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Effect of Storage Time and Temperature on the Survival of *Clostridium botulinum* Spores in Acid Media[1]

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Clostridium botulinum type A and type B spores were stored in tomato juice (pH 4.2) and citric acid-phosphate buffer (pH 4.2) at 4, 22, and 32°C for 180 days. The spore count was determined at different intervals over the 180-day storage period. There was no significant decrease in the number of type A spores in either the tomato juice or citric acid-phosphate buffer stored for 180 days at 4, 22, and 32°C. The number of type B spores did not decrease when storage was at 4°C, but there was an approximately 30% decrease in the number of spores after 180 days of storage at 22 and 32°C.

The limiting pH for *Clostridium botulinum* spore germination, growth, and toxin production has been investigated in a number of different foods (4, 5, 9). These studies and others have established that *C. botulinum* spores cannot germinate, grow, and produce toxin if the medium pH is less than 4.6. Since in acid foods the pH is relied upon to prevent germination and growth of *C. botulinum*, the heat processes used for these foods have not been designed to destroy *C. botulinum* spores. The concern over a botulinum hazard in tomato products (1-3, 8) and the recent research in this area (4, 6) prompted us to investigate the effect of storage time and temperature on the survival of *C. botulinum* spores in acid media.

(This work was taken in part from a thesis submitted by Theron E. Odlaug to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.)

MATERIALS AND METHODS

Spores. Cultures of a *C. botulinum* type A strain, A16037, and a type B strain, B15580, were obtained from the Center for Disease Control in Atlanta, Ga. These strains were implicated in outbreaks of botulinum intoxication involving home-canned tomato products as the toxin-carrying vehicle (1, 2). The procedure for preparing spore crops from these strains was described previously (6).

Tomato juice (pH 4.2). One-quart (ca. 1-liter) jars of commercially glass-packed tomato juice were purchased to be used throughout this study. The jars were held at 4°C until used. Before each heating test, each jar was removed from the refrigerator, washed, dried, flamed on the top and sides, and opened with a disk cutter (Difco) under a laminar-flow hood. The entire contents of the jar were poured into a sterile flask and then dispensed aseptically in 100-ml amounts into milk dilution bottles.

Buffer (pH 4.2). McIlvaines citric acid-phosphate buffer (pH 4.2) was prepared by mixing 58.6 ml of a stock solution of 0.1 M citric acid with 41.4 ml of a stock solution of 0.2 M disodium phosphate (Na_2HPO_4). Stock solutions were prepared with distilled water. The prepared buffer was dispensed in 100-ml amounts into milk dilution bottles and autoclaved for 15 min at 121.1°C.

Spore inoculation and storage conditions. Each bottle was inoculated with 1.0 ml of spore suspension such that there were about 10^5 spores per 100 ml of test substrate. Each bottle was then shaken. There were 12 bottles with tomato juice, 6 containing type A spores and 6 containing type B spores. There were also 12 bottles with the citric acid-phosphate buffer, 6 containing type A spores and 6 containing type B spores. After inoculation, the 24 bottles were heated at 80°C for 30 min.

The bottles with the test substrates and spores were stored at 4, 22, and 32°C.

Spore count procedure. Tests were carried out to determine the number of viable spores at 0, 12, 20, 47, 79, 99, 125, and 180 days. On the day of the test, 2 ml from each of the bottles was placed in separate test tubes. The tubes were heated at 80°C for 10 min. Triplicate 0.1-ml portions from each tube were plated with yeast extract agar (6). The plates were incubated in an anaerobic jar under a hydrogen-carbon dioxide atmosphere for 72 h at 32°C and then counted with the aid of a colony counter (Bactronic).

