Bacterial spores are in cryptobiosis during the dormant period, which can change vegetative cells upon exposure to favorable conditions and eventually cause serious illness through ingestion of spore-contaminated food (Leuschner et al., 1999; Reissbrodt et al., 2002; Roszak and Colwell, 1987). The most common spore-forming bacteria causing foodborne disease are aerobic *Bacillus* spp. and anaerobic *Clostridium* spp. such as *B. cereus*, *C. perfringens*, and *C. botulinum* (Cho et al., 2007; Lee et al., 2007; Shin et al., 2008). Other *Bacillus* spores such as *B. subtilis*, *B. coagulans*, *B. licheniformis* are the causes of food spoilage (Cazemier et al., 2001). However, spores are extremely resistant to heat and other external stresses, which is primarily attributed to their ultra-structural characteristics (Nicholson et al., 2002; Oomes et al., 2007).

Heat treatment triggers an activation, germination, and destruction of dormant spores through a complex multistage process (Collado et al., 2006; Iciek et al., 2006). The activated spores are more susceptible to heat than the dormant spores. However, the inactivation of dormant spores during thermal processing is not clearly explained by general inactivation processes due to the nature of the heterogeneous populations (Davey and Kell, 1996). Some spores are directly heat-inactivated without an activation stage, while others are heat-injured or remain in a dormant state for a longer heating time (Abraham et al., 1990; Esty and Meyer, 1922; Vary and Halvorson, 1965). Therefore, understanding the heat-induced inactivation of bacterial spores with special regard to physiological, biochemical, and molecular properties is essential for developing an appropriate kinetic model elucidating the thermal effect on spore inactivation.

Mathematical predictive modeling is used as a tool for quantifying the efficacy of food preservation technologies for the inactivation of microorganisms. Heterogeneous populations are present during thermal treatment, including an injured, activated, germinated, or inactivated state of bacterial spores (Iciek et al., 2006; Sapru et al., 1993). There have been few studies of mathematical models for heterogeneous bacterial spores (Abraham et al., 1990; Sapru et al., 1993). It is important to describe the kinetic patterns of spore inactivation including variability in thermal resistance. Therefore, the objective of this study was to quantitatively describe the population heterogeneity based on a kinetic approach to thermal inactivation of bacterial spores. In this study, *B. amyloliquefaciens* TMW 2.479 Fad 82 and *B. subtilis* KACC 10854 were used to represent heat-resistant and heat-sensitive spores, respectively.
A strain of *Bacillus amyloliquefaciens* TMW 2.479 Fad 82 was provided by Dr. Michael Gänzle of the Department of Agricultural, Food and Nutritional Science, the University of Alberta (Edmonton, AB, Canada). A strain of *Bacillus subtilis* KACC 10854 was obtained from the Korean Agricultural Culture Collection (KACC; Suwon, Korea). The strains were cultivated aerobically in tryptcase soy broth supplemented with 0.1% yeast extract (TSBYE; BD, Becton, Dickinson and Co., Sparks, MD, USA) at 30°C (*B. subtilis*) and 32°C (*B. amyloliquefaciens*) for 24 h. After the cultivation, the cultures were used to induce sporulation.

The fresh culture (100 µl) of *B. subtilis* or *B. amyloliquefaciens* was spread-plated on tryptcase soy agar (TSA, BD, Becton, Dickinson and Co.) containing 10 ppm of MnSO₄ and incubated until at least 95% of bright dormant spores was observed by phase-contrast microscopy. After incubation, spores on the plates were collected with 10 ml of sterile distilled water and washed five times by differential centrifugation ranging from 2,000 to 8,000 × g for 20 min each at 4°C. The collected spores were resuspended in deionized water to approximately 10⁹ (CFU/ml).

The thermal inactivation of spores suspended in deionized water was determined at 105°C using sterile capillary tubes (0.8 to 1.1 mm; Kimble, Vineland, NJ, USA). The capillary tubes containing 50 µl of spore suspension were submerged in a 10-L thermostatically controlled circulating oil bath (JSOB-100T, JSR, Korea) which was maintained at the desired temperature (105°C) for 0, 5, 10, 20, and 30 min. After thermal treatment, the capillary tubes were immediately cooled in an ice bath to avoid further inactivation.

The heat-treated samples were serially (1 : 10) diluted with 0.1% peptone water and pour-plated in duplicate for three replicates on tryptcase soy agar (TSA) to enumerate total viable spores. The plates were incubated at 30°C or 32°C for 24 to 48 h. The viability of spores below the detection limit was determined by enrichment culture in double-strength TSB, and no-growth was assigned at 0 for statistical analysis. The heat-treated spores were pour-plated on TSA containing the predetermined concentration of 7.5% NaCl to estimate the number of heat-injured spores. In order to further evaluate heat-activated spores, the heat treatment was followed by additional heat shock at 80°C for 10 min.

The kinetic parameters for spore inactivation were analyzed using linear and Weibull models. D values were calculated at the linear portion of survival curves:

\[ \log \frac{N_D}{N_{D0}} = -t \]  \hspace{1cm} (1)

where \( N_{D0} \) and \( N_D \) are the numbers of dormant spores at time 0 and \( t \), respectively. \( N_{D0} \) and \( N_D \) do not include the number of activated spores or the injured spores, which were excluded by heat-shock and 7.5% NaCl, respectively.

The Weibull model was used to describe thermal inactivation of spores suspended in deionized water (Peleg and Cole, 1998):

\[ \log \frac{N_D}{N_{D0}} = -b \cdot t^n \]  \hspace{1cm} (2)

where \( b \) and \( n \) are the scale and shape factors, respectively.

Rate constants for *B. amyloliquefaciens* and *B. subtilis* were calculated by the following Sapru model (Sapru et al., 1993):

\[ N_D = N_{D0} \exp[(k_{d1} - k_a)t] \]  \hspace{1cm} (3)

\[ N_A = N_{A0} \exp(-k_{g2}t) + \frac{k_a}{k_a + k_{d1} - k_{g2}} \]

\[ N_{D0}[1 - \exp(k_{g2} - k_{d1} - k_a)t]\exp(-k_{g2}t) \]  \hspace{1cm} (4)

where \( N_D \) is the number of dormant spores at time \( t \), \( N_{A0} \) is the number of activated spores at time 0, \( N_A \) is the number of activated spores at time \( t \), and \( k_a \) is the activation rate of dormant spores, \( k_{d1} \) is the inactivation rate of dormant spores, and \( k_{g2} \) is the inactivation rate of activated spores. The direct microscopic count includes the initial number of dormant spores (\( N_{D0} \)) and is the initial number of activated spores (\( N_{A0} \)). The inactivation process of bacterial spores was assumed as follows:

\[ N_f \leftrightarrow k_{d1} N_D \leftrightarrow k_a N_A \leftrightarrow k_{g2} N_l \]

where \( N_f \), \( N_D \), \( N_A \), \( k_{d1} \), \( k_a \), \( k_{g2} \), and \( N_l \) are the number of spores inactivated after activation and the number of spore inactivated without activation, respectively.

The dipicolinic acid (DPA) content released from bacterial spores during thermal treatment was estimated by using the colorimetric assay (Janssen et al., 1958) with slight modifications. A 1-ml sample was centrifuged at 3,000 × g for 20 min and the supernatant was mixed with a fresh reagent containing 1% Fe(NH₄)₆(SO₄)₂6H₂O and 1% ascorbic acid in 0.5 M acetate buffer at pH 5.5. The absorbance of the mixtures was measured at 440 nm by using a Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The untreated sample was autoclaved at 121°C for
20 min to determine the total DPA content. A standard curve was generated at 5, 10, 20, 40, 60, 80, and 100 μg ml⁻¹ of pyridine-2,6-dicarboxylic acid as prepared for the sample.

The spore inactivation curves were analyzed using Nonlinear Curve Fitting Function of Microcal Origin® 7.5 (Microcal Software, Inc., Northampton, MA, USA). Data were analyzed using the Statistical Analysis System software (SAS 8.0, SAS Institute, Inc., Cary, NC, USA). Significant mean differences were calculated by Fisher’s Least Significant Difference (LSD) at p<0.05.

