TECHNICAL REPORT
NATICK/TR-01/003

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EFFECT OF HYDROSTATIC PRESSURE ON THE INDUCTION AND INACTIVATION OF BACTERIAL SPORES PHASE 1

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December 2000

Final Report
February 1998 - January 1999

Approved for Public Release; Distribution is Unlimited

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Soldier Systems Center
Natick, Massachusetts 01760-5018

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
1-12-00	Phase 1 - Final	1/2/98 - 1/31/99

4. TITLE AND SUBTITLE
Effect of Hydrostatic Pressure on the Induction and Inactivation of Bacterial Spores - Phase 1

5a. CONTRACT NUMBER
DAAK 60-97-R-9601

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
W13118-7323-3100/165

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
*Food Microbiology Laboratory
Depatrment of Animal Science
University of Wyoming
Laramie WY 82071

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
**U.S. Army Soldier and Biological Chemical Command
Soldier Systems Center
Natick, MA 01760-5018

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
NATICK/TR-01/003

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Destruction of microbial cells and bacterial spores by hydrostatic pressure is being envisioned as a nonthermal novel technique in food preservation. A combination of 50000 psi (345 MPa) at 50°C could kill over 6 log cycles of foodborne pathogenic and spoilage bacteria within 5 min. In contrast, even 200,000psi at 80°C for 60min will not be able to inactivate important bacterial spores to the same level. However, bacterial spores can be induced to germinate at a relatively low pressure range, and then the germinated and over-growing spores can be destroyed by one or more methods used in destroying the bacterial cells. To achieve this goal it would be necessary to determine the pressurization parameters that would induce maximum germination of spores. In the Phase I study, the frequency of germination induction of 2 *Bacillus* and 4 *Clostridium* species (that include both food spoilage and pathogen) following exposure to several pressurization-temperature combinations was examined. Depending upon a treatment, pressurization-temperature combinations caused both inactivation and induction of spores, which were some what directly proportional; but the *Bacillus* spores were more sensitive than the clostridial spores. The pressurized spores continued to germinate during subsequent storage with higher germination at 25°C that at 4°C as determined by the increase in inactivation by low heat treatment.

15. SUBJECT TERMS

HYDROSTATIC PRESSURE	INDUCTION	FOOD PRESERVATION	PROCESSED FOODS
BACTERIAL SPORES	INACTIVATION	FOODBORNE PATHOGENS	STORAGE
NOVEL TECHNIQUE	MICROBIAL CELLS	PATHOGENIC BACTERIA	GERMINATION

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Anthony Sikes
Unclassified	Unclassified	Unclassified	SAR	30	19b. TELEPHONE NUMBER *(Include area code)* (508) 233-4362

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PREFACE

This study was conducted from February 1998 through January 1999 by Mssrs. Norasak Kalchayanand and Bibek Ray, University of Wyoming, under the supervision of Anthony Sikes and Patrick Dunne, Combat Feeding Program, U.S. Army Soldier and Biological Chemical Command, Natick, MA. The work was funded under the project (DAAK 60-97-R-9601) titled "Inactivation of Pathogenic and Spoilage Bacterial Spores in Processed Meat Products by a Combination of Hydrostatic Pressure, Temperature, Biopreservatives, Reduced Water Activity and pH".

Mssrs. Kalchayanand's and Ray's research was designed to ascertain answers to the following questions: (1) do hydrostatic pressure, pressurization temperature and incubation time and temperature induce spore germination of *Clostridium* and *Bacillus* species; (2) do pressure-induced spores of *Clostridium* and *Bacillus* species become sensitive to bacteriocin-based biopreservatives along with lysozyme and EDTA; (3) does hydrostatic pressure in combination with bacteriocin-based biopreservatives inactivate spores in processed meat products?

This research, which was divided into 3 phases, was initiated on 1 Feb 98. This report summarizes results from Phase 1, which ended 31 Jan 99.

EFFECT OF HYDROSTATIC PRESSURE ON THE INDUCTION AND INACTIVATION OF BACTERIAL SPORES PHASE 1

Introduction

Hydrostatic pressurization provides an alternative to thermal processing of foods. The effect of high hydrostatic pressure on bacterial cells is basically targeted on cell membrane. Hydrostatic pressure increased permeability of the cell membrane causing cell death (Hoover et al., 1989). Most spoilage and pathogenic bacteria were killed at pressure ranging from 30,000 to 50,000 psi at 25°C (Kalchayanand et al., 1998). However, *Staphylococcus aureus* and *Enterococcus faecalis* were only killed at pressures about 88,000 psi (Shigehisa et al., 1991).

