition, pH, and organic chelators on suicidal germination has not been clarified sufficiently.

In this study, liquid food models contaminated with B. subtilis spores were employed to study the effect of food components under acidic pH conditions (pH 4.0 to pH 7.0) on their suicidal germination at 100 MPa and 65°C for 30 min. For that purpose, sodium chloride (NaCl) as taste conditioner and calcium lactate (Ca(C$_2$H$_5$O$_3$)$_2$; Ca lactate) as mineral intensifier were added to the system as well as calcium chloride (CaCl$_2$) and sodium lactate (NaC$_2$H$_3$O$_2$; Na lactate) to clarify the contributions of Na$^+$, Ca$^{2+}$, Cl$^-$, and Lactate$^-$ . Lactate ion was expected to chelate Ca ion.

*Recall* NBRC 111470 known as the strain 168 was obtained from National Institute of Technology and Evaluation (Chiba, Japan). The bacterial strain was stored at -80°C in 0.9% saline solution with glycerol added to be 20%. After thawing, the solution was inoculated into 20 ml sporulation medium (pH 7.5), which contained 0.8% Nutrient Broth (Difco Nutrient Broth, Becton, Dickinson and Company, Franklin Lakes, U. S.A.) as well as 2.0 mM MgCl$_2$, 1 mM CaCl$_2$, 0.01 mM MnCl$_2$, 1 mM FeCl$_3$, 27.0 mM KCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The culture media was incubated at 37°C with agitation at 130 rpm for 24 h. After the incubation, the culture containing spores and vegetative cells was heated at 65°C for 30 min to kill the vegetative cells. The spores were collected by centrifugation (2000 x g, 25°C, 10 min), and the resulting pellet was then resuspended in 40 ml of sterile distilled water. This procedure was repeated twice to wash out non-spore residues in the supernatant as much as possible. After three times of centrifugation, the resulting pellet was resuspended in 5 ml sterile distilled water. The suspension was again heated at 65°C for 30 min to kill the rest of vegetative cells.

Distilled water and aqueous solutions of sodium chloride (NaCl), calcium chloride (CaCl$_2$), sodium lactate (Na lactate) and calcium lactate (Ca lactate) were used for suspending the spores after pH adjustment using hydrochloric acid (HCl) and/or sodium hydroxide (NaOH) and subsequent filter sterilization. Concentration of the solutions was fixed at 139 mM, which was sufficiently higher than the concentrations of HCl and NaOH added for the pH adjustment. Their mixture solutions were also prepared by mixing each solution as described in Table 1. Autoclaved distilled water (pH 6.8) was used without pH adjustment. All the reagents were of analytical grades.

The spore suspension in water (1 ml) was mixed with each of 9 ml of the matrix solutions, and final solution pH was measured for each solution (pH 4.0±0.1, 5.0±0.1, 6.0±0.1, and 7.0±0.1; hereafter, simply pH 4, pH 5, pH 6, and pH 7). Initial counts of spore suspension in the tested matrices were 7.4±0.0 log CFU/ml (mean ± standard deviation). Spore suspension (1.5 ml) was heat-sealed into a sterile polypropylene bag (80 mm x 80 mm). The suspension in the bag was subjected to MHHP treatment by using a MHHP food processor (TFS 5, Toyo Kouatsu, Inc., Hiroshimia, Japan) using water as pressure medium. Pressure chamber (φ 80 mm × D 140 mm) was maintained at 40.0±1.0°C or 65.0±1.0°C by a heater hermetically inserted in the chamber. Two bags were immersed in the pressure chamber and compressed up to 100±2

### TABLE 1. Composition of the solutions I, II, III, and IV used for suspending spores.

| solution | additives       | solute in ratio (·) |
|----------|-----------------|---------------------|
| NaCl     | 139 mM NaCl     | Na⁺ 0 1 0           |
| Na lactate | 139 mM NaC$_2$H$_3$O$_2$ | Ca$^{2+}$ 1 1 2    |
| I        | 139 mM NaCl     | 1 1 2 1             |
| II       | 139 mM NaC$_2$H$_3$O$_2$ | 1 0.5 1 1      |
| III      | 139 mM NaCl     | 1 1 3 0             |
| IV       | 139 mM NaCl     |                     |
MPa at 1.1 MPa/sec, and the pressure was held for 30 min. After decompression at 10 MPa/sec, one of the
MHHP-treated bags was further immersed in a water bath at 65.0±2.0°C for 30 min to kill germinated
spores. As a control, MHT treatment was performed by immersing the bag in a water bath at 65.0±2.0°C for 60
min. After the treatments, the bags were cooled to ambient temperature. The spore suspension in each of
the bags was subjected to subsequent bacterial counts.