This study describes the thermal inactivation of B. amyloliquefaciens spores and B. subtilis spores, including dormant, activated, injured, and inactivated spores, during thermal treatment at 105°C. The survival curves of B. amyloliquefaciens and B. subtilis spores were fitted with linear and Weibull functions as shown in Fig. 1. The initial numbers of B. amyloliquefaciens and B. subtilis spores suspended in deionized water were 2.81 × 10⁸ CFU/ml and 2.45 × 10⁸ CFU/ml, respectively. B. amyloliquefaciens and B. subtilis spores were reduced by 1.17 and 7.29 log CFU/ml, respectively, after 30 min of thermal treatment at 105°C. The number of surviving B. subtilis spores was approximately 10 CFU/ml after 30 min of thermal treatment, while that of B. amyloliquefaciens spores was still more than 7 log CFU/ml. The survival curve of B. subtilis spores exhibited more obvious curvature than that of B. amyloliquefaciens spores, showing that the Weibull distribution was a better fit than the log-linear model (Fig. 1). No shouldering phase was observed, indicating the direct lethal effect of thermal treatment on the inactivation of B. subtilis spores rather than a cumulative effect. As shown in Table 1, the degree of thermal resistance of B. amyloliquefaciens was much higher than that of B. subtilis. The D values are highly negatively correlated with b values (r = −0.984). The inactivation pattern of B. subtilis spores exhibited upward concavity as indicated by the shape factor (n=0.53). The observation of a highly negative correlation between D and n values suggests that D and b values can be used as reliable indicators of spore resistance (Table 1). The Weibull model could provide a quantitative estimate of the inactivation of microorganisms under various treatment conditions and fit different inactivation patterns, including downward concave (n>1), upward concave (n<1), and linear curves (n=1) (Chen and Hoover, 2003; Peleg and Normand, 2004).

The thermal-induced activation of spores and the release of DPA from spores were observed as shown in Fig. 2. The log reductions of B. subtilis spores treated at 105°C were not significantly different from those treated at 105°C followed by additional heat shock, while the numbers of B. amyloliquefaciens spore were significantly reduced after additional heat shock. The amounts of DPA released from B. amyloliquefaciens and B. subtilis were increased up to 74 and 98%, respectively, after 30 min thermal treatment (Fig. 2). Difference in the number of B. amyloliquefaciens spores between thermal treatment alone and thermal treatment followed by heat shock might result from the thermal-induced germinated or injured spores which were completely inactivated during the heat shock. Thermal treatment induces diverse changes in spore physiology, leading to non-equal susceptibility of spore populations (Abraham et al., 1990). The most notable DPA release occurred at the beginning of thermal treatment (Fig. 2). This is in good agreement with previous reports that the DPA released from bacterial spores varied in heat resistance, suggesting the in-
increase in the susceptibility of heat-treated spores may be due to the change in permeability (Church and Halvorsen, 1959; Kort et al., 2005; Mishiro and Ochi, 1966). The amount of DPA released is used as a reliable indicator of spore germination because the DPA released from the core is associated with the first stage of spore germination (Paidhungat et al., 2002).

Accurate kinetic description of the spore inactivation process is needed to characterize the stages, including activated, germinated, and injured spores. Table 2 shows the rate constants of \textit{B. amyloliquefaciens} and \textit{B. subtilis} spores treated at 105°C. \textit{B. amyloliquefaciens} spores had the same rate constants \((k_a \text{ and } k_{d1})\) as 0.07, while \(k_{d1}\) was faster than \(k_a\) for \textit{B. subtilis} spores. Values of \(k_{d1}\) (0.07 1/min, 0.92 1/min) and \(k_{d2}\) (0.16 1/min, 1.29 1/min) were significantly different for both \textit{B. amyloliquefaciens} and \textit{B. subtilis} spores \((p<0.05)\). Most spores were rapidly heat-inactivated through the activation (Table 2). This is in agreement with the results from Abraham et al. (1990), who reported that the activation was the rate determining reaction during the thermal inactivation.

In conclusion, this study could help explain the population heterogeneity of bacterial spores during thermal inactivation. Inactivation kinetics including population heterogeneity of bacteria spores could be of great assistance in predicting microbiological safety in the food industry. Therefore, the results provide useful information to develop predictive models for quantitative microbiological risk assessment.

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**References**