Bacterial spores are certainly more resistant to inactivation by hydrostatic pressure than vegetative cells (Cheftel, 1995). The ungerminated endospores of *Bacillus* and *Clostridium* can survive pressures above 176,000 psi (Hoover, 1993, Sale et al., 1970, and Timson and Short, 1965). The mode of action of pressure on bacterial spores is still unknown, but spores are induced for germination at pressures between 7,250 and 43,500 psi (Sale et al., 1970, Timson and Short, 1965, and Wuytack et al., 1997). It is not known whether pressure induces "spore activation" similar to reversible heat activation or triggers germination irreversibly (Smelt, 1998). However, germinated spores lose their exceptional resistance to heat, radiation, desiccation, electrical shock and chemical agents (Gould, 1970).

The objectives of these studies were to determine: (a) the effect of pressures, and pressurization temperatures on activation to germination of *Bacillus* and *Clostridium* spores, (b) the effect of incubation time and incubation temperature following pressurization on germination

of *bacillus* and clostridial spores, and (c) the effect of pressure and temperature on inactivation of bacterial spores of both genera.

METHODS AND MATERIALS

Bacterial strains, media and growth conditions

Bacillus cereus ATCC 10876 (UW food microbiology stock collection), *Bacillus stearothermophilus* ATCC 12980 (Dr. Sikes, Food Engineering, Natick, MA) were grown aerobically in antibiotic assay mediumSupplemented (AAMS) with 0.1% soluble starch but without antibiotic (Feeherry et al., 1987) at 30° and 55°C, respectively. *Clostridium laramie* ATCC 51254 , *Clostridium perfringens* 1027, isolated *Clostridium* sp. From hydrostatic-pressure-pasteurized roast beef (all from UW food microbiology stock collection), and *Clostridium sporogenes* PA 3679 (Dr. Sikes, Food Engineering, Natick, MA) were grown anaerobically in reinforced clostridial medium (RCM; Difco, Detroit, MI) at 10° and 37°C, respectively.

Identification of isolated *Clostridium* from hydrostatic-pressure-pasteurized roast beef during storage at 25°C

The purified isolated *Clostridium* was grown in RCM at 37°C for 16 to 18 h. Cells were harvested by centrifugation (Beckman, Fullerton, CA) at 6,000 x g for 5 min at 25°C, washed twice with sterile 0.1% peptone solution supplemented with 0.05% L-cysteine hydrochloride (Sigma, St. Louis, MO). Cells were resuspended in API anaerobe basal medium and twenty-one biochemical tests were determined using API 20A system (bioMérieux Vitek, Inc., Hazelwood, MO). Test strip was incubated anaerobically at 37°C for 24 to 36 h. The test results were confirmed with the bioMérieux computer profile for its identification. The isolate confirmed to the profile of *Clostridium tertium* (see the Results section)

Bacterial spores preparation

B. cereus was streak on mannitol egg yolk polymyxin agar (MYP; Mehlman, 1984) but without polymyxin B sulfate. Plates were incubated aerobically at 30°C for 48 h. Spores were washed 6 times with sterile deionized water and collected by centrifugation at 8,000 x g for 10 min at 4°C. Spores were resuspended with sterile deionized water and stored at 4°C until use.

B. stearothermophilus was prepared as the method described by Feeherry et al. (1987). Spores were resuspended with sterile water and stored at 4°C until use.

C. laramie was grown anaerobically in RCM at 10°C for 14 to 28 d. Spores were collected by centrifugation at 8,000 x g for 10 min at 4°C. The pellet was washed 6 times, resuspended with sterile deionized water and stored at 4°C until use.

C. perfringens was grown in sporulation broth (Mehlman, 1984) supplemented with 200µl/ml of 1-Methylxanthine (Sigma, St. Louis, MO; Sack and Thompson, 1977). The culture was incubated at 37°C for 48 h under anaerobic conditions using GasPak anaerobic systems (Becton Dickinson, Cockeysville, MD). Spores were collected by centrifugation at 8,000 x g for 10 min at 4°C. The pellet was washed 6 times, resuspended with sterile deionized water, and stored at 4°C until use.

