

# Thermal Resistance of Spores from pH Elevating Strains of *Bacillus licheniformis*

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## ABSTRACT

The thermal resistance of *Bacillus licheniformis* spores from strains originally isolated from home-canned tomatoes was examined. In tomato puree at pH 4.4,  $D_{95}$  and  $D_{100}$  values of 4.5 and 2.0 min, respectively, were obtained. The  $z$  value was 14.9 °C. The  $D_{95}$  in pH adjusted tomato puree increased at the rate of 50% per pH unit over the range pH 4–5.5. The  $D_{95}$  in pH 7.2 buffer was 7.8 min. The *B. licheniformis* spores could survive the USDA raw pack process for home-canned tomatoes and elevate the pH to greater than 5.2 under aerobic but not anaerobic conditions.

## INTRODUCTION

THERE HAS BEEN continued concern about the adequacy of recommendations for the home canning of high acid foods, especially tomatoes. Although outbreaks of botulism involving acid foods are rare and account for less than 5% of the botulism outbreaks between 1899 and 1975, virtually all of these outbreaks were due to home-canned foods (Odlag and Pflug, 1978). The evidence that foods with pH  $\leq$  4.6 inhibit growth and toxin production by *Clostridium botulinum* is compelling (Ito and Chen, 1978). Raatjes and Smelt (1979) reported that *C. botulinum* can grow and produce toxin in laboratory media at pH  $<$  4.6. This occurs only when the *C. botulinum* spores are coinoculated with *Bacillus sp.* into media containing protein rich substrates—a situation nonexistent in canned vegetables.

Sapers et al. (1978a) have shown that new tomato cultivars are not usually low in acidity and that the rare occurrence of high pH ( $\geq$  4.7) tomatoes is due to combinations of factors such as cultivar, location, degree of ripeness, and presence of decay. The probability that the pH of an uncontaminated jar of tomatoes exceeds 4.8 has been estimated by Powers and Goodwin (1978) to be 0.0014. The feasibility of acidifying home-canned tomatoes was investigated by Sapers et al. (1978b) and is still under debate.

Post-process contamination by molds can raise the pH to a level that permits *C. botulinum* growth and toxin production. Huhtanen et al. (1976) demonstrated that tomato juice inoculated with *Cladosporium sp.* or *Penicillium sp.* had pH values approaching neutrality near the mold mat and supported growth and toxin production by *Clostridium botulinum*. Similar results were reported by Odlag and Pflug (1979) using *Aspergillus gracilis*. Mundt (1978) has shown that most molds in a variety of genera can raise the pH of tomato juice.

*B. licheniformis* was isolated from about 30% of the home-canned tomatoes examined by Fields et al. (1977), who reported that many isolates elevate the pH of tomato serum.

We were interested in determining if these relatively high numbers were due to poor canning practices or if heat resis-

tant spores could survive an approved process. The objectives of this study were to determine the thermal resistance of *B. licheniformis* spores in tomato puree, to ascertain if they could survive the recommended USDA raw pack home canning method and to identify conditions under which pH elevation occurs.

## EXPERIMENTAL

### Test organisms

*B. licheniformis* NRRL NRS 1264 was obtained from Dr. L.K. Nakamura (USDA, Northern Regional Research Center, Peoria, IL). Strains of *B. licheniformis* originally isolated from home-canned tomatoes were the gift of Dr. M.L. Fields (Univ. of Missouri, Columbus, MO) who designated them as strains 075-T-09, 011-T-11, 110-T-05, and 015-T-03. These cultures were maintained on nutrient agar slants at 5°C.

Spores of each *B. licheniformis* strain were produced in 100 ml Trypticase Soy Broth (TSB) (BBL) with shaking (200 rpm) at 30°C. When microscopic examination showed that 90% or more of the cells had sporulated, the spores were harvested by centrifugation at 10,000  $\times g$  for 10 min at 3°C, resuspended in 80 ml of cold sterile distilled water, and treated with 0.8 ml of a 1% lysozyme solution (22,000  $\mu$ /mg, A grade, Calbiochem). After 48 hr at 4°C, no vegetative cells were observed. The spores were centrifuged and washed twice with sterile distilled water, resuspended in a final volume of 100 ml, and stored at 5°C.

Vegetative inocula were prepared by inoculating 100 ml of TSB from the stock cultures and incubating overnight with shaking (200 rpm) at 30°C.

### pH Effects

The lowest pH limit at which *B. licheniformis* spores could initiate growth was determined by inoculating  $10^6$  spores/ml of each strain into tomato puree with the pH adjusted in 0.2 unit increments by addition of 0.1N HCl or NaOH and incubating the samples at 37°C.

Tomato puree used throughout these studies was prepared by blending Roma VF tomatoes with an equal volume of distilled water and sterilizing at 15 psi for 15 min. The pH of the uninoculated tomato puree was 4.4.

