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Physiological Responses of *Bacillus amyloliquefaciens* Spores to High Pressure

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Abstract Pressure inactivation behavior of *Bacillus amyloliquefaciens* spores was investigated in deionized water. The spores of *B. amyloliquefaciens* were subjected to 105°C and 700 MPa. The magnitude of the decrease in viability after pressure treatment was similar to that after pressure treatment followed by heat shock. The increase of dipicolinic acid (DPA) release was correlated with the spore inactivation, and the hydrophobicity did not significantly change during the pressure-assisted thermal processing (PATP). Lag phase duration increased with increasing pressure process time. The mechanisms of spore germination and inactivation during the PATP were related to a complex physiological process.

Keywords: PATP, *Bacillus amyloliquefaciens*, DPA, hydrophobicity, lag phase duration

Since bacterial endospores are highly resistant to conventional thermal processing [13, 21, 32], the application of high pressure combined with heat can be a more practical approach to ensuring microbiological food safety. However, there still remains a challenging question of whether the bacterial endospores are completely inactivated by using pressure-assisted thermal processing (PATP) to achieve satisfactory sterilization. In order to approach this question, it is necessary to first determine the spore inactivation mechanisms with regard to structural and physiological changes during the PATP.

The spore inactivation is more complicated at high pressure combined with thermal treatment and also linked to other complex metabolites. Pressure inactivation mechanisms are mainly divided into two types, namely structural damage and physiological damage [4, 22]. The outer spore membrane is believed to be a primary target site under high pressure processing, which may increase pressure sensitivity resulting from morphological and physiological changes of bacterial spores [6, 37]. A hydrophobic surface causes spore aggregation and increase resistant to high temperature and pressure [15, 33]. The fact that spore inactivation decreases with increasing inoculum is evidence that pressurization causes hydrophobic clumps resulting in a spore tailing phenomenon [8, 35].

Many researchers have reported that high pressure at elevated temperature increases the spore inactivation, leading to the leakage of intracellular substances such as metallic ions, dipicolinic acid (DPA), and small acid-soluble spore proteins (SASPs), the irreversible hydrolysis of core and cortex, and the decrease of intracellular pH [9, 14, 26, 29]. DPA, a unique component of bacterial endospores, is released from the core during the first stage of spore germination [19]. The Ca^{2+}-DPA complex activates zymogen as an effective germinant of bacterial endospores [20]. The amount of DPA release is often used as a reliable indicator of spore germination [10, 17, 19, 34].

Relatively fewer studies, however, have been reported on the spore germination and inactivation regarding DPA release, hydrophobic surface, and injury recovery when treated with PATP, specifically higher than 100°C and 500 MPa. Therefore, the objective of this study, by taking a systematic approach to determining the mechanisms of spore inactivation as a function of pressure holding times, was to investigate physical and physiological changes in surviving spores during the PATP at 105°C and 700 MPa.

The strain of *Bacillus amyloliquefaciens* TMW 2.479 Fad 82 was initially isolated from ropy bread [23] and kindly provided by Dr. Michael Gänzle of Lehrstuhl für Technische Mikrobiologie, Technische Universität München (Freising, Germany). The strain of *Bacillus amyloliquefaciens* Fad 82 used in this study has been reported as being highly resistant to pressure [17]. The strain was cultivated aerobically in trypticase soy broth supplemented with 0.1% yeast extract (TSBY, Difco, Detroit, MI, U.S.A.) at

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32°C for 24 h. After the second cultivation in TSBY, cultures were used to induce sporulation.

The fresh cultures (100 μl) were plated on trypticase soy agar (TSA, Difco, Detroit, MI, U.S.A.) containing 10 ppm of $MnSO_4$ (Fisher Scientific, Pittsburgh, PA, U.S.A.) and incubated at 32°C until more than 95% sporulation was observed by microscopic examination. Spores were collected by flooding the surface with 10 ml of sterile distilled water and scraping the colonies with a sterile glass spreader. Each spore suspension was washed five times by differential centrifugation ranging from 2,000 to 8,000 ×*g* for 20 min each at 4°C, sonicated for 10 min (SM275HT, peak power 270W, Crest Ultrasonic, ETL Testing Laboratories, INC., Cortland, NY, U.S.A.), and heated at 80°C for 10 min to destroy any remaining vegetative cells and avoid interfering with vegetative cells and germinated spores throughout the study [1]. The spore pellet was resuspended in deionized water to approximately 10^9 (CFU/ml) and stored at 4°C prior to use within 1 month.