Viable counts of *B. subtilis* cells in control and treated samples were determined by direct plating method.
Samples were serially diluted using sterile saline, and each of the diluted solution (0.1 ml) was plated in triplicate
on standard method agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). All the plates were incubated
at 37.0±1.0°C for 24 h. Viable cell counts of *B. subtilis* were calculated from the number of colonies formed on
a plate. Experimental results were expressed as mean and standard deviation of three independent trials.
Statistical significance was verified by Dunnett’s test (*p* < 0.05) or Tukey-Kramer’s method (*p* < 0.05).

The spores suspended in water or aqueous solution of NaCl, CaCl₂, Na lactate, or Ca lactate under acidic
conditions (pH 4 to pH 7) were subjected to an MHT treatment at 0.1 MPa and 65°C for 60 min to under-
stand their resistance to the MHT treatment (Fig. 1). As for water suspension as a control, viable cell counts
reduced by less than 0.1 log at each pH and no significant difference from the initial counts was observed
with the counts after the MHT treatment (*p* ≥ 0.05). In the other solutions, slight (at highest less than 0.9 log) but
significant reduction in the viable cell counts was observed with each of the solutions at pH 4 and pH 5.
When phosphate buffer solution was acidified from pH 6.7 to pH 5.2, *D₉₀* value of *B. cereus* spores in the solution
was reduced from 6.7 min to 2.4 min, indicating reduced heat resistance at low pH (Moussa-Boudjema et al., 2006). The reduced heat resistance at pH 4 and pH 5 in this study agreed with the report.

In MHHP-induced germination, spores may germinate under pressure holding (or pressurized) condition
and ungerminated spores may germinate after decompression (Reineke et al., 2012). Although injury of
spore induced by MHHP treatment has not been clari-
fied, some of the ungerminated spores might include
MHHP-injured spores, which would potentially be
pasteurized in the subsequent heating by MHT treat-
ment (65°C, 30 min). Accordingly, the efficacy of
MHHP-induced germination, which may include injury,
was evaluated by applying the MHT treatment to
pasteurize ungerminated and/or injured spores after
MHHP+MHT treatment (100 MPa, 40°C or 65°C, 30 min).
The spores suspended in water (pH 6.8) were treated
by the simultaneous combination of MHHP and MHT
(Fig. 2). MHHP treatment at 40°C slightly but signifi-
cantly reduced the spore survivors by 0.6 log (*p* <
0.05). Furthermore, viable cell counts of survivors after
subsequent MHT treatment (65°C, 30 min) decreased

![FIG. 1](image1.png)

**FIG. 1.** Effect of pH on the reduction from the initial counts (*N₀*) to viable cell counts (*Nₓ*) of *B. subtilis* spores treated by MHHT treatment at 65°C and 0.1 MPa for 60 min. The spore was suspended in water (■), NaCl solution (□), CaCl₂ solution (▲), Na lactate solution (■), or Ca lactate solution (■). Results were mean values of three independent trials ± standard deviation. Significant difference in the reduction of viable cell counts between control (water) and the solutions was evaluated by Dunnett’s test, and data set with a significant difference from control data (*p* < 0.05) was indicated using an asterisk.

![FIG. 2](image2.png)

**FIG. 2.** Effect of temperature during MHHP treatment on the viable cell counts of *B. subtilis* spores in distilled water (pH 6.8) after MHHP+MHT treatment (100 MPa, (A) 40°C / (B) 65°C for 30 min) and subsequent MHT treatment (65°C for 30 min). The bars showed the initial counts (■), the viable cell counts after MHHP+MHT treatment (▲), and those after MHHP+MHT treatment and the subsequent MHT treatment (□), respectively. Results were mean values of three independent trials ± standard deviation. For each set of 40°C or 65°C, significant difference in viable cell counts before and after the treatments was evaluated by Tukey-Kramer’s method, and data marked with different letters indicate a significant difference (*p* < 0.05).
promotive effects were not significant at pH 6.8. Since mechanism of spore suspension to water and the tested solutions was evaluated by Dunnett's test and data set with a significant difference from control data ($p < 0.05$) was indicated using an asterisk.

![FIG. 3](image)

**FIG. 3.** Effect of NaCl, CaCl$_2$, Na lactate, and Ca lactate on the reduction from the initial counts ($N_0$) to viable cell counts ($N_v$) of *B. subtilis* spores treated by MHHP+MHT treatment (100 MPa, 65°C, 30 min). The spore was suspended in water (■), NaCl solution (□), CaCl$_2$ solution (▲), Na lactate solution (●), or Ca lactate solution (▪). Results were mean values of three independent trials ± standard deviation. Significant difference in the reduction of viable cell counts between control (water) and the solutions was evaluated by Dunnett’s test, and data set with a significant difference from control data ($p < 0.05$) was indicated using an asterisk.

