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Thermal Inactivation and Injury of *Bacillus stearothermophilus* Spores

FLORENCE E. FEEHERRY, DONALD T. MUNSEY, AND DURWOOD B. ROWLEY*

Microbiology Branch, Science and Advanced Technology Directorate, U.S. Army Natick Research, Development and Engineering Center, Natick, Massachusetts 01760-5020

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Aqueous spore suspensions of *Bacillus stearothermophilus* ATCC 12980 were heated at different temperatures for various time intervals in a resistometer, spread plated on antibiotic assay medium supplemented with 0.1% soluble starch without (AAMS) or with (AAMS-S) 0.9% NaCl, and incubated at 55°C unless otherwise indicated. Uninjured spores formed colonies on AAMS and AAMS-S; injured spores formed colonies only on AAMS. Values of D, the decimal reduction time (time required at a given temperature for destruction of 90% of the cells), when survivors were recovered on AAMS were 62.04, 18.00, 8.00, 3.33, and 1.05 min at 112.8, 115.6, 118.3, 121.1, and 123.9°C, respectively. Recovery on AAMS-S resulted in reduced decimal reduction time. The computed *z* value (the temperature change which will alter the D value by a factor of 10) for spores recovered on AAMS was 8.3°C; for spores recovered on AAMS-S, it was 7.6°C. The rates of inactivation and injury were similar. Injury (judged by salt sensitivity) was a linear function of the heating temperature. At a heating temperature of ≤118.3°C, spore injury was indicated by the curvilinear portion of the survival curve (judged by salt sensitivity), showing that injury occurred early in the thermal treatment as well as during logarithmic inactivation (reduced decimal reduction time). Heat-injured spores showed an increased sensitivity not only to 0.9% NaCl but also to other postprocessing environmental factors such as incubation temperatures, a pH of 6.6 for the medium, and anaerobiosis during incubation.

Denny (9) reported that as early as 1913, Barlow had demonstrated that thermophilic bacteria were the prime cause of spoilage of canned corn. Although thermal processes have been developed which generally ensure the safety of low-acid (pH of ≥4.6) canned foods from spoilage by *Clostridium botulinum* (16) and *Clostridium sporogenes* (23), thermophilic *Bacillus stearothermophilus* spores can and do survive thermal processing in a commercially sterile product (4, 9, 23). Since many products cannot withstand the heat treatment required to inactivate thermophilic bacterial spores, other measures are required to prevent spoilage. Such measures include the control of contamination of ingredients by thermophilic organisms, rapid cooling to 43°C after thermal processing, and controlled storage.

During the thermal process, spores may be inactivated, sublethally injured, or uninjured. The exponential death rate resulting from the heating of spores forms the basis of calculations used in thermal processing in the food industry (23); however, the injured spore population is not always considered in these calculations. Sublethal injury caused by various food-processing techniques affects either the germination or the outgrowth capability of the spore as manifested by a requirement for nonnutritive germination stimulants, a modified optimal temperature for enumeration, an increased sensitivity to inhibitors, and altered nutritional requirements (1). Spore injury may have beneficial effects in food processing by increasing the sensitivity of spores to food ingredients such as sodium chloride. Such injury may play a role in reducing spoilage of thermally processed foods by thermophilic bacteria.

The purpose of this study was to determine the thermal resistance of *B. stearothermophilus* spores, the extent of the injured spore population at various processing temperatures, and the significance of the injured spore population in the spoilage of thermally processed low-acid canned foods.

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MATERIALS AND METHODS

Spore preparation. The medium of Cook and Brown (6) was used to prepare the spores of *B. stearothermophilus* ATCC 12980. This medium had the following composition (in grams per liter): Bacto-Tryptone (Difco Laboratories, Detroit, Mich.), 3.0; Bacto-Peptone (Difco), 6.0; yeast extract (Difco), 3.0; Lab Lemco beef extract (Oxoid Ltd., London, England), 1.5; and agar (Difco), 15.0 for slants or 25.0 for Fernbach flasks. It also included 1 μg of Mn^{2+} per ml.

Stock cultures were maintained on agar slants on the medium of Cook and Brown at 5°C. Growth was washed from stock slants with water (10 ml), heated at 75°C for 10 min, and diluted (100-ml total), and 3 ml of the diluted suspension was inoculated onto a solid agar (250-ml) surface in a 2,800-ml Fernbach flask. Incubation was at 55°C for 4 days.

