APPLIED MICROBIOLOGY, Nov., 1965 Vol. 13, No. 6
 Printed in U.S.A.

Thermal Inactivation Characteristics of *Bacillus subtilis* Spores at Ultrahigh Temperatures[1]

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Received for publication 27 April 1965

ABSTRACT

EDWARDS, J. L., JR. (North Carolina State University at Raleigh), F. F. BUSTA, AND M. L. SPECK. Thermal inactivation characteristics of *Bacillus subtilis* spores at ultrahigh temperatures. Appl. Microbiol. **13**:851–857. 1965.—The thermal inactivation characteristics of *Bacillus subtilis* A spores suspended in skim milk with the use of large-scale ultrahigh temperature (UHT) processing equipment were investigated in terms of survival as measured with two plating media. Data on survival immediately after UHT treatments were recorded in temperature-survivor curves, time-survivