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Screening Foods for Processing-Resistant Bacterial Spores and Characterization of a Pressure- and Heat-Resistant *Bacillus licheniformis* Isolate

JUHEE AHN[1] AND V. M. BALASUBRAMANIAM[2]*

[1]*Department of Medical Biomaterials Engineering, Kangwon National University, Chuncheon, Gangwon 200-701, Republic of Korea; and* [2]*Department of Food Science and Technology, The Ohio State University, 2015 Fyffe Road, Parker Food Science Building, Columbus, Ohio 43210-1007, USA*

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ABSTRACT

This study was carried out to isolate pressure- and heat-resistant indicator spores from selected food matrices (black pepper, red pepper, garlic, and potato peel). Food samples were processed under various thermal (90 to 105°C) and pressure (700 MPa) combination conditions, and surviving microorganisms were isolated. An isolate from red pepper powder, *Bacillus licheniformis,* was highly resistant to pressure-thermal treatments. Spores of the isolate in deionized water were subjected to the combination treatments of pressure (0.1 to 700 MPa) and heat (90 to 121°C). Compared with the thermal treatment, the combined pressure-thermal treatments considerably reduced the numbers of *B. licheniformis* spores to less than 1.0 log CFU/g at 700 MPa plus 105°C and at 300 to 700 MPa plus 121°C. The inactivation kinetic parameters of the isolated *B. licheniformis* spores were estimated using linear and nonlinear models. Within the range of the experimental conditions tested, the pressure sensitivity (z_P) of the spores decreased with increasing temperature (up to 121°C), and the temperature sensitivity (z_T) was maximum at atmospheric pressure (0.1 MPa). These results will be useful for developing a combined pressure-thermal inactivation kinetics database for various bacterial spores.

Pressure-assisted thermal processing (PATP) of low-acid foods continues to attract industrial interest because this method can effectively inactivate harmful pathogenic and spoilage spores *(3, 4, 19, 24)*. The process involves simultaneous application of high pressures (up to 700 MPa) and temperatures (90 to 120°C) to a preheated food *(2, 14, 15)*. However, relatively few studies have addressed the inactivation kinetics of various pressure- and heat-resistant spores. Therefore, databases containing kinetics parameters determined under well-defined process conditions for various target pathogenic and spoilage spores would be beneficial for food processors.

Similar to observations for thermal processing, strains of *Clostridium botulinum* produce the most PATP-resistant pathogenic spores *(2, 20)*. Reddy et al. *(16)* reported that *C. botulinum* BS-A and 62-A suspended in phosphate medium decreased by 2 to 3 log units when subjected to 827 MPa for 20 min at an average process temperature of 75°C. Margosch et al. *(9)* reported that *C. botulinum* TMW2.357 suspended in Tris-histidine buffer was reduced by 2.4 log units after treatment for 23 min at 800 MPa and an average process temperature of 87°C. Therefore, nonpathogenic surrogate spores with similar or higher processing resistance than that of *C. botulinum* are needed for the validation of low-acid PATP methods *(20)*. *Bacillus stearothermophilus, Thermoanaerobacterium thermosaccharolyticum, Bacillus amyloliquefaciens* Fad 82, and *B. amyloliquefaciens* Fad 11/2 were among the potential PATP surrogate spores evaluated *(1, 3, 4, 9, 14)*. Nevertheless, more studies are needed to understand pressure and heat resistance of various spoilage and pathogenic spores isolated from food sources.

Several spore-forming nonpathogenic bacteria were tested for suitability as surrogates for *C. botulinum* spores for in-factory validation of PATP. Availability of multiple surrogate spores provides a food processor with the opportunity to select the most compatible microorganism for the tested food and processing environment. This study was initiated to screen various foods for hardy bacterial spores. A selected PATP-resistant spore-forming bacterium was further investigated, and the kinetics of inactivation of its spores by PATP was elucidated.

MATERIALS AND METHODS

Preliminary experiment. Experiments were conducted to isolate potential PATP-resistant spores from black pepper, red pepper, garlic, and potato, foods likely to be contaminated with bacterial spores. These products were purchased from a local supermarket. Total aerobic bacterial counts in the tested products were 1×10^3 to 5×10^5 CFU/g. The populations of indigenous anaerobic microbiota were $<4 \times 10^4$ CFU/g. Ten grams of food sample was mixed with 90 ml of Trypticase soy broth (TSB; BD, Sparks, MD) in a pouch. Sample pouches were then subjected to a

* Author for correspondence. Tel: 614-292-1732; Fax: 614-292-0218; E-mail: balasubramaniam.1@osu.edu.

TABLE 1. *Typical pressure-assisted thermal processing settings used in this study*

Process temp (°C)	Process pressure (MPa)	Glycol bath temp (°C)	Sample temp (°C)[a]		
			During preheating (T_1)	Immediately before pressurization (T_2)	During pressure holding (T_3–T_4)
90	300	90	67.2 ± 0. 6	75.1 ± 0.8	91.2 ± 0.2
	500	90	48.3 ± 0.9	63.7 ± 0.6	91.0 ± 0.1
	700	90	45.6 ± 0.5	52.2 ± 0.8	90.8 ± 0.3
105	300	105	71.3 ± 0.7	86.6 ± 1.4	105.7 ± 0.3
	500	105	61.5 ± 1.1	76.6 ± 0.9	105.6 ± 0.3
	700	105	45.6 ± 0.7	58.5 ± 1.2	105.8 ± 0.2
121	300	110	72.2 ± 0.8	99.3 ± 0.7	120.2 ± 0.2
	500	110	62.7 ± 0.6	93.0 ± 0.7	120.8 ± 0.2
	700	110	47.2 ± 0.8	83.6 ± 0.6	121.3 ± 0.3

[a] Data are means ± standard deviations of three independent trials of various combinations of pressure, temperature, and holding time.

combined thermal (90°C) plus pressure (700 MPa) treatment for 3 min using pilot scale equipment (QFP-6 high pressure processor, Flow Autoclave Systems, Inc., Columbus, OH). The treated samples were incubated aerobically or anaerobically at 37°C for 24 to 48 h and plated on Trypticase soy agar (TSA; Difco, BD). Additional higher temperature (100 and 105°C) plus pressure (700 MPa) treatments were conducted to isolate a pressure- and heat-resistant colony.

Identification of the isolated spore-forming bacterium. The biochemical profile of the isolated spore was initially determined with a commercial kit (API 50 CH, bioMérieux Inc., Durham, NC) following the manufacturer's instructions. The ability of isolates to utilize carbohydrates was determined using the manufacturer's software (Apiweb, bioMérieux) and third-party software (*Bacillus*-ID, Microgen Bioproducts Ltd., Camberley, UK). Partial sequence of the isolate's 16S rDNA was determined at the *Bacillus* Genetic Stock Center (BGSC; Ohio State University, Columbus). The colonies obtained from TSA plates were aerobically cultured in 2 ml of Luria-Bertani broth (Difco, BD) at 37°C with shaking (150 rpm) for 20 h. The culture was washed three times in 1.5 ml of buffer (10 mM Tris-HCl, pH 8.5) by centrifugation (8,000 × *g* for 30 s), and the resulting pellet was resuspended in 0.5 ml of the same buffer. Glass beads (SI-BG01, Scientific Industries, Inc., Bohemia, NY) were added and mixed in a bead beater apparatus (Disruptor Genie, Scientific Industries) for 1 min in accordance with the protocol prescribed by the BGSC. The suspension was then centrifuged at 8,000 × *g* for 5 min. For PCR amplification, the cell lysate (0.5 μl) was used as a template with the FastStart High Fidelity PCR system (Roche Applied Science, Indianapolis, IN). Part of the isolate's 16S rDNA was amplified using universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16R1093 (5′-GTTGCGCTCGTTGCGGGACT-3′) *(6)* and 34 PCR cycles (Minicycler, MJ Research, Inc., Watertown, MA) of denaturation at 90°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 65 s. The PCR products (0.5 μl) were cloned with a commercial kit (TOPO TA cloning kit, Invitrogen, Carlsbad, CA). The resulting plasmid was sequenced with the F, R, T3, and T7 promoter primers available at the Plant Microbe Genomics Facility (Ohio State University, Columbus).

***Bacillus licheniformis* spore preparation.** Spores of the PATP-resistant isolate (identified as *B. licheniformis*) were prepared as described by Rajan et al. *(14)* with modifications. Fresh culture (100 μl) of the isolated strain was spread plated on TSA supplemented with 10 ppm of $MnSO_4$ (Fisher Scientific, Pittsburgh, PA) and incubated aerobically at 37°C for 10 days until more than 98% sporulation was observed by microscopic examination. Spores were collected by flooding the surface with 10 ml of 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. The suspension was then sonicated for 10 min (peak power of 270 W; SM275HT, Crest Ultrasonic, ETL Testing Laboratories, Inc., Cortland, NY) and heated at 80°C for 10 min to destroy any remaining vegetative cells. The spore pellet was resuspended in deionized water to approximately 10^9 CFU/ml and stored at 4°C. The prepared spore crop was stored at 4°C and utilized for inactivation studies within 30 days of preparation.

Thermal inactivation of the isolated spores. Inactivation of the isolated spores suspended in deionized water (1.5×10^8 CFU/ml) was determined at 90, 105, and 121°C using custom-fabricated aluminum tubes (11 mm cylindrical diameter, 42 mm long, wall thickness of 3 mm). Aluminum tubes containing the spore suspension were submerged into a 28-liter circulating oil bath maintained at the desired target temperature. The sample temperature was recorded using a K-type thermocouple (Omega Engineering, Stamford, CT) connected to a data logger (IOtech, Cleveland, OH). The come-up times were approximately 3.7 min at 90°C, 3.3 min at 105°C, and 3.0 min at 121°C. A set of aluminum tubes was removed at various time intervals from the oil bath. The heat-treated spore sample was then immersed in an ice-water bath to avoid further inactivation.

High-pressure microbial kinetic tester. PATP experiments were carried out using custom-fabricated high-pressure equipment (Pressure Tester Unit PT-1, Avure Technologies, Kent, WA). With this unit, the samples can be subjected to a maximum pressure and heat combination of 700 MPa and 130°C. The bath surrounding the pressure chamber was maintained at a suitable temperature (Table 1) so that isothermal process conditions could be maintained. Propylene glycol (57-55-6, Avatar Corp., University Park, IL) was used as the pressure transmitting fluid. The sample temperature and chamber pressure were recorded every second with a K-type thermocouple sensor (model KMQSS-04OU-7, Omega Engineering) and pressure transducer (model 3399 093 006, Tecsis, Frankfurt, Germany). A data acquisition computer was used to record the data. The pressure device had a pressurization rate of 22.3 MPa/s, and the depressurization occurred in less than 1 s regardless of the pressurization level.

PATP of the isolated spores. The initial spore population (N_0) suspended in deionized water was approximately 1.4×10^8 CFU/ml. Pouches (5 by 2.5 cm) made from sterile stomacher bags (Fisher Scientific) were used for packaging spore suspensions. Aliquots (1.2 ml) of the aqueous spore suspensions were individually packaged in the pouches and heat sealed with an impulse heat sealer (American International Electric, Whittier, CA). Packages were then placed inside a 10-ml capacity syringe (model 309604, BD, Franklin Lakes, NJ) as a sample carrier. Water was used as the pressure transmitting fluid within the syringe. The syringe was covered with two layers of insulating material to minimize heat exchange with the surrounding environment. Prior to pressurization experiments, the sample carrier containing the spores was preheated (T_1) in a water bath (Isotemp 928, Fisher Scientific) (Table 1). The preheated sample was immediately loaded into the pressure chamber. Pressurization was initiated when the sample temperature reached a predetermined value (T_2) (Table 1) estimated as described in an earlier study *(14)*. Actual process temperature was defined as the average temperature (T_3 to T_4) during the pressure holding time (Table 1). Depending on the target process temperature and pressure, ΔT_H was estimated on a trial-and-error basis. The inoculated samples were treated under different pressure (0.1, 300, 500, and 700 MPa) and heat (90, 105, and 121°C) combination conditions for various hold times. Process hold times were adjusted so that adequate data could be collected for subsequent inactivation kinetics studies. After the treatment, samples were immediately immersed in an ice bath. Microbial analysis of the samples was conducted within 3 h after treatment. Pouches containing the spore samples were opened aseptically, and viable spore counts were determined.

Enumeration of surviving spores. Total viable spores of heat- or PATP-treated samples were directly determined by the pour plating method on TSA. The inoculated plates were incubated aerobically at 37°C for 24 to 48 h. The number of surviving spores in samples with low spore counts were determined by mixing the samples with double-strength TSB (9 ml), incubating the sample for 24 h, streaking on TSA, incubating the plates for 24 to 48 h, and examining for visible growth.

Estimation of kinetics inactivation parameters. The spore inactivation kinetics parameters were estimated using log-linear and Weibull distribution models. The decimal reduction time (*D*-value) was calculated at the initial linear portion of the survivor curve (immediately after the come-up time) using the equation

$$\log\left(\frac{N}{N_0'}\right) = -\frac{t}{D} \tag{1}$$

where N_0' is the initial spore count measured immediately after process (thermal or pressure) come-up time, and N is the spore count after exposure to the thermal or pressure-thermal treatment for a specific time (t).

The adjusted *D*-values for nonlinear survival curves were estimated by the following equation *(14)*:

$$b = \frac{t}{D}^{1-n} \tag{2}$$

where b and n are the scale and shape factors, respectively, which were obtained from the Weibull distribution model (equation 8).

The temperature coefficient z_T (°C) at constant pressure (the temperature change required at constant pressure to achieve a 10-fold change in *D*-value) was estimated as the negative reciprocal of the slope resulting from plotting log D against temperature. Similarly, the pressure coefficient, z_P (MPa) at constant temperature (the pressure required at constant temperature to achieve a 10-fold change in the *D*-value) was estimated as the negative reciprocal of the slope resulting from plotting log D against pressure.

TABLE 2. *Bacterial survival in black pepper powder (BP), red pepper powder (RP), garlic powder (GP), and potato peel (PP) treated with different pressure-thermal combinations and cultured at 37°C on TSA plates*[a]

Treatment	BP	RP	GP	PP
90°C + 700 MPa	+, −	+, +	−, +	+, +
100°C + 700 MPa	−, −	+, +	−, +	−, +
105°C + 700 MPa	−, −	+, +	−, −	−, −

[a] Results for duplicate samples, growth (+) or no growth (−).

The reaction rate constant k (1/min) was calculated from the following reciprocal relation with *D*-value:

$$D = \frac{2.303}{k} \tag{3}$$

The temperature dependence on the inactivation of the spores was determined using the Arrhenius equation:

$$\ln k = -\left(\frac{E_a}{RT}\right)_P + \ln A \tag{4}$$

where E_a is the energy of activation (J/mol), R is the universal gas constant (8.314 J/mol/K), T is the temperature (Kelvin), and A is a preexponential factor. A plot of $\ln(k)$ against $1/T$ is a straight line where the slope is $(-E_a/R)$ and the y intercept is $\ln(A)$. E_a describes the effect of temperature changes on the reaction rate (at constant pressure). A higher E_a signifies that the reaction is temperature sensitive.

The pressure dependence on the inactivation of the isolated spores can be determined using the apparent activation volume *(13)*:

$$\left(\frac{d\ln k}{dP}\right)_T = -\frac{\Delta V}{RT} \tag{5}$$

where ΔV is the activation volume (cm^3/mol), R is the universal gas constant (8.314×10^{-6} m^3/MPa/K/mol), and T is the temperature (Kelvin). ΔV signifies the net effect of pressure reactions causing physiological changes at constant temperature *(10)*.

The activation enthalpy and activation entropy (a measure of the thermodynamic disorder of a physical system) can be calculated from the Eyring equation by nonlinear regression:

$$\ln k = \ln\left(\frac{k_B T}{h}\right) - \left(\frac{\Delta H + RT}{R}\right)\cdot\frac{1}{T} \tag{6}$$

where ΔH is the activation enthalpy (kJ/mol), k_B is the Boltzmann constant (1.381×10^{-23} J/K), h is Planck's constant (6.626×10^{-34} J/s), T is the temperature (Kelvin), and R is the gas constant (8.314 J/mol/K). According to transition state theory, a plot of $\ln(k)$ versus $1/T$ has a slope of $-(\Delta H + RT)/R$. For most reactions, $\Delta H \gg RT$ and $\Delta H \approx E_a$. Combining equations 4 and 6, we obtain

$$\Delta S \approx R\cdot\ln\left(\frac{Ah}{k_B T}\right) \tag{7}$$

where ΔS is the activation entropy (J/mol/K).

Weibull model parameter estimation. The Weibull model is commonly used to evaluate bacterial resistance to heat and pressure *(1)*. The Weibull model is described as

$$\log\left(\frac{N}{N_0'}\right) = -bt^n \tag{8}$$

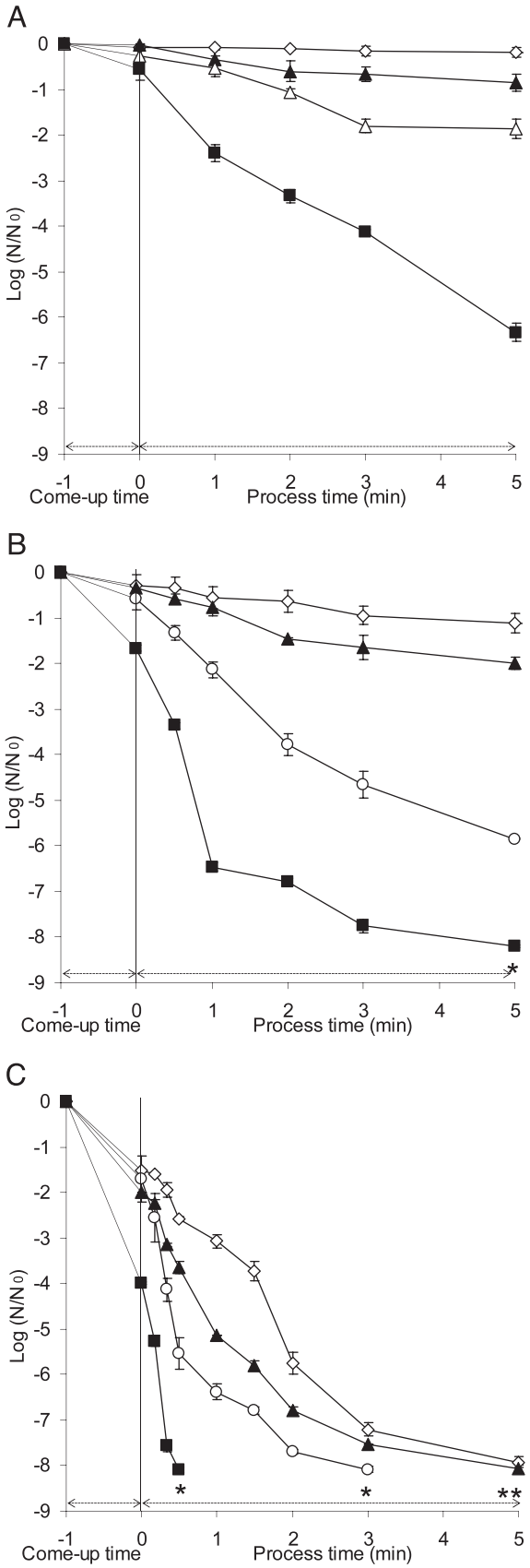

FIGURE 1. *Log-transformed surviving* Bacillus licheniformis *spores suspended in deionized water and subjected to 0.1 MPa (◇), 300 MPa (▲), 500 MPa (○), and 700 MPa at 90°C (A), 105°C (B), and 121°C (C). Dashed lines are changes in counts during come-up time. Asterisk indicates negative results from the enrichment culture.*

where b and n are the scale and shape factors, respectively. Curves were fitted and the model parameters were estimated using nonlinear curve fitting software (Origin 7.5, Microcal Software Inc., Northampton, MA).

The regression coefficient of determination (R^2) and mean square error (MSE) were used to evaluate the goodness of fit. A relatively large R^2 value is indicative of well-fitting models, and a smaller MSE indicates a better fit for the data. The MSE values were calculated from the following equation:

$$\mathrm{MSE} = \frac{\sum (\text{predicted} - \text{observed})^2}{n - p} \quad (9)$$

where n and p are the number of observations and the number of parameters, respectively.

Statistical analysis. Data were analyzed using the Statistical Analysis System software (SAS 8.2, SAS Institute Inc., Cary, NC). The general linear model module included the effects of treatment and strain. Significant mean differences were calculated by Fisher's least significant difference at $P < 0.05$. All experiments were independently conducted three times on different days with duplicate samples for each thermal or combined pressure-thermal treatment.

RESULTS AND DISCUSSION

Phenotypic and genotypic characterization of pressure- and heat-resistant spores. All food samples treated at 90°C and 700 MPa for 3 min and incubated aerobically produced survivors on TSA (Table 2). Colonies recovered from samples treated at the lowest temperature (90°C and 700 MPa) were morphologically different from colonies recovered from samples treated at higher temperatures (100 and 105°C). At 100°C and 700 MPa, bacterial growth was detected by enrichment cultures of red pepper powder, garlic powder, and potato peel. Only red pepper samples produced growth when the process intensity was further increased to 105°C and 700 MPa (Table 2). The bacterium isolated from red pepper powder was gram positive, aerobic, and spore forming. The colonies did not grow at 60°C and had a beige, rough, mucoidal, and lichen-like appearance. The carbon substrate fermentation profile indicated a match for *B. licheniformis*. The isolate produced acetoin, arginine dihydrolase, indole, and β-galactosidase. It also fermented mannitol, mannose, rhamnose, sorbitol, and sucrose but did not metabolize raffinose. The isolate grew slowly in the absence of oxygen. The 16S rDNA sequences were used to confirm the identity of the isolate. Partial 1,108-bp sequences of the 16S rDNA gene were compared with entries in the database at the BGSC, further confirming the identity as *B. licheniformis,* which was more than 99% similar in its 16S rDNA sequence.

B. licheniformis is a saprophytic spore-forming bacterium widespread in nature, specifically in soil. In the fermentation industry, *B. licheniformis* has been commonly used to produce enzymes such as proteases, α-amylases, penicillinase, pentosanase, cycloglucosyltransferase, keratinase, and β-mannanase *(17, 23)*. Its optimum growth conditions range from 30 to 55°C. Mesophilic spore-forming *B. licheniformis* is closely related to *B. amyloliquefaciens, Bacillus subtilis,* and *Bacillus pumulis,* which are part of the *B. subtilis* group *(17)*.

TABLE 3. *Log-linear and Weibull model kinetics parameters for* Bacillus licheniformis *spores suspended in deionized water during thermal processing or pressure-assisted thermal processing*[a]

Temp (°C)	Pressure (MPa)	Linear *D*-value (min)[b]	Weibull model[c]			
			b	*n*	R^2	MSE
90	0.1	154 D	0.03 A	0.64 BCD	0.96	0.01
	300	5.3 C	0.37 AB	0.53 ABC	0.98	0.01
	500	2.1 B	0.51 B	0.79 DE	0.90	0.10
	700	0.83 A	1.6 C	0.77 DE	0.99	0.06
105	0.1	5.6 D	0.30 AB	0.51 AB	0.99	0.01
	300	2.2 B	0.58 B	0.69 CD	0.95	0.01
	500	0.72 A	1.7 C	0.74 DE	0.99	0.03
	700	0.22 A	3.8 F	0.38 A	0.92	0.35
121	0.1	0.43 A	2.4 D	0.68 BCD	0.93	0.26
	300	0.31 A	2.8 E	0.54 ABC	0.95	0.15
	500	0.14 A	4.2 G	0.44 A	0.94	0.44
	700	0.10 A	8.1 H	0.89 E	0.95	0.14

[a] Kinetics parameters were estimated based on three independent trials of thermal processing and pressure-assisted thermal processing experiments. Within a column, means with different letters are significantly different at $P < 0.05$.
[b] Calculated at the initial linear portion of inactivation curve.
[c] The smaller the MSE (mean square error) values and higher the R^2 (regression coefficient) values, the better the model fits the data.