The cultures from approximately 25 Fernbach flasks, containing a mixture of cell forms, were harvested by scraping the agar surface with a bent glass rod and rinsing off the growth with water. The combined suspensions were centrifuged at 1,000 × *g* for 30 min in a swinging-bucket rotor in a refrigerated centrifuge (International Equipment Co., Div. Damon Corp., Needham Heights, Mass.) at 5°C. The supernatant was decanted, and the pellet was washed by suspension in chilled water and recentrifugation. This washing procedure was repeated twice. Subsequently, the

* Corresponding author.

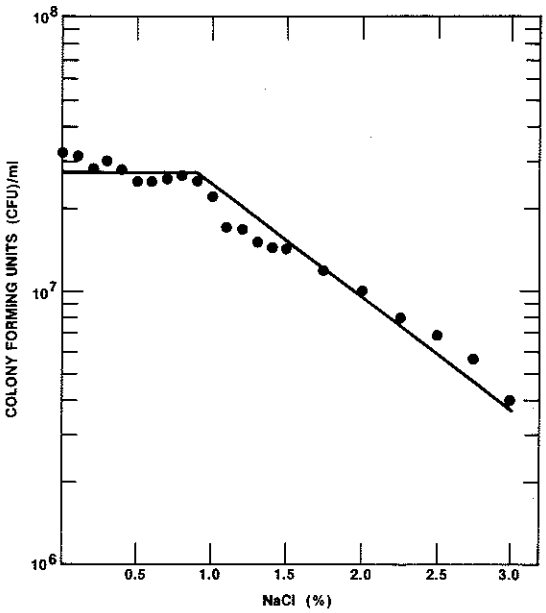

FIG. 1. Effect of NaCl concentration in a recovery medium (AAMS) on the uninjured spores of *B. stearothermophilus* ATCC 12980. Heat-activated (100°C for 15 min) spores were plated on AAMS supplemented with various concentrations of NaCl and incubated at 55°C for 24 h. The values plotted were the average of three experiments.

washed pellets were suspended in 200 ml of 0.05 M potassium phosphate (pH 7.0) containing lysozyme (100 μg/ml) and incubated with stirring at 37°C for 1 h. Enzyme-treated spores were washed with water four times to remove vegetative debris.

Twenty spore suspensions prepared as described above were lyophilized, pooled, (ca. 8 g of spores), and stored in an evacuated desiccator over silica gel at 5°C. The lyophilized spores had no detectable vegetative cells, no spores in sporangia, and less than 5% germinated spores.

Spore activation. Aqueous suspensions of spores (1.0 mg of lyophilized spores per ml of sterile distilled water or ca. 2.0×10^8 spores per ml) were mixed thoroughly with a Vortex mixer and filtered through a coarse sintered-glass funnel (Pyrex; Corning Glass Works, Corning, N.Y.) to remove spore clumps. Spores were activated by being heated at 100°C for 15 min in a free-flowing-steam cabinet (G. H. Wahmann Manufacturing Co., Baltimore, Md.) and were subsequently chilled in an ice bath until used.

Heat treatment. A volume (0.5 ml) of the activated spore suspension was placed in a stainless steel cup (inside diameter, 17.4 mm; height, 13 mm) and heated in a Biological Indicator Evaluator Resistometer (Joslyn Valve Co., Macedon, N.Y.) at different temperatures for various times. After being heated, these cups were aseptically dropped into tubes (11 by 203 mm) containing 9.5 ml of chilled sterile distilled water as the first diluent.

Recovery conditions. Diluted samples (0.1 ml) were spread plated (duplicate plates) on antibiotic assay medium supplemented with 0.1% soluble starch (AAMS; 6), which had the following composition (in grams per liter): Bacto-Peptone (Difco), 6.0; Bacto-Tryptone (Difco), 4.0; yeast extract (Difco), 3.0; Lab Lemco beef extract (Oxoid), 1.5; dextrose, 1.0; soluble starch (Difco), 1.0; and agar (Difco), 15.0 (pH 6.9). This was the optimal or nonselective medium for growth under aerobic conditions. The number of CFU on this medium measured growth of the total viable population (uninjured and injured spores). The addition of NaCl to concentrations as high as 0.9% to AAMS (AAMS-S) had little effect on the number of colonies formed by unstressed cells (Fig. 1). Increasing the concentration of NaCl to more than 0.9% caused a progressive decrease in the number of CFU per milliliter. Colonies formed on this selective medium represented only the uninjured spore population, i.e., those cells not sensitive to 0.9% NaCl.

Incubation temperatures ranged from 40 to 65°C, with 55°C being the optimal temperature of incubation. Incubation time was approximately 24 h except at low temperatures, such as 40 and 45°C, at which the time increased to 6 to 7 days and 2 to 3 days, respectively.

In some experiments, the pH of AAMS or AAMS-S was lowered with HCl before the medium was autoclaved. Since autoclaving may affect the pH, all values were determined after autoclaving.

Incubation was under aerobic conditions unless otherwise noted. Anaerobic conditions (spread plates) were maintained with GasPak (BBL Microbiology Systems, Cockeysville, Md.).

Calculations. The percentage of spores injured was calculated according to the following equation:

$$\text{injured cells (\%)} = I = \left(1 - \frac{\text{CFU/ml}_{\text{selective}}}{\text{CFU/ml}_{\text{nonselective}}}\right) \times 100$$

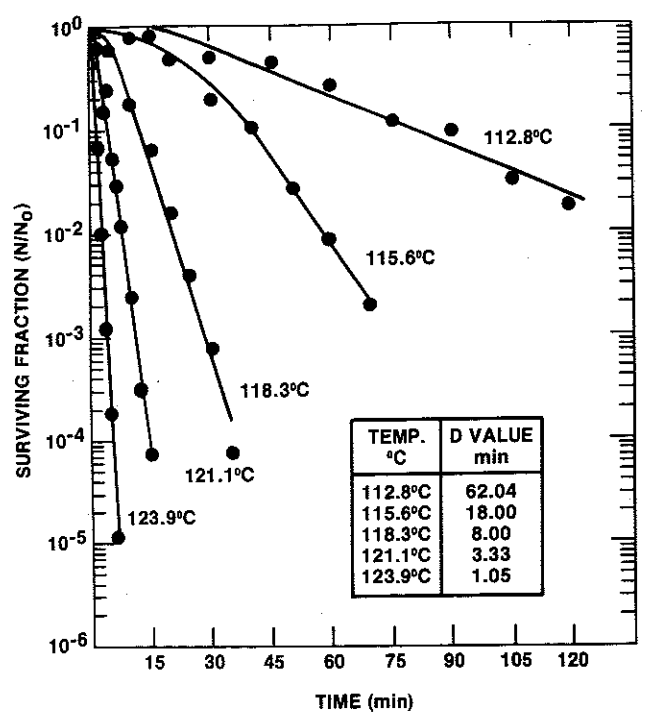

FIG. 2. Thermal inactivation of spores of *B. stearothermophilus* ATCC 12980. Heat-activated (100°C for 15 min) spores were treated at various temperatures for various times, plated on AAMS, and incubated at 55°C for 24 h. The surviving fraction (N/N_0) was the number of viable spores at a given time divided by the number of spores at time zero. *D* values for the rectilinear portion of the curve were computed by the least-squares method.

where the term selective means either selective medium (AAMS-S), selective temperature of incubation, selective pH (<6.9), or anaerobic conditions and the term nonselective means either nonselective medium (AAMS), nonselective temperature of incubation (55°C), nonselective pH (6.9), or aerobic conditions.

***D* and *D'* values.** The *D* and *D'* values measure the time (in minutes) required at a given temperature for destruction of 90% of the cells when the survivors were recovered on AAMS or AAMS-S, respectively.

All experiments were repeated two to three times, and the results represent the average of all experiments. In all figures except Fig. 1, the best straight line was determined by the least-squares method.

RESULTS

Thermal inactivation. The kinetics of thermal inactivation of spores of *B. stearothermophilus* in aqueous suspension are shown in Fig. 2. The survival curves had an initial curvilinear portion preceding a logarithmic decline when spores were heated at 112.8, 115.6, or 118.3°C, but no such pattern was evident when spores were heated at 121.1 or 123.9°C. The *D* values ranged from 62.04 min at 112.8°C to 1.05 min at 123.9°C.