C. sporogenes was grown under anaerobic conditions in 0.6% trypticase peptone (BBL, Cockeysville, MD) and 0.1% dextrose (Sigma, St. Louis, MO) at 35°C as described by Uehara et al. (1965). Spores were collected, washed 6 times, resuspended with sterile deionized water and stored at 4°C until use.

C. tertium was grown in RCM at 25°C for 36 to 48 h under anaerobic conditions. Spores were harvested by centrifugation at 8,000 x g , washed 6 times, resuspended with sterile deionized water, and stored at 4°C until use.

Heat treatment

A. Mild heat treatment

Bacterial spores were suspended in sterile 0.1% peptone solution (pH 7.0±0.2) and 3 ml were transferred into sterile cryovials (Simport, Quebec, Canada) in duplicate. Each vial was vacuum-sealed in a plastic bag (Nasco, Ft. Atkinson, WI) and subjected to mild heat either at 27° or 52°C for 5 min to ensure that the temperature at the middle of the vial reached either 25° or 50°C before hydrostatic pressure treatment. Controls were subjected to the samemild heat treatment, cooled in an ice-bath, and incubated at either 4° or 25°C without hydrostatic pressure treatment

B. Spore activation

Controls were not subjected to hydrostatic pressure treatment but heat activated as follows: *B. cereus* was activated at 70°C for 10 min; *B. stearothermophilus* was activated at 80°C for 30 min; *C. laramie* was activated at 60°C for 10 min; *C. perfringens* and *C. sporogenes* were activated at 70°C for 20 min and isolated *Clostridium* (*C. tertium*) was activated at 70°C for 15 min.

C. Germinated spore inactivation

After hydrostatic pressure treatment, the spores were incubated either at 4° or 25°C for 1 or 24 h. The spores were previously heat treated to inactivate germinated spores using the same time and temperature for each type of bacterial spores as used for spore activation.

Hydrostatic pressure treatment

Spores were subjected to an initial mild heat treatment before placing into the preheated (25° or 50°C) fluid (Hydrolubric 2; Houghton International, Valley Forge, PA) in the pressurization chamber (10 x 4 inches internal diameter; Engineered Pressure System, Wilmington, MA) 1 or 2 min to equilibrate the temperature of spore suspension in the vials. Spore suspensions were pressurized at

20,000, 40,000, 50,000, 60,000 or 70,000 psi for 5 min. After pressurization, spore suspensions were cooled in an ice-bath and incubated at either 4° or 25°C for 1 or 24 h before enumeration.

Determination of germination and enumeration

The pressurized spore suspensions were heat treated to inactivate the germinated spores as described previously. The heat-resistant ungerminated spores were enumerated by pour plating using agar media. *B. cereus* and *B. stearothermophilus* were pour plated using AAMS agar and aerobically incubated for 48 h at 30° and 55°C, respectively. *C. laramie*, *C. perfringens*, *C. sporogenes*, and isolated *Clostridium* species (*C. tertium*) were pour plated using RCM agar (Barnes et al., 1963) with 1% glucose and incubated anaerobically at 10° and 37°C, respectively. The germinated spores induced by hydrostatic pressure were calculated as follows:

$$\% \text{ Germinated spore} = \frac{\text{Total number of spores} - \text{Number of heat-resistant ungerminated spores}}{\text{Total number of spores}} \times 100$$

RESULTS AND DISCUSSION

Identification of the *Clostridium* species, isolated from hydrostatic–pressure-pasteurized roast beef during storage at 25°C

This *Clostridium* species was isolated only from several samples of hydrostatic-pressure-pasteurized roast beef without biopreservatives during storage studies of the previous project, DJ10:# DAAK60-93-K-0003. The isolates were purified and twenty-one biochemical tests were performed using bioMérieux API 20A. The biochemical reactions of the isolate were presented in Table 1. Based on the biochemical reaction profile, they were identified as *Clostridium tertium*. *C. tertium* cells were gram-positive, spore former and catalase negative. Spores are oval, terminal and distend the cells. Growth was found at 25°, 37°, 45° or 50°C, but not at 4° or 10°C. Indole formation, urease, esculin hydrolysis and gelatin hydrolysis were negative. Nitrate reductiase and starch hydrolysis was positive. Arabinose, glycerol, raffinose, sorbitol. rhamnose and trehalose were not fermented. Acid production was found from glucose, mannitol, lactose, sucrose, maltose, salicin. xylose, cellobiose, mannose and melezitose. According to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1984), *C. tertium* is relatively nonpathogenic and generally occurs in soil, feces or colonic healthy adults, infection in a calf, and infections related to the intestinal tract.