Pressure-assisted thermal processing (PATP) experiments were carried out using a custom-fabricated high pressure tester (PT-1, Avure Technologies, Kent, WA, U.S.A.). Propylene glycol (57-55-6, Avatar Corp., University Park, IL, U.S.A.) was used as the pressure transmitting fluid. The sample temperature and chamber pressure were recorded every second during the entire treatment cycle using a K-type thermocouple sensor (Model KMQSS-04OU-7; Omega Engineering, Stamford, CT, U.S.A.) and pressure transducer (Model 3399 093 006, Tecsis, Frankfurt, Germany). A data acquisition computer equipped with relevant hardware (Daq-Board/2000 16-Bit, 200 kHz PCI card, DBK 81 7-Channel thermocouple expansion card, DBK 203-expansion card; IOtech, Cleveland, OH, U.S.A.) and software (DasyLab 7.00.04; National Instruments Corp., Austin, TX, U.S.A.) was used to record the data. The PT-1 pressure tester had a come-up of 0.58 min and the depressurization occurred in less than 2 sec.

The harvested spores were inoculated in sterile deionized water at levels between approximately 10^2 and 10^8 CFU/ml. Deionized water is representative of a germinant-free medium. The inoculated suspensions (2 ml each) were individually packaged in sterile plastic bags (5×2.5 cm; 01-002-57; Fisher Scientific, Pittsburgh, PA, U.S.A.) and sealed using an impulse heat sealer (American International Electric, Whittier, CA, U.S.A.). The packaged samples were then placed in a sample carrier consisting of a 10-ml capacity polypropylene syringe (Model 309604; Becton, Dickinson and Company) covered with two layers of insulating material. Water was used as the pressure transmitting fluid within the syringe. Prior to pressurization experiments, the sample carrier containing the spores was preheated in a water bath (Isotemp 928; Fisher Scientific) at 47°C for 5 min immediately prior to pressurization. The samples were subjected to 700 MPa for 0, 1, 2, 3, 5, 7, and 10 min holding times and constant heating rate for 0.58 min of the come-up time starting at 58°C of initial temperature, followed by holding at 105°C of the target temperature. The initial temperatures of the samples were estimated based on the respective knowledge of compression heating factors [3]. After high-pressure treatment, samples were immediately cooled in an ice-bath to avoid further inactivation.

Total viable spores of PATP-treated samples were directly determined by the pour-plating method and the enrichment culture. The spores suspended in deionized water were directly taken without further dilution (1:1) or serially (1:10) diluted with 0.1% peptone water, and a 1-ml aliquot of each sample was pour-plated in duplicate on trypticase soy agar (TSA, Difco, Detroit, MI, U.S.A.). The plates were incubated at 32°C for 24 to 48 h. Spore inactivation and germination were compared with log (N/N_0) calculated from the pressure treatment and followed by heat shock. N_0 and N denote the numbers of untreated bacterial spores (control) and the number of bacterial spores with or without heat-shock treatment after pressure treatment, respectively.

The relative hydrophobicity of bacterial spores suspended in deionized water was determined by bacterial adherence to chloroform assay [27]. Spores were suspended in potassium phosphate buffer (pH 7.0) at an absorbance of 0.8 to 1.0 (10^8 CFU/ml) at 540 nm. Spore suspensions (3 ml) were mixed with 0.5 ml of chloroform (Fisher Scientific, Pittsburgh, PA, U.S.A.). The mixture was vortexed for 1 min in round-bottom test tubes (100×13 mm i.d., 1.0 mm wall thickness) and allowed to stand for 5 min to separate into the hydrophilic aqueous phase and hydrophobic chloroform phase. The aqueous phase was measured at 540 nm using a Spectronic 20 GENESYS spectrophotometer (Bausch & Lomb, Rochester, N.Y., U.S.A.). The hydrophobicity index (HI) was calculated as follows;

$$HI(\%)=\frac{A_0 - A}{A_0} \times 100 \quad (1)$$

where A_0 and A are the initial and aqueous absorbance, respectively.