Spore resistance during thermal processing or PATP. At a given processing temperature, pressure treatment accelerated *B. licheniformis* spore inactivation compared with thermal processing alone (Fig. 1), and spore inactivation increased with increasing pressure ($P < 0.05$). Although come-up times during PATP treatment were shorter (0.2 to 0.6 min) than those for thermal treatment (3.0 to 3.7 min), greater spore inactivation during the PATP come-up time was observed. This finding highlights the importance of documenting population changes during come-up times during PATP. Similar observations on spore lethality during the come-up time were made for various *Bacillus* and *Clostridium* spores by Margosch et al. *(9)* and Rajan et al. *(14)*. When the samples were treated at 105°C and 500 MPa and at 121°C and 300, 500, or 700 MPa, spores inactivation was biphasic, with rapid initial inactivation followed by tailing during extended holding times. Spores were completely inactivated at 105°C and 700 MPa after 5 min of treatment (Fig. 1B), and the population decreased by ~5 log units when treated at 105°C and 0.1 MPa for 60 min. Similarly, the times needed for complete inactivation (>8-log reduction as confirmed by an enrichment technique) at 121°C and 500 MPa and at 121°C and 700 MPa were 3 and 0.5 min, respectively (Fig. 1C).

Comparison of inactivation parameters for thermal processing and PATP. Thermal treatments (90, 105, and 121°C) at 0.1 MPa had the highest *D*-values and lowest Weibull parameter *b* values compared with PATP (Table 3). As expected, *D*-values decreased with increasing temperature and pressure and were inversely related to the *b* values. The results were similar to those of Rajan et al. *(14),* who reported an inverse relationship between *b* and *D* for the PATP inactivation kinetics of *B. amyloliquefaciens* spores. The estimated Weibull factor *n* values for PATP treatment ranged from 0.38 to 0.79, consistent with the upward concavity of the plots (Table 3 and Fig. 1). An *n* value closer to 1 signifies first order kinetics. In contrast, a PATP survivor curve with sharp tailing would have a lower *n* value, indicating nonlinear kinetics *(14)*. The *D*-values of the isolated *B. licheniformis* spores in this study were much larger (Table 3) than those reported by Janštová and Lukášová *(8).* These authors estimated *D*-values for *B. licheniformis* spores isolated from milk as 3.7 min at 95°C, 1.3 min at 105°C, and 0.2 min at 120°C. Palop et al. *(12)* reported *D*-values of *B. licheniformis* spores (Spanish Type Culture Collection 4523) of 4.2 min at 99°C, 2.2 min at 102°C, and 0.004 min at 120°C in McIlvaine buffer (0.1 M citric acid and 0.2 M disodium hydrogen phosphate) at pH 7.

Pressure and thermal coefficients of *B. licheniformis* spores. *B. licheniformis* spores were more sensitive to temperature changes at atmospheric pressure ($z_T = 12.2$°C at 0.1 MPa) than at elevated pressures ($z_T = 33.8$°C at 700 MPa) (Table 4). The z_T values of *B. licheniformis* spores at 0.1 MPa was in the range of z_T values similar to those reported for heat-resistant spores of *B. stearothermophilus* ($z_T = 10$°C), *T. thermosaccharolyticum* ($z_T = 7.2$ to 10°C), and *Clostridium sporogenes* ($z_T = 8.8$ to 11.1°C). The calculated z_T value (33.8°C) for *B. licheniformis* spores at 700 MPa was similar to the published values for spores of *B. stearothermophilus* (34.5°C) and *B. amyloliquefaciens* (26.8°C) under similar process conditions *(14, 18)*. The inactivation of the isolated spore had a significantly higher E_a (225.1 kJ/mol) at 0.1 MPa than at 700 MPa (81.7 kJ/mol). This finding suggests that pressure enhanced the spore lethality within the range of conditions studied. The E_a values for inactivation of *B. subtilis*, *B. stearothermophilus*, and *C. botulinum* spores at atmospheric pressure were reported as 318, 283, and 343 kJ/mol, respectively *(7)*. Although the isolated *B. licheniformis* spores appeared to be very temperature sensitive at 0.1 MPa, the number of *B. licheniformis* spores was reduced at higher pressures (300 to 700 MPa). This finding suggests that the combination of

TABLE 4. *Temperature coefficients (z_T and E_a) for* Bacillus licheniformis *spores suspended in deionized water at different pressures and temperatures (90 to 121°C)*[a]

Process pressure (MPa)	z_T (°C)	E_a (kJ/mol)	ΔS (J/mol/K)
0.1	12 ± 0.8 A	225 ± 14 B	339 ± 37 B
300	25 ± 1.7 B	108 ± 7.9 A	9.0 ± 20 A
500	26 ± 2.0 B	105 ± 8.0 A	8.5 ± 21 A
700	34 ± 0.5 C	82 ± 0.9 A	−47 ± 2.4 A

[a] Values are means ± standard deviations. A higher z_T (or lower E_a) value implies less sensitivity to temperature change. Within a column, means with different letters are significantly different at $P < 0.05$.

TABLE 5. *Pressure coefficients (z_P and ΔV) for* Bacillus licheniformis *spores suspended in deionized water at different temperatures and pressures (0.1 to 700 MPa)*[a]

Process temp (°C)	z_P (MPa)	ΔV (cm^3/mol)
90	312 ± 18 A	−23 ± 1.3 A
105	499 ± 11 B	−15 ± 0.1 B
121	801 ± 45 C	−7.0 ± 0.4 C

[a] Values are means ± standard deviations. A higher z_P (or less negative ΔV) value implies less sensitivity to pressure change. Within a column, means with different letters are significantly different at $P < 0.05$.

high pressure and high temperature enhanced the lethality of the isolated *B. licheniformis* spore.

Activation entropy (ΔS) represents the difference between entropy of the transition state and the sum of the entropies of the reactants and provides information about the level of order in the system. In the current study, ΔS values decreased from 339.5 to −46.7 J/mol/K (Table 4). A positive change in ΔS indicates that the system has become more ordered and the reaction is fast. A negative ΔS value suggests that the activated complex in the transition state is more ordered than the reactants in the ground state; the reaction is slower than normal under these circumstances *(21)*. The calculated ΔS for PATP treatments were significantly smaller than those for thermal treatment (Table 4). Thus, high pressure (700 MPa or higher) likely has a limited lethal effect on *B. licheniformis* spore inactivation. The z_P values significantly increased from 312.5 MPa at 90°C to 801 MPa at 121°C (Table 5). These results suggest that *B. licheniformis* spores became increasingly less sensitive to pressure changes as the processing temperature increased. Therefore, the pressure effect on spore lethality was more pronounced at the lower processing temperature (90°C) than at the higher temperature (121°C). This finding agrees closely with previous observations *(13)*. The activation volume (ΔV) values were negative, indicating that pressure has a lethal effect on *B. licheniformis* spores. According to Le Chatalier's principle, an increase in pressure results in an equilibrium shift toward a decrease in volume to minimize the change *(5)*. The absolute ΔV value at a constant 90°C was significantly larger than that at 105 and 121°C, suggesting that the microbial inactivation rate with a greater absolute ΔV value is more pressure dependent *(11, 13, 22)*. As a result, pressure inactivation of the isolated *B. licheniformis* spores increases more rapidly at a lower temperature when pressure levels increase from 0.1 to 700 MPa.

In conclusion, PATP effectively inactivated the isolated *B. licheniformis* spores under the high pressure and temperature conditions tested. A similar approach may help isolate spores resistant to combined pressure and thermal treatment in various natural food matrices. The combination of elevated pressure and temperature helped to significantly reduce the processing time in comparison with traditional thermal processing. Weibull kinetics parameters (b and n) adequately described PATP microbial lethality. More studies are necessary to comprehensively understand related spore inactivation mechanisms and the protective effect of various food matrices.

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