Effect of hydrostatic pressure, pressurization temperature, and incubation time and temperature on spore activation to germination of *Bacillus* species

Without hydrostatic pressure treatment at 25°C (control), both spores of *B. cereus* and *B. stearothermophilus* did not germinate following subsequent incubation for 1 or 24 h at either 4° or 25°C (Table 2 and 3). Six percent and 25% of self-germinated spores were found, when spores of *B. cereus* were incubated for 24 h at 4° and 25°C, respectively (Table 2).

Table 1. Biochemical identification of *Clostridium* sp. isolated from hydrostatic-pressure-pasteurized roast beef stored at 25°C.

Characteristics	Clostridial isolated[a]
Gram reaction	+
Spore formation	+, oval, terminal with extend
Indole production	-
Urease	-
Catalase	-
Esculin hydrolysis	-
Gelatin hydrolysis	-
Starch hydrolysis	+
Nitrate reductase	+
Acid production from	
Glucose	+
Mannitol	+
Lactose	+
Sucrose	+
Maltose	+
Salicin	+
Xylose	+
Arabinose	-
Glycerol	-
Cellobiose	+
Mannose	+
Melezitose	+
Raffinose	-
Sorbitol	-
Rhamnose	-
Trehalose	-
Growth at °C	
4	-
10	-
25	+
37	+
45	+
50	+

[a]Confirmed as *Clostridium tertium*

Table 2. Influence of hydrostatic pressure and temperature on germination of *B. cereus*

Pressurized temp (°C)	Incubation time (h)	Incubation temp (°C)	% spore germination following pressurization (5 min; pH 7.00 at (psi)					
			0	20,000	40,000	50,000	60,000	70,000
25	1	4	0	99	99	99	99	99
25	24	4	6	99	99	99	99	99
25	1	25	0	99	99	99	99	99
25	24	25	25	99	99	99	99	99
50	1	4	6	99	99	99	99	99
50	24	4	33	99	99	99	99	99
50	1	25	6	99	99	99	99	99
50	24	25	56	99	99	99	99	99

Table 3. Influence of hydrostatic pressure and temperature on germination of *B. stearothermophilus*

Pressurized temp (°C)	Incubation time (h)	Incubation temp (°C)	% spore germination following pressurization (5 min; pH 7.00 at (psi)					
			0	20,000	40,000	50,000	60,000	70,000
25	1	4	0	42	90	88	85	90
25	24	4	0	45	90	88	88	88
25	1	25	0	80	90	92	94	95
25	24	25	25	85	94	96	96	99
50	1	4	0	99	98	98	97	97
50	24	4	0	99	99	98	97	98
50	1	25	0	99	99	98	98	99
50	24	25	20	99	99	99	98	98

When spores were heat treated at 50°C for 5 min, higher self-germination of *B. cereus* spores, 33% and 56%, were detected after incubated for 24 h either at 4° or 25°C, respectively (Table 2). Spores of *B. stearothermophilus* underwent self-germination after incubated at 25°C for 24 h (Table 3). However, spores of *B. stearothermophilus* germinated only 20%, when spores were incubated at 25°C for 24 h. Pressurization of spores of both *B. cereus* and *B. stearothermophilus* either at 25° or 50°C initiated more spores to germinate (Table 2 and 3). Ninety-nine percent of *B. cereus* spores were germinated either pressurized temperature at 25° or 50°C. Raso et al., (1998) pressurized *B. cereus* spores at 100,000 psi for 1 min at 40°C and found that 4.5 log cycles of spores were germinated. Increasing incubation temperature from 4° to 25°C and pressure from 20,000 to 70,000 psi induced *B. stearothermophilus* spores to germinate by 97 to 99%. These data indicated that pressurization induced spore germination of *Bacillus* species.

Effect of hydrostatic pressure, pressurization temperature, and incubation time and temperature on spore activation to germination of *Clostridium* species

There was no germination of *C. laramie* spores following exposure to 25° or 50°C, when spores were subsequently incubated at 4° or 25°C for either 1 or 24 h (Table 4). Normally, spores of *C. laramie* sporulate within 10 to 14 d under anaerobic conditions at 10° or 15°C (Kalchayanand et al., 1993). When *C. laramie* spores were pressurized for 5 min from 20,000 to 70,000 psi at 25°C and then incubated for 1 or 24 h either at 4° or 25°C, spore germination increased with increase in pressure, incubation time and temperature. Thirty-two percent of spores were germinated when spores were pressurized at 70,000 psi and incubated at 4°C for 24 h (Table 4). Pressurization at 60,000 or 70,000 psi induced spores to germinate by 33% after 24 h at 25°C.