The pH elevating capability of each strain was determined by inoculating 10 ml of tomato puree with 0.1 ml of an overnight TSB culture. The pH of the samples was determined by use of a pH meter with a microprobe combination pH electrode (Fisher) after incubation at 30°C for 1 and 6 wk.

In order to determine if pH elevation was due to acid utilization per se or was the result of normal aerobic metabolism, Phenol Red Broth Base (Difco) containing 0.5% glucose, 0.5% citric acid, or 0.25% glucose + 0.25% citric acid was adjusted to pH 5.1 and inoculated with *B. licheniformis* 075-T-09 overnight cells (3% v/v). The inoculated cultures were incubated on a shaker maintained at 30°C; pH was monitored at intervals during the incubation period.

### Spore thermal resistance

Survival curves at 95° and 100°C were determined by inoculating 0.1 ml of spore suspension ( $\sim 10^7$ /ml) from each strain into 9.9 ml of tomato puree pre-equilibrated to the specified temperature, pipetting a 1 ml aliquot into 9 ml diluent at appropriate intervals, and counting the number of survivors on spread plates of Trypticase Soy Agar (TSA, Difco) after 48 hr at 37°C. Survival curves at 85° and 90°C were also determined for strains NRRL-NRS 1264 and 075-T-09. Decimal reduction times (D values) were calculated from the survival curves after determining the lines of best fit by least squares linear regression.

The effect of pH on the heat resistance of *B. licheniformis* 075-T-09 spores were investigated by determining the D<sub>95</sub> in tomato puree adjusted to pH 4.0, 4.4, 5.0, 5.4, and 7.2.

#### Test pack experiments

Pint jars of Roma VF tomatoes were inoculated along the vertical axis with 1 ml of *B. licheniformis* 075-T-09 spores or a mixed spore suspension containing strains 075-T-09, 011-T-11, 110-T-05, and 015-T-03. These jars and uninoculated controls were shaken by hand and processed according to the USDA recommended procedure for raw pack tomatoes (USDA, 1975). Pre- and post-process plate counts were done on TSA plates. The seals on half of the jars were broken under a laminar flow hood to simulate leakers. The simulated leakers and jars with intact seals were incubated at 30°C.

## RESULTS & DISCUSSION

### pH Effects

*B. licheniformis* strains 075-T-09, 110-T-05, and 015-T-03 grew in sterilized tomato puree at pH 4.2 but not at 4.0; strain 011-T-11 had a lower limit of pH 4.4. This slight difference from the results of Fields et al. (1977) is not surprising considering the multiplicity of factors that affect the limits of microbial growth in a given food system. Spores of the reference strain NRRL NRS 1264 could not initiate growth at pH 4.4.

The ability of *B. licheniformis* strains to elevate pH is demonstrated in Table 1. The values after 7 days of incubation at 30°C tended to be higher than those reported by Fields et al. (1977) after 5 days at 35°C. This difference may be due to the higher pH of the starting material (pH 4.4 vs 4.2) and a longer incubation time. Two of the twelve replicates had pH > 4.6 after 7 days without visible signs of microbial growth. Inoculum levels as low as 70/ml of *B. licheniformis* vegetative cells were sufficient to initiate growth and elevate pH. *B. licheniformis* NRRL NRS 1264 could not be recovered from the tomato puree after 7 days, suggesting that the low pH was bacteriocidal.

*B. licheniformis* 075-T-09 elevated the pH of Phenol Red Broth Base from pH 5.1 to above pH 7.0 regardless of the carbon source. This occurred most rapidly with glucose and was delayed by the addition of citric acid. When citric

Table 1—pH of tomato puree inoculated with *B. licheniformis* and incubated at 30°C

<i>B. licheniformis</i> strain	pH of tomato puree <sup>a</sup>	
	1 wk	6 wk
075-T-09	5.2	8.5
011-T-11	5.2	8.5
110-T-05	5.5	8.4
015-T-03	5.0	8.4
NRRL NRS 1264	4.4 <sup>b</sup>	4.4 <sup>b</sup>
uninoculated	4.4 <sup>b</sup>	4.4 <sup>b</sup>

<sup>a</sup> Average of triplicates mixed prior to measurement  
<sup>b</sup> No growth observed.

acid was the sole carbon source, pH elevation was delayed even longer but did occur. This indicates that the pH elevation was not due to acid utilization per se.

Elevation of pH by members of the *B. subtilis* group is a well known phenomenon and has, for example, been reported by Anker et al. (1948) in studies of bacitracin production. Since carbohydrate metabolism by *B. licheniformis* yields the neutral products 2,3-butanediol and glycerol, pH elevation is most likely due to end products of amino acid and protein metabolism.