Thermal injury determined by sensitivity to salt or temperature of incubation. The kinetics of thermal injury (as judged by inability to form colonies on AAMS-S) compared with inactivation at two temperatures, 115.6 and 121.1°C, are shown in Fig. 3. At 115.6°C, it was evident that injury preceded inactivation. There was an initial curvilinear portion in the survival curve which indicated that some spores were injured but not inactivated. For example, there was ca. 83% survival on AAMS when the spores were heated for 10 min at 115.6°C. However, 68% of these surviving spores were injured, as judged by their inability to form colonies on AAMS-S. Rapid inactivation and injury resulted from exposure to 121.1°C; thus, no curvilinear portion was evident. The *D'* values at 112.8, 118.3, and 123.9°C were 44.04, 5.36, and 0.82 min, respectively (data not shown). The smaller *D'* values are pseudo *D* values (not true *D* values) and are an indication of injury.

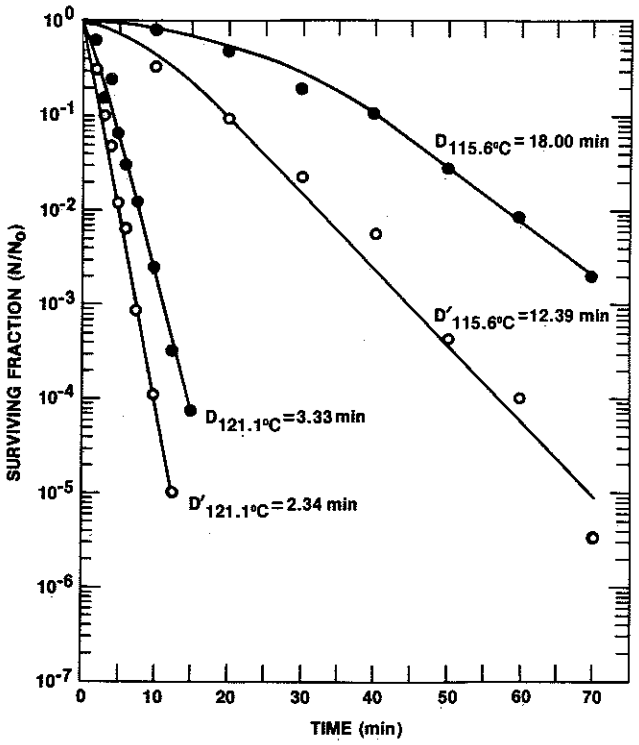

FIG. 3. Thermal inactivation and injury of spores of *B. stearothermophilus* ATCC 12980. Heat-activated spores (100°C for 15 min) were treated at 115.6 or 121.1°C for various times, plated on AAMS without (●) or with (○) 0.9% NaCl, and incubated at 55°C for about 24 h. The *D* and *D'* values were computed as described in the legend to Fig. 2. The surviving fraction (N/N_0) was the number of viable spores at a given time divided by the number of spores at time zero.

TABLE 1. Effect of incubation temperature on recovery of uninjured and thermally injured spores of *B. stearothermophilus*

Incubation conditions		Spores recovered (CFU/ml)[a]			% Injured cells[d]
Temp (°C)	Time (days)	Unactivated (10^8)	Activated (10^8)[b]	Heat treated (10^6)[c]	
40	6–7	1.35	2.61	3.28	62.3
45	2–3	1.38	2.76	4.86	44.1
55	1	1.52	2.50	8.70	0
65	1	1.31	2.49	6.57	24.5

[a] On AAMS.
[b] An aqueous suspension of spores was heated at 100°C for 15 min.
[c] Activated spores were heated at 121.1°C for 6 min, resulting in a spore population of inactivated, injured, and uninjured cells.