Table 4. Influence of hydrostatic pressure and temperature on germination of *C. Laramie*

Pressurized temp (°C)	Incubation time (h)	Incubation temp (°C)	% spore germination following pressurization (5 min; pH 7.00 at (psi)					
			Control	20,000	40,000	50,000	60,000	70,000
25	1	4	0	0	3	3	22	30
25	24	4	0	3	8	8	27	32
25	1	25	0	11	22	25	30	33
25	24	25	0	14	25	25	33	33
50	1	4	0	0	0	0	7	20
50	24	4	0	0	0	0	0	3
50	1	25	0	13	6	6	10	23
50	24	25	0	0	10	7	7	7

However, pressurization up to 50,000 psi followed by incubation at 4°C for 24 h did not initiate spore germination (Table 4). Pressurization at 70,000 psi for 5 min at 50°C activated spore germination by only 23%. This inconsistency in pressure-induced of *C. laramie* spores to germination may be due to denaturation of core protein by a combination of pressure and temperature. High pressure induced protein denaturation by ionization and the formation of ionic bonds between charged groups causing change in protein solubility (Timson and Short, 1965). Murrel and Wilis (1977) proposed that the transformation of a dormant to a germinating spore involved core hydration or a decrease core viscosity.

Spores of *C. perfringens* incubated at 25°C did not initiate germination after 24 h incubation at either 4° or 25°C; only 4% germinated after 24 h of incubation at 25°C (Table 5). The percentage of *C. perfringens* spores germinated increased when pressurized temperature and incubation time were increased. Gould and Sale (1970) postulated that the optimum temperature for initiation of spore germination differed at different pressures. They also found that as the pressure was increased, the optimum temperature for germination increased. Pressurized *C. perfringens* spores at 20,000 psi for 5 min at 25°C caused spores to germinated by 35% after incubated at 25°C for 24 h. Fifty-two percent of *C. perfringens* were germinated, when spores were pressurized at 20,000 psi for 5 min at 50°C after being incubated at 25°C for 24 h (Table 5). However, pressurization at either 60,000 or 70,000 psi for 5 min at 50°C did not greatly increase the initiation of spore germination (52%). This indicates that the temperature for induction of spore germination differs at different pressures.

C. sporogenes spores did not germinate when spores were first exposed to 25°C for 5 min and then incubated 1 or 24 h either at 4° or 25°C. Pressurization at 50°C for 5 min, initiated more spore germination than pressurization at 25°C.

Table 5. Influence of hydrostatic pressure and temperature on germination of *C. perfringens*

Pressurized temp (°C)	Incubation time (h)	Incubation temp (°C)	% spore germination following pressurization (5 min; pH 7.00 at (psi)					
			Control	20,000	40,000	50,000	60,000	70,000
25	1	4	0	4	8	17	21	29
25	24	4	0	17	17	21	29	29
25	1	25	0	8	17	21	25	25
25	24	25	4	35	17	22	32	26
50	1	4	0	4	4	16	20	24
50	24	4	4	12	36	40	36	44
50	1	25	4	4	8	16	28	28
50	24	25	40	52	48	44	52	52

This agreed with the results of Gould and Sale (1970) who found that the amounts of spore germination increased when heat-activated spores were pressurized. More than 80% of *C. perfringens* were induced to germination when spores were pressurized at 70,000 psi for 5 min at 50°C and incubated either at 4° or 25°C for 24 h (Table 6).

C. tertium spores did not germinate when spores were held at 25°C for 5 min and then incubated 1 or 24 h at either 4° or 25°C. Only 11% of spores were germinated after mild heat treatment at 50°C for 5 min and 24 h incubation at either 4° or 25°C (Table 7). Increase in the magnitude of pressures, pressurization temperatures, and incubation times and temperatures activated more *C. tertium* spores to germinate. Fifty-four percent of spores were germinated, when spores were pressurized either at 60,000 psi for 5 min at 25°C and incubated for 24 h at 25°C or at 70,000 psi for 5 min at 25°C and incubated for 1 h at 25°C. Ninety-nine percent pressure-induced germinated spores were achieved by pressurization at 60,000 psi for 5 min at either 25° or 50°C and incubation at 4° or 25°C for 1 or 24 h (Table 7).