### Thermal resistance of *B. licheniformis* spores

D value for *B. Licheniformis* spores in tomato at pH 4.4 averaged 2.0 min at 100°C and 4.5 min at 95°C. There was no significant difference ( $p \geq 0.95$ ) in the D values of the five strains. Representative curves are presented in Figure 1.

The similarity of the heat resistance of the spores from 075-T-09 and NRRL NRS 1264 is shown by the high correlation coefficient for the plot of log D vs. temperature when the D values for both strains are combined and regressed (Fig. 2). This plot yields a z value of 14.9°C. This suggests that the *B. licheniformis* strains isolated by Fields and coworkers do not have abnormally high heat resistance, but can, as previously noted, initiate growth at a lower pH than the reference strain.

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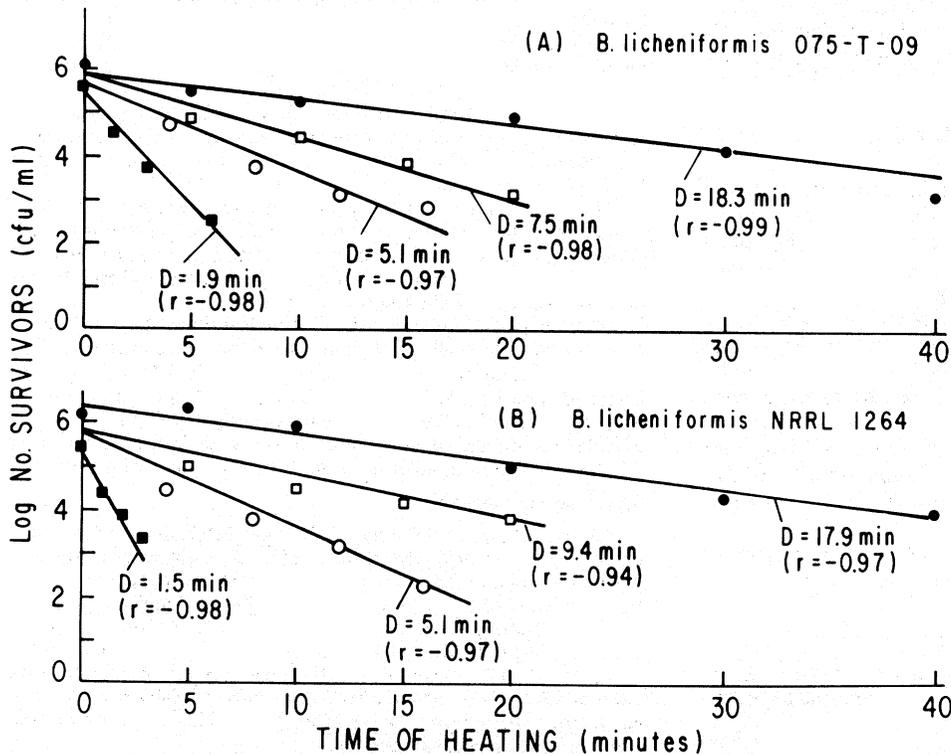


Fig. 1—Survival curves for spores from *B. licheniformis*; (A) strain 075-T-09; (B) strain NRRL NRS 1264; ○ = 85°C, □ = 90°C, ● = 95°C, ■ = 100°C.

The  $D_{95}$  of *B. licheniformis* 075-T-09 in pH adjusted tomato puree increased at the rate of 50% per pH unit over the range pH 4–5.5 (Fig. 3). This increase is of the same order of magnitude as the increases observed by Xezones and Hutchings (1965) for *C. botulinum* spores suspended in a tomato based product. Their data, however, would predict a much greater increase in resistance near pH 7 than was observed in this study. In Butterfield's buffer (0.00031M  $\text{KH}_2\text{PO}_4$  adjusted to pH 7.2), *B. licheniformis* 075-T-09 had a  $D_{95} = 7.8$  min, similar to the value (7.2 min) in tomatoes at the same pH. Burgos et al. (1972) have reported *B. licheniformis* to have a  $D_{99}$  of 3–5.5 min in Ringers solution depending on the length of sonication. Using  $z = 14.9^\circ\text{C}$ , this is equivalent to a  $D_{95}$  of 5.6–10.2 min and is consistent with our data.

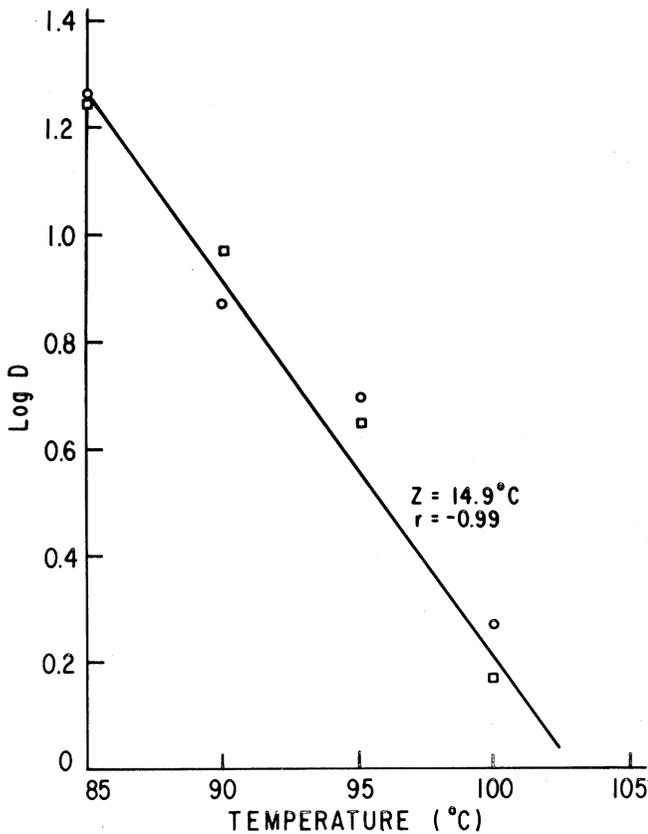


Fig. 2—Thermal death time curve for spores from *B. licheniformis* 075-T-09 (○) and NRRL NRS 1274 (◻).

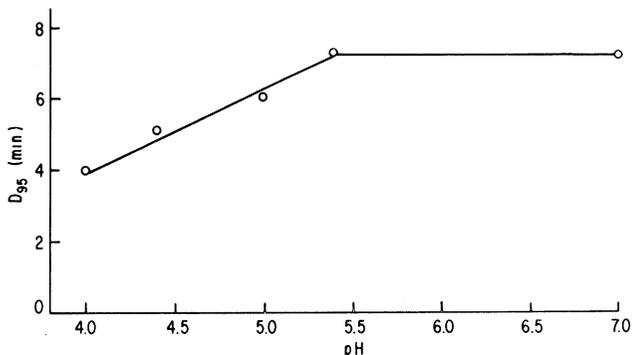


Fig. 3—Effect of pH on the thermal resistance of *B. licheniformis* 075-T-09 spores in pH adjusted tomato puree at  $95^\circ\text{C}$ .

## Test pack experiments

The inoculated jars had initial counts of  $2.6 \times 10^5$  or  $7.2 \times 10^5$  spores/ml of *B. licheniformis* 075-T-09 or mixed strains, respectively. The uninoculated control had a pre-process count of  $5.1 \times 10^2$  cfu/ml indigenous bacteria. Post-process counts were  $4.4 \times 10^1$ /ml for strain 075-T-09, and  $1.7 \times 10^2$ /ml for the mixed strain inoculum. No viable microorganisms could be recovered from the uninoculated control. These results indicate that the USDA recommended raw pack process provides more than a 3 D process with respect to *B. licheniformis* spores.

The surviving spores grew out and raised the pH to greater than 5.2 in the jars that were simulated leakers. The jars with intact seals showed no growth or pH elevation after 90 days at  $30^\circ\text{C}$ . *B. licheniformis* was isolated from these inoculated jars at levels equal to those found immediately post process. Odlaug and Pflug (1977) reported that *C. botulinum* spores do not die off when held in tomato juice at pH 4.2 for as long as 180 days.

Wentz et al. (1967) suggested that bacitracin produced by *B. licheniformis* can inhibit *C. botulinum* type F in a particular ecological niche. In preliminary experiments where *C. botulinum* 62A spores were inoculated into filter sterilized tomato serum whose pH had been elevated to 7.2 by the aerobic growth of *B. licheniformis* 075-T-09, we observed luxuriant growth of *C. botulinum* under anaerobic conditions. This indicates that this *B. licheniformis* strain neither produces inhibitors to nor depletes essential nutrients required for the germination and outgrowth of *C. botulinum* spores.

Given the low incidence of botulism outbreaks in acid foods, the hazard posed by pH elevating *B. licheniformis* spores must be small. A long chain of events consisting of the presence and survival of both *C. botulinum* and *B. licheniformis* spores, a poor jar seal, permissive post-process temperature, failure to heat prior to consumption, and an unobservant consumer must occur to effect a botulinal hazard. The probability of such a concurrence of events is small but not zero. Such a chain of events may have been responsible for the outbreaks that resulted in two deaths reported by Slocum et al. (1941) in which aerobic bacilli were isolated from the botulinic home-canned tomatoes.

Coinoculation experiments with *B. licheniformis* and *C. botulinum* spores in tomatoes are underway in this laboratory.

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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