The colorimetric assay of Janssen *et al.* [10] with minor modifications was used to determine the DPA content in bacterial spores. A 5-ml sample was centrifuged at 3,000×*g* for 20 min and 4 ml of the supernatant was mixed with 1 ml of freshly prepared reagent containing 1% $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1% ascorbic acid in 0.5 M acetate buffer at pH 5.5. The absorbance was measured at 440 nm using a Spectronic 20 GENESYS spectrophotometer (Bausch & Lomb, Rochester, N.Y., U.S.A.). The untreated sample was autoclaved at 121°C for 25 min to determine the total DPA content. A standard curve was prepared at 5.0, 10.0, 20.0, 40.0, 60.0, 80.0, and 100.0 mg/ml of pyridine-2,6-dicarboxylic acid as described for the sample.

The physiological heterogeneity of the spores after high-pressure treatment was determined with the assumption that a germinated spore has a shorter lag time to recover than an injured spore. Untreated (control) and pressure-treated (0, 1, 2, 3, and 5 min) samples were diluted to the desired inoculum (10 CFU/ml) in double-strength TSBY and all inoculated tubes were incubated for 0, 4, 8, 12, 24, 36, and 48 h at 32°C. The number of spores in batch culture was determined by the pour-plating method using TSA.

The kinetic parameters for the bacterial growth were analyzed using the modified Gompertz equation [16];

$$\log N = A + C \cdot \exp[-\exp(-B \cdot (t - M))] \quad (2)$$

The lag phase duration (LPD) and maximum growth rate (μ_{max}) were calculated as follows;

$$LPD = M - \left(\frac{1 + e^{1 - e^{BM}}}{B}\right) \quad (3)$$

$$\mu_{max} = \frac{B \cdot C}{e} \quad (4)$$

where N, A, B, C, and M are the viable spore counts (CFU/ml) at time t, inoculum level (CFU/ml), the growth rate at the inflection point (1/h), the number of log cycles of spore growth (CFU/ml), and the time required to reach the maximum growth rate (h), respectively.

The spore growth curves were analyzed using Nonlinear Curve Fitting Function of Microcal Origin 7.5 (Microcal Software Inc., Northampton, MA, U.S.A.). Each experiment was performed in three replicates. All data were analyzed using the Statistical Analysis System software (SAS). The General Linear Model (GLM) and least significant difference (LSD) procedures were used to compare means. Significant mean differences were calculated by Fisher's Least Significant Difference (LSD) at $p<0.05$.

The initial number of *B. amyloliquefaciens* Fad 82 spores suspended in deionized water was 1.68×10^8 CFU/ml. During the come-up time from 47 to 105°C and from 0.1 to 700 MPa, the initial population was not significantly reduced (Fig. 1). The spores of *B. amyloliquefaciens* Fad 82 was rapidly inactivated by more than 6 log at the first 3 min of pressure holding time, while the inactivation curves showed a distinct level of tailing after 3 min of holding time. The number of surviving spores was more than 50 CFU/ml after 5 min of holding time. These results indicate that PATP inactivation of *B. amyloliquefaciens* Fad 82 spores is a direct lethal event rather than a cumulative effect [36]. The tailing phenomenon may be resulted from genetic variables in spore resistance and aggregation of spore suspension during PATP treatment [5]. In order to evaluate pressure-induced spore germination, the number of the inactivated spores after PATP and the number of the germinated spores after additional heat shock following

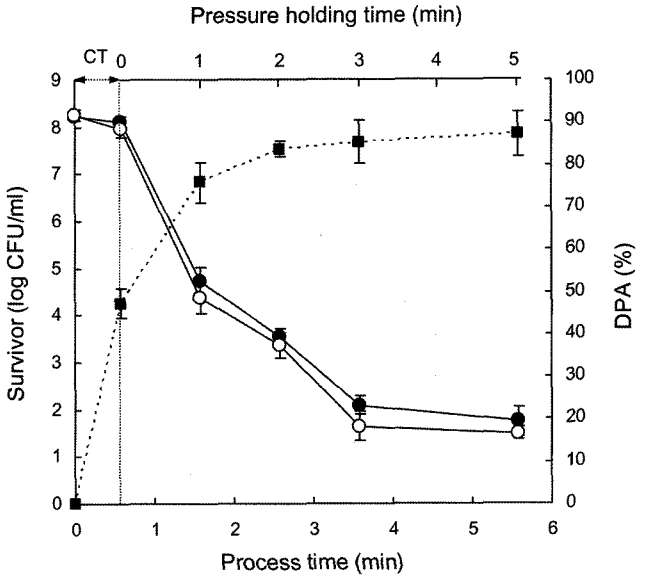

Fig. 1. DPA release (■, - - -) and inactivation curves (●, —; ○, —) of *B. amyloliquefaciens* Fad 82 spores treated at 105°C and 700 MPa (●, —) followed by heat shock at 80°C for 10 min (○, —). PATP (105°C and 700 MPa) had 0.58 min come-up time. CT is the process come-up time.