Germination depending on solution pH and its counterions. DPA in the core of *B. subtilis* spore is mineralized with cations such as Ca$^{2+}$, Na$^+$, K$^+$, and Mg$^{2+}$, and the mineralization of DPA plays important roles in heat resistance and germination ability of the spores (Setlow, 2006). Demineralization of DPA reduced heat resistance of spores (Bender et al., 1985; Marquis et al., 1985; Beaman et al., 1986; Palop et al., 1999) and suppressed germination (Nishihara et al., 1981; Inukai et al., 1984). Moreover, demineralization of DPA in the core of spore can be induced by acidification of spore suspension to low pH (Alderton et al., 1964; Nishihara et al., 1981; Inukai et al., 1984; Bender et al., 1985; Marquis et al., 1985; Beaman et al., 1986; Palop et al., 1999). Therefore, it will be reasonable that Na ion at a low pH of 4 may induce the demineralization which suppresses the germination. On the other hand, it may be speculated that Ca ion rather promoted the suicidal germination of the spores since the survived spores in CaCl$_2$ solutions at pH 4-7 after MHHT+MHT treatment were slightly less than those in water. Since mechanism regarding the promotive effect of Ca ion on the suicidal germination of the spores was unclear in this study, further investigation will be indispensable.

Another suppressive effect was observed with Na lactate (Fig. 3). Significant suppression of suicidal germination was observed with Na lactate solutions at pH 5 and pH 6 ($p < 0.05$), and suppressive tendency was observed with Na lactate solutions at pH 4 and pH 6. 

![FIG. 4](image)

**FIG. 4.** Effect of NaCl, CaCl$_2$, Na lactate, and Ca lactate on the reduction from the viable cell counts ($N_{vi}$) of *B. subtilis* spores treated by MHHP+MHT treatment (100 MPa, 65°C, 30 min) to viable cell counts ($N_{vi}$) of *B. subtilis* spores treated by subsequent MHT treatment (0.1 MPa, 65°C, 30 min) after MHHP treatment. The spore was suspended in water (■), NaCl solution (□), CaCl$_2$ solution (▲), Na lactate solution (●), or Ca lactate solution (▪). Results were mean values of three independent trials ± standard deviation. Significant difference in the reduction of viable cell counts between control (water) and the solutions was evaluated by Dunnett’s test and data set with a significant difference from control data ($p < 0.05$) was indicated using an asterisk.
The suppressive effect of Na lactate on suicidal germination (Fig. 3) seemed to be cancelled and the viable cell counts were not significantly different (p ≥ 0.05) between in water and in the solutions I, II, III, and IV all of which were supplemented with Ca ion. Therefore, Ca ion may have promotive effect on suicidal germination, which may also be supported the observation in Fig. 3: the viable cell counts were more reduced in CaCl₂ solution than in water although no significant difference in viable cell counts was observed between water and CaCl₂ solution. The viable cell counts in Ca lactate were not significantly different (p ≥ 0.05) from those in water, implicating possible balance between suppressive effect of lactate and promotive effect of Ca.

Suicidal germination at pH 4 in the solutions I, II, and III containing lactate was suppressed in comparison with the solution IV containing no lactate (Fig. 5). Therefore, it can be speculated that lactate might have suppressed the suicidal germination. The ratio of protonated lactate can be calculated by Henderson-Hasselbach equation using acid dissociation constant (pKa). Since pKa of lactic acid is 3.86, the ratio of protonated form (lactic acid) is calculated to be 42% at pH 4, 7% at pH 5, less than 1% at pH 6 and pH 7. Lactic acid may form more calcium complex at acidic pH 4 than at neutral pH ( Bazin et al., 1995). Therefore, calcium ion as a possible promoter of suicidal germination might have formed more calcium lactate complex at pH 4 than pH 5 – 7, and the complex might have reduced the availability of free calcium ion. The suppressed suicidal germination at pH 4 in the solutions I, II, and III might be ascribed to the formation of calcium lactate at pH 4.

The effect of solution pH and Na⁺, Ca²⁺, Cl⁻, and Lactate⁻ on the suicidal germination was partly revealed in this study. In conclusion, Na and lactate ions showed suppressive effect on suicidal germination of B. subtilis spores at pH 4. The suppression of suicidal germination by Na ion at pH 4 was partly cancelled by coexisting Ca ion. The role of Ca lactate complex at low pH such as pH 4 should further be studied from a viewpoint of Ca ion availability. Since the MHHP+MHT treatment has a potential to reduce bacterial spores in food with reduced quality deterioration at MHT, further study will be necessary to understand and control the suicidal germination in terms of pH, coexisting ion species and organic chelators, and other relevant operation parameters. Effect of food matrix and bacterial species other than B. subtilis on the suicidal germination should also be studied in the future.

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