RESULTS

The mean plate counts for each set of test conditions (two bottles) as a function of storage time are shown in Table 1 for spores of strains A16037 and B15580 in tomato juice, and in Ta-

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TABLE 1. *Mean plate counts for Clostridium botulinum spores stored in tomato juice (pH 4.2)*

Spore type	Incubation temp (°C)	Count per 0.1 ml of test substrate after days of storage: 0	12	22	47	79	99	125	180
A16037	4	113 (2.051)[a]	114 (2.051)	110 (2.038)	112 (2.050)	104 (2.012)	113 (2.046)	110 (2.039)	114 (2.057)
A16037	22	103 (2.011)	114 (2.052)	111 (2.044)	114 (2.051)	110 (2.038)	109 (2.035)	111 (2.046)	110 (2.039)
A16037	32	109 (2.034)	121 (2.080)	117 (2.060)	104 (2.017)	102 (2.006)	100 (2.000)	98 (1.989)	100 (2.000)
B15580	4	128 (2.104)	144 (2.156)	140 (2.143)	131 (2.116)	129 (2.108)	134 (2.125)	128 (2.105)	126 (2.099)
B15580	22	141 (2.146)	133 (2.122)	133 (2.122)	117 (2.066)	118 (2.069)	99 (1.993)	103 (2.012)	96 (1.981)
B15580	32	143 (2.147)	129 (2.109)	129 (2.109)	118 (2.067)	114 (2.053)	106 (2.020)	91 (1.956)	90 (1.949)

[a] Mean of logarithms is given in parentheses.

TABLE 2. *Mean plate counts for Clostridium botulinum spores stored in citric acid-phosphate buffer (pH 4.2)*

Spore type	Incubation temp (°C)	Count per 0.1 ml of test substrate after days of storage: 0	12	22	47	79	99	125	180
A16037	4	114 (2.055)[a]	117 (2.068)	110 (2.041)	115 (2.061)	112 (2.049)	117 (2.068)	114 (2.055)	116 (2.066)
A16037	22	113 (2.053)	108 (2.033)	115 (2.061)	112 (2.051)	112 (2.051)	114 (2.057)	111 (2.045)	110 (2.041)
A16037	32	112 (2.051)	118 (2.074)	114 (2.055)	111 (2.046)	108 (2.031)	112 (2.050)	108 (2.033)	105 (2.021)
B15580	4	142 (2.152)	144 (2.157)	140 (2.146)	136 (2.133)	140 (2.146)	140 (2.145)	132 (2.121)	140 (2.145)
B15580	22	148 (2.170)	140 (2.146)	126 (2.099)	114 (2.057)	114 (2.055)	109 (2.037)	94 (1.975)	94 (1.975)
B15580	32	146 (2.166)	139 (2.142)	120 (2.077)	119 (2.075)	121 (2.083)	104 (2.017)	95 (1.977)	88 (1.947)

[a] Mean of logarithms is given in parentheses.

TABLE 3. *Analysis of variance table for Clostridium botulinum type A16037 spores in tomato juice (pH 4.2)*

Term	df[a]	Sum of squares	Mean square	F[b]
Temp (T)	2	3.5913E-03[c]	1.7956E-03	1.604
Storage time (S)	7	7.7585E-03	1.1084E-03	0.9902
T × S	14	1.1896E-02	8.4972E-04	0.7591
Error	24	2.6864E-02	1.1194E-03	
Total	47	5.0109E-02		

[a] df, Degrees of freedom.
[b] Not significant at $\alpha = 0.01$.
[c] E-03 = 10^{-3}.

ble 2 for spores of strains A16037 and B15580 in citric acid-phosphate buffer.

In analyzing the data, the means of the logarithms were used. Two-way analyses of variance for the four experiments are presented in Table 3 for the strain A16037 spores stored in tomato juice, in Table 4 for the strain A16037 spores in citric acid-phosphate buffer, in Table 5 for strain B15580 spores in tomato juice, and in Table 6 for the strain B15580 spores stored in citric acid-phosphate buffer.

The analysis of variance for the *C. botulinum* type A spores (Tables 3 and 4) showed that there was no significant change in spore level over the 180-day test period, regardless of the storage temperature.

The analysis of variance (Tables 5 and 6) for the *C. botulinum* type B spores showed that there was a significant effect of temperature and storage time on type B spore viability. The mean square for linear regression was calculated for each temperature over the storage period. These values were used in computing the *F*-values shown in Table 7. This analysis showed that the effect of storage time at 4°C was not significant for the type B spores in

tomato juice or in citric acid-phosphate buffer. However, there was a significant decrease in spore levels at 22 and 32°C for both test substrates.