PATP were determined (Fig. 1). The log reductions of *B. amyloliquefaciens* Fad 82 spores at PATP treatment were not significantly different from those at PATP treatment followed by additional heat shock throughout the pressure holding time ($p>0.05$). Difference in the number of *B. amyloliquefaciens* Fad 82 spores between PATP and PATP followed by heat shock corresponds to the number of pressure-induced germinated spores, which were further inactivated during the heat shock. The number of heat inactivated spores following pressure treatment may include not only germinated spores but also sublethally injured spores. Heat and high pressure are known to trigger dormant bacterial spores to activate and germinate, and the germinated spores become more susceptible to other treatments including high pressure [18, 25, 30]. In our study, no considerable germination was observed at 105°C and 700 MPa, because pressure-induced germination is more likely to occur at relatively lower pressure between 200 and 500 MPa [11, 34]. A spore inactivation mechanism with regard to germination has been well proposed as a basis of the recent scientific consensus [2]; the germination system is not activated at less than 70°C, activated between 70 and 95°C, and damaged at more than 95°C. Unlike the germination at lower than 95°C and 500 MPa, bacterial spores undergo a different inactivation mechanism at high pressures (700 MPa) and temperatures (105°C) [2, 17, 34]. The amount of DPA released from *B. amyloliquefaciens* Fad 82 spores was increased up to 87% after 5 min pressure holding time, which occurred mostly within 2 min of holding time. The observation that the release of DPA

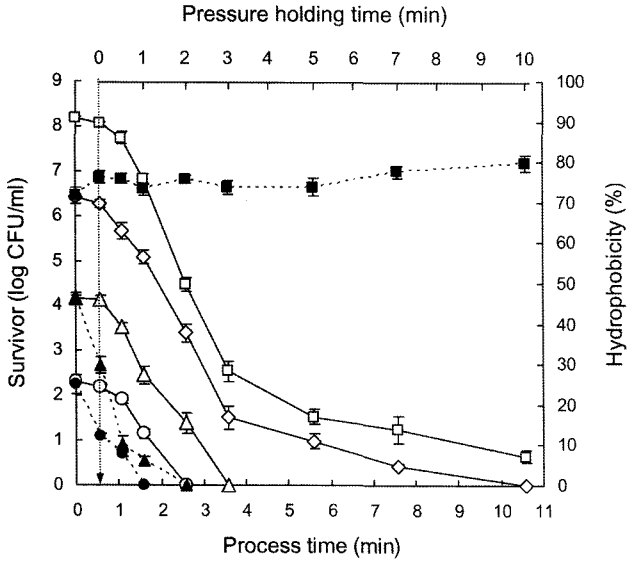

Fig. 2. Hydrophobicity (■, - - -) and inactivation curves of *B. amyloliquefaciens* Fad 82 spores at the initial population of (○, —) 2.00×10^2 CFU/ml; (△, —) 1.48×10^4 CFU/ml, (◇, —) 2.59×10^6 CFU/ml, (□, —) 1.48×10^8 CFU/ml during the first 105°C and 700 MPa treatment, and inactivation curves of the survival of *B. amyloliquefaciens* Fad 82 spores at the initial population of (●, - - -) 1.78×10^2 CFU/ml, (▲, - - -) 1.50×10^4 CFU/ml during the second 105°C and 700 MPa treatment. The arrow indicates the come-up time (0.58 min).

was significantly increased as *B. amyloliquefaciens* Fad 82 spores was more inactivated suggests that the DPA may be an important role in pressure and heat resistance of bacterial spores [28].