[d] Injured cells (%) $= \left(1 - \frac{\text{CFU/ml}_{x°C}}{\text{CFU/ml}_{55°C}}\right) \times 100.$

There was at least 95% inactivation of the spore population when spores were heated at 121.1°C for 6 min (Fig. 3). Furthermore, 95% of the surviving 5% of spores was injured, as judged by the inability of cells to grow on AAMS-S. When sensitivity to incubation temperature was used as a criterion of injury, the same degree of injury was not shown (Table 1). Incubation temperatures did not affect the total number of CFU of either unactivated or activated spores per ml, except that the incubation time for colony visualization increased at lower temperatures. However, incubation temperature did have an effect on the ability of heat-treated spores to produce colonies on AAMS. For example, spores heated at 121.1°C for 6 min formed fewer colonies at 40 than at 55°C, and ca. 62% of the surviving fraction was injured, as judged by inability of cells to grow at 40°C. Even a prolonged incubation of 14 days at 40°C did not increase the number of recoverable cells (data not shown). The results indicate that the inhibitory effect of temperature on the recovery of heat-injured cells was most pronounced at 40°C (Table 1).

Effect of pH or anaerobiosis on the recovery of heated spores. The use of AAMS recovery medium (pH 6.6) instead of unadjusted medium (pH 6.9) had no effect on the percent recovery of activated spores of *B. stearothermophilus*. As the pH of the recovery medium was reduced to less than 6.6, the percent recovery progressively decreased, so that at pHs 6.4, 6.2, 6.0, and 5.8, the percentage of spores that formed colonies was 62, 38, 20, and 1%, respectively.

Heat-injured spores showed an increased sensitivity not only to 0.9% NaCl and certain incubation temperatures, but also to two other conditions (pH of 6.6 and anaerobiosis) which had no effect on the recovery of activated spores (Table 2). This sensitivity of heat-injured spores was also demonstrated by reduced *D'* values of 2.34 min (with NaCl), 1.54 min (with anaerobiosis), and 2.36 min (at pH 6.6) (Table 3). When the pH of the recovery medium was less than 6.6,

TABLE 2. Effect of various selective conditions of recovery on *B. stearothermophilus* spores[a]

Min at 121.1°C	% Injury[b] with:			
	0.9% NaCl	6.6 pH	Anaerobiosis (GasPak)	Incubation at 40°C
0.0	0.0	0.0	0.0	0.0
2.0	70.8	37.9	62.0	ND
4.0	85.7	55.4	93.0	ND
6.0	91.3	76.5	98.2	62.3
7.5	95.7	89.9	99.7	ND

[a] Spores were activated by heating at 100°C for 15 min.

[b] $\% \text{ Injury} = \left(1 - \frac{\text{CFU/ml}_{\text{selective conditions}}}{\text{CFU/ml}_{\text{nonselective conditions}}}\right) \times 100$, where selective conditions are those defined above and nonselective conditions means either AAMS, 55°C, pH of 6.9, or aerobic conditions.

the D' values resulted not only from inactivation and injury but also from pH inhibition. The presence of 0.9% NaCl in the recovery medium or anaerobiosis further reduced the D' values.

Estimation of heating time to attain 90 to 95% injury. To compare the extent of injury at various heating temperatures when at least 90% of the total population was thermally inactivated, the time required to achieve 90 to 95% injury (detected by salt sensitivity) was plotted versus the heating temperature. The logarithm of the time required to attain 90 to 95% injury was a linear function of the temperature of heating and ranged from 120 min at 112.8°C to 2 min at 123.9°C (Fig. 4).

Thermal destruction curves. D and D' values were plotted versus temperature to generate z values, the changes in temperature which would alter the D or D' value by a factor of 10 (Fig. 5). In the temperature range of 115.6 to 121.1°C, a z value of 8.3°C was obtained for spores recovered on AAMS compared with a z value of 7.6°C for spores recovered on AAMS-S. The rates of inactivation (z values) and injury (the area between the two curves) were similar.

DISCUSSION

The thermal resistance of aqueous suspensions of *B. stearothermophilus* as characterized by D and z values confirmed other work (2, 7, 11, 17, 18, 24), indicating that these spores are extremely heat resistant and that a 12D process for *C. botulinum* would not provide a 5D process as used for *B. stearothermophilus* (D. J. Goeke, paper presented at the Second Annual Eastern Research Highlights Conference, National Canners Association, Washington, D.C., 1977). The z value reported in our study was 8.3°C, whereas a range of 6.9 to 7.8°C has been reported for various strains of *B. stearothermophilus* (17). Briggs (2) and Pflug and Smith (20) published z values of 7.0 and 8.4°C for aqueous suspensions of spores of strains NCIB 8919 and ATCC 7953, respectively.

The addition of a stressful substance (e.g., NaCl or antibiotics) to a good medium (13) or the creation of certain

TABLE 3. Effects of various factors on *B. stearothermophilus* spores[a]

Recovery medium	D' values (min) at pH[b]:			
	6.2	6.4	6.6	6.9
Aerobic				
AAMS	1.11	1.57	2.36	3.33[c]
AAMS-S	0.79	1.07	1.46	2.34
Anaerobic				
AAMS	0.73	0.95	1.15	1.54

[a] Activated (100°C for 15 min) aqueous spore suspensions were heated at 121.1°C, diluted, plated, and incubated at 55°C for 1 to 2 days aerobically and for 3 to 4 days anaerobically.

[b] Of recovery medium.

[c] D (not D') value obtained by recovery of heated spores under optimal conditions.

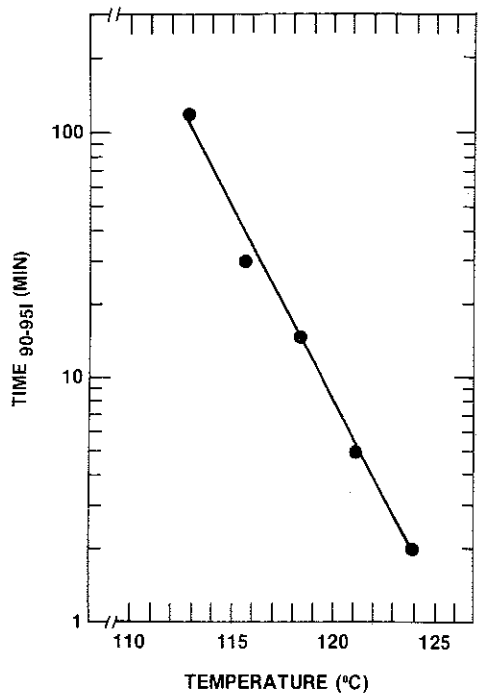

FIG. 4. Effect of heating temperature on the time required to attain 90 to 95% injury (90-95I) of spores of *B. stearothermophilus* ATCC 12980.

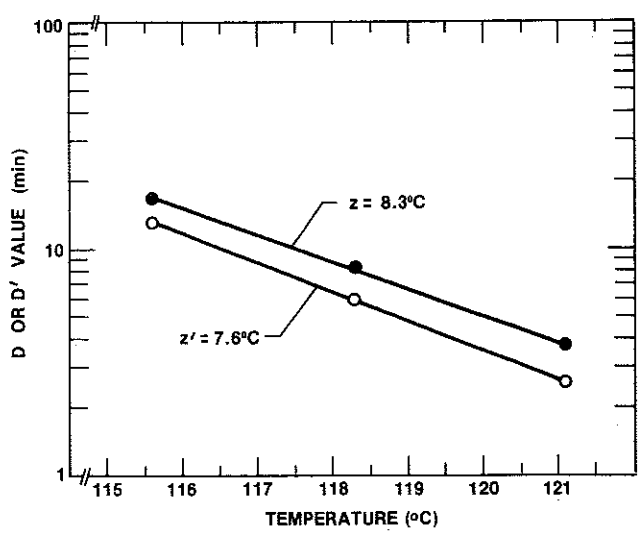

FIG. 5. Thermal destruction curve for spores of *B. stearothermophilus* ATCC 12980. D and D' values were based on the recovery at 55°C of total survivors (uninjured and injured) on AAMS (●) and uninjured survivors on AAMS-S (○).

stressful conditions has been used to assay spore injury. In our studies, the presence of thermally injured spores was routinely judged by the inability of these spores to form colonies in the presence of 0.9% NaCl. Cook and Gilbert (8), Briggs and Yazdany (3), and Labbe (15) previously demonstrated that heated spores of *B. stearothermophilus* are sensitive to NaCl in the recovery medium.

Many researchers (2, 10, 14, 18) have demonstrated that the survival curves of spores heated at temperatures below 121.1°C frequently consist of an initial curvilinear portion succeeded by a logarithmic decline. The curvilinear portions may result from the effect of spore clumping, an activation process counteracting the inactivation process, or the existence of an intermediate state of thermal susceptibility between the normal viable state and death (e.g., injured cells capable of repair). In our studies, all spore suspensions were filtered (with a sintered-glass filter) to remove any spore clumps and activated under experimentally determined optimal conditions (100°C for 15 min) before any heating experiments determining survival were done. Therefore, we believe that the intermediate state of thermal susceptibility is just another way of referring to the injured spore population. The injured spore population capable of repair accounts for the curvilinear portions of our survival curves.

What is the significance of the injured spore population? Clearly, injured spores are sensitive to any one of an array of typical postprocessing factors such as NaCl, temperature, pH, and anaerobiosis, all of which result in fewer survivors as evidenced by reduced D and z values (Fig. 3; Table 3). Cook and Gilbert (8) suggested that the presence of 1 to 3% NaCl may play an important role in the control of spoilage of low-acid (pH ≥4.6) canned foods by thermophilic bacteria. Spore injury and subsequent sensitivity to the surrounding environment are among the numerous factors (e.g., nitrite and brine concentrations, pH, storage temperature) associated with the inhibition of *C. botulinum* and reduced thermal requirements in cured meats (5).

Although the recovery of unstressed *B. stearothermophilus* spores was not affected by anaerobic conditions, 93% of those spores surviving 121.1°C for 4 min were unable to proliferate under these conditions (Table 2). Thus, injury should play a role in the delay or reduction of spoilage of hermetically sealed foods by thermophilic bacteria.

Fewer heat-treated *B. stearothermophilus* spores were recovered at ≤45°C than at 55°C (Table 1). This inability of injured spores to outgrow at lower temperatures may be of benefit in the common practice of rapidly cooling thermally processed cans to a temperature of 43°C to aid in the elimination of spoilage by thermophilic bacteria (9).

The phenomenon of autosterilization (19) or sterilization by spore deactivation (21) has been reported for flat-sour thermophiles, e.g., *B. stearothermophilus*. Pearce and Wheaton (19) have defined the term autosterilization as the dying out or loss of viability of spoilage spores which have never grown in the product. This loss occurred at a suboptimal incubation temperature of 21.1°C. It is conceivable that spore injury plays an important role in autosterilization.

It does appear that thermal injury reduces the possibility of *B. stearothermophilus* spoilage of foods with a pH of ≤6.6 or containing ≥0.9% NaCl or of foods that are hermetically sealed or stored at ≤45°C. The effects of the interactions of these postprocessing conditions on the recovery of injured spores were not investigated in these studies. We did demonstrate the effect of pH of the medium alone or in combination with NaCl or anaerobiosis on the decimal reduction time of heated *B. stearothermophilus* spores (Table 3). When the recovery medium had a pH of ≤6.6 and contained 0.9% NaCl or was incubated under anaerobic conditions, the reduced D' values resulted not only from injury, but also from inhibition by pH alone and in combination with NaCl or anaerobic conditions.

Gombas (12) appropriately stated "Preventing proliferation of injured spores is a double-edged sword." He further emphasized that one must ensure that (i) all of the surviving population is injured, (ii) all injured spores are inhibited by the postprocess treatments, and (iii) repair of the injured spores is prevented. After repair, an injured pathogen or spoilage microorganism has the potential to cause a health hazard or spoilage.

It is evident that a low indigenous thermophilic spore population must be maintained in the formulation by the use of ingredients with minimal levels of thermophilic spores and by appropriate cleaning of vegetables (22), since if the spore level is too high, it is impossible to ensure that all thermophilic spores are either inactivated or injured during a thermal process (e.g., 6 min at 121.1°C). G. R. Haldeman (M.S. thesis, Pennsylvania State University, University Park, 1978) demonstrated that low numbers (<2/g) of thermophilic spores were capable of causing spoilage in canned mushrooms. In some of our studies (data not reported), cans containing ca. one *Clostridium thermosaccharolyticum* spore per 100 g of beef strips with green peppers and gravy spoiled within 4 days at 65°C.

The actual significance of injury and treatment interactions reducing spoilage by thermophilic bacteria must eventually be verified by inoculated-pack studies dealing with the product(s) causing concern.

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