Effect of hydrostatic pressure and temperature on inactivation of bacterial spores

Table 8 presents the influence of hydrostatic pressure on the inactivation of *Bacillus* and *Clostridium* spores at two pressurization temperatures (25° or 50°C). The degree of inactivation, however, depended on bacterial strain. Among the strains tested, spores of *Bacillus* species were inactivated by hydrostatic pressure more than spores of *Clostridium* species. Spores of *B. cereus* were the most sensitive to hydrostatic pressure, whereas *C. sporogenes* spores were the most resistant to hydrostatic pressure. More than 3 log cycles were achieved when spores were pressurized at 20,000 psi for 5 min at 25°C. *C. sporogenes* spores were not inactivated even spores were pressurized at 70,000 psi for 5 min at 50°C.

Table 6. Influence of hydrostatic pressure and temperature on germination of *C. sporogenes*

Pressurized temp (°C)	Incubation time (h)	Incubation temp (°C)	% spore germination following pressurization (5 min; pH 7.00 at (psi)					
			0	20,000	40,000	50,000	60,000	70,000
25	1	4	0	0	4	4	0	4
25	24	4	0	0	0	0	4	4
25	1	25	0	0	0	9	18	31
25	24	25	9	18	14	9	4	14
50	1	4	25	25	38	25	25	25
50	24	4	25	40	40	50	50	82
50	1	25	50	50	50	50	50	50
50	24	25	65	76	79	65	68	81

Table 7. Influence of hydrostatic pressure and temperature on germination of *C. tertium*

Pressurized temp (°C)	Incubation time (h)	Incubation temp (°C)	% spore germination following pressurization (5 min; pH 7.00 at (psi)					
			0	20,000	40,000	50,000	60,000	70,000
25	1	4	0	27	27	36	45	45
25	24	4	0	27	27	36	45	45
25	1	25	0	27	36	45	45	54
25	24	25	9	27	36	45	54	54
50	1	4	0	11	56	96	99	99
50	24	4	11	11	76	95	99	99
50	1	25	11	11	50	95	99	99
50	24	25	11	11	79	94	99	99

There was no optimum inactivation pressure or optimum pressurization temperature observed in this studies. Basically, increase in pressure and pressurization temperature caused more lethal effect especially to spores of *B. cereus*, *B. stearothermophilus* and *C. tertium*. Pressurization at 70,000 psi for 5 min at 50°C destroyed spores of *B. cereus*, *B. stearothermophilus* and *C. tertium* by 5.6, 1.5 and 2.7 log cycles (Table 8).

Table 8. Inactivation of bacterial spores by hydrostatic pressure and temperature

Bacterial strain	Pressurization temperature (°C)	log_{10} spore reduction/ml following pressurization [(5 min; pH 7.0) at (psi)]		
		20,000	50,000	70,000
B. cereus	25	3.3	3.5	3.7
	50	5.0	5.5	5.6
B. stearothermophilus	25	0.2	0.9	1.0
	50	2.0	1.6	1.5
C. laramie	25	0.1	0.2	0.2
	50	0.1	0.1	0.1
C. perfringens	25	0.0	0.1	0.2
	50	0.0	0.1	0.2
C. sporogenes	25	0.0	0.0	0.0
	50	0.1	0.1	0.0
C. tertium	25	0.1	0.2	0.2
	50	0.1	1.4	2.7

CONCLUSIONS

1. Hydrostatic pressure induced spore germination of *Bacillus* and *Clostridium* species, and more so with *Bacillus*.
2. Increased pressure, pressurization temperature along with incubation time and temperature initiated more spores to germinate, except for *C. Laramie*.
3. Hydrostatic pressure also had a lethal effect on *Bacillus* and *Clostridium* spores, but the inactivation was uneven. *B. cereus* spores were the most sensitive to hydrostatic pressure, whereas *C. sporogenes* spores were the most resistant to pressure. Pressurization at higher tempatures caused more spores to be destroyed.
4. The pressure resistance of *C. sporogenes* PA3679 spores may raise safety concerns about the validity of hydrostatic pressurization of foods. Good manufacturing practice (GMP) and low temperature storage are still a must

This document reports research undertaken at the U.S. Army Soldier and Biological Chemical Command, Soldier Systems Center, and ha been assigned No. NATICK/TR-01/003 in a series of reports approved for publication.

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