The effect of different inoculum levels and cellular hydrophobicity on the inactivation of *B. amyloliquefaciens* Fad 82 spores was observed during PATP (Fig. 2). Fig. 2 also includes the resistant characteristics of the PATP resistant spores surviving after the first pressurization. Biphasic inactivation curves were observed at high inoculum levels during the PATP, which is described by a fast decline at the first phase and a slight tail at the second phase. The remaining survivors are more likely to be resistant to heat and pressure, resulting in the tailing phenomenon. Researchers attempted to explain tailing using vitalistic theory [5, 24], which states that the natural spore population exhibits heterogeneous resistance to heat and pressure. The extended tailing up to 10 min holding time was observed at a higher level of inoculum (approximately 10^8 CFU/ml), whereas no surviving spores were detected at a lower level of inoculum (approximately 10^2 and 10^4 CFU/ml) after 3 min of holding time. The finding that spore resistance increases with increasing inoculum level is the evidence that PATP may cause hydrophobic clumps resulting in an extensive tailing phenomenon [8]. Hydrophobic surface properties cause spore aggregation and increase resistance to high pressure and temperature [7, 15, 33]. However, in our study, the hydrophobicity was not significantly increased with an increase in pressure process time. Relative hydrophobicity of spores of *B. subtilis* (55 to 80%), *B. stearothermophilus* (70 to 75%), *B. cereus* (87 to 94%), *B. coagulans* (87 to 97%), and *B. licheniformis* (87 to 92%) increased during thermal treatment at 85°C for 10 min [7, 33]. Hydrophobic characteristics varied among *Bacillus* spp. and *Clostridium* spp., broadly ranged from 19 to 95% [12, 33]. The survivors from the tailing portion were retreated at 105°C and 700 MPa. The control (no PATP treatment) spores were more resistant to heat and pressure than the tail survivors. During the come-up time, the control spores were not significantly inactivated, while the tail survivors were reduced by more than 1 log CFU/ml from the initial numbers of 2 and 4 log CFU/ml. Unlike the control spores, the tail survivors did not retain a PATP-resistant characteristic.

The PATP-resistant *B. amyloliquefaciens* Fad 82 spores treated at 105°C and 700 MPa were collected at the same initial level and cultivated to determine their recovery characteristics, which was whether spore inactivation is involved in PATP-induced germination or PATP-induced injury as a function of pressurization time. The growth of the untreated and PATP-treated spore exponentially increased up to 8 log CFU/ml through germination and outgrowth after 48 h incubation. Lag times of *B. amyloliquefaciens* Fad 82 were estimated from the modified Gompertz

Table 1. Gompertz parameters and derived parameters of *B. amyloliquefaciens* spores cultivated in TSB after 105°C and 700 MPa.

Time (min)	A	B	C	M	μ_{max}	LPD	r^{2d}
0.00	1.04	0.20	^{ab}6.56	^{c}9.09	0.48	^{b}4.02	0.998
0.58	1.02	0.18	^{a}6.70	^{c}10.41	0.44	^{b}4.75	0.998
1.58	1.11	0.19	^{c}6.13	^{b}12.83	0.44	^{a}7.61	0.998
2.58	1.06	0.19	^{abc}6.41	^{ab}13.49	0.45	^{a}8.12	0.997
3.58	1.02	0.16	^{ab}6.57	^{a}14.92	0.40	^{a}8.80	0.999
5.58	1.08	0.18	^{bc}6.18	^{ab}14.47	0.40	^{a}8.79	0.999
LSD^e	0.14	0.05	0.42	1.91	0.13	1.53	

$^{a-c}$Means with different superscript letters within a column are significantly different at $p<0.05$.
^{d}The regression coefficient.
^{e}The least significant difference.

equation, as shown in Table 1. The control and 0 min PATP-treated spores had a similar lag time, while 1, 2, 3, and 4 min PATP-treated spores showed a longer lag phase. No significant differences in the growth rate were observed in *B. amyloliquefaciens* Fad 82 ($p<0.05$). The appearance of increased lag phase at the late period of treatment of 105°C and 700 MPa indicates that injured spores may be more dominantly present than geminated spores. Injured spores require a certain amount of time for recovery mechanisms, which include membrane repair, phospholipid synthesis, ribosomal RNA synthesis, DNA repair, and protein synthesis [4, 31]. *B. amyloliquefaciens* Fad 82 spores showed the extended lag phase compared with untreated spores, which is in agreement with the report of Margosch *et al.* [17] that *B. licheniformis* TMW 2.492 treated with 800 MPa and 70°C for 4 min had longer detection than untreated spores. The inactivation of *B. amyloliquefaciens* Fad 82 spores by high pressure is more likely to be associated with PATP-induced injury than PATP-induced germination. These results provide not only an interesting insight on the spore inactivation mechanisms but also useful information for optimizing the PATP.

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