DISCUSSION

Citric acid-phosphate buffer (pH 4.2) was used as one of the storage media, since citric acid is the most common acidulant used in the food industry (8), and citric acid is present in appreciable quantities in tomatoes.

Information on the effect of an acid medium on *C. botulinum* spore viability is scarce in the literature. Ito et al. (5), Huhtanen et al. (4), and Townsend et al. (9), in studying the limiting pH for growth of *C. botulinum*, did not report the level of spores remaining in those tubes with no growth at the end of the test period.

The results of this study show that *C. botulinum* spores can survive in an acid medium for long periods of time. The numbers of type A spores used in this study did not significantly decrease over the test period. The numbers of type B spores decreased by about 30% in the 180-day period.

These results indicate that if an acid food is contaminated with *C. botulinum* spores, these spores are capable of surviving for long periods of time, even though the pH is less than 4.6. The spores are capable of germinating and growing when the pH of the growth medium increases to a level above 4.6 (4, 6, 8). Viable *C. botulinum* spores in acid foods are not considered to be hazardous to adults if the pH remains below 4.6. However, acid foods may be a source of *C. botulinum* spores in infant botulism (7).

TABLE 4. *Analysis of variance table for Clostridium botulinum type A16037 spores in citric acid-phosphate buffer (pH 4.2)*

Term	df[a]	Sum of squares	Mean square	F[b]
Temp (T)	2	1.3672E-03	6.8360E-04	2.757
Storage time (S)	7	1.6515E-03	2.3593E-04	0.9516
T × S	14	4.5421E-03	3.2444E-04	1.309
Error	24	5.9507E-03	2.4794E-04	
Total	47	1.35115E-02		

[a] df, Degrees of freedom.
[b] Not significant at $\alpha = 0.01$.

TABLE 5. *Analysis of variance table for Clostridium botulinum type B15580 spores in tomato juice (pH 4.2)*

Term	df[a]	Sum of squares	Mean square	F
Temp (T)	2	4.2067E-02	2.1034E-02	28.87[b]
Storage time (S)	7	9.7973E-02	1.3996E-02	19.21[b]
T × S	14	3.7163E-02	2.6545E-03	3.644[b]
Error	24	1.7485E-02	7.2853E-04	
Total	47	0.19469		

[a] df, Degrees of freedom.
[b] Significant at $\alpha = 0.01$.

TABLE 6. *Analysis of variance table for Clostridium botulinum type B15580 spores in citric acid-phosphate buffer (pH 4.2)*

Term	df[a]	Sum of squares	Mean square	F
Temp (T)	2	6.9463E-02	3.4731E-02	141.6[b]
Storage time (S)	7	0.1108	1.5830E-02	64.54[b]
T × S	14	4.3526E-02	3.1090E-03	12.68[b]
Error	24	5.8866E-03	2.4528E-04	
Total	47	0.22968		

[a] df, Degrees of freedom.
[b] Significant at $\alpha = 0.01$.

TABLE 7. *F-tests for Clostridium botulinum B15580 spores stored in tomato juice (pH 4.2) and citric-acid phosphate buffer (pH 4.2)*

Substrate	Storage temp (°C)	Mean square		F
		Analysis of variance error[a]	Linear regression[b]	
Tomato juice (pH 4.2)	4	7.2853E-04	1.9199E-03	2.64
	22	7.2853E-04	4.9657E-02	68.16[c]
	32	7.2853E-04	6.8851E-02	94.51[c]
Citric acid-phosphate buffer (pH 4.2)	4	2.4528E-04	3.7604E-04	1.53
	22	2.4528E-04	6.2864E-02	256.29[c]
	32	2.4528E-04	7.0036E-02	285.53[c]

[a] Degrees of freedom = 24.
[b] Degrees of freedom = 1.
[c] Significant at $\alpha = 0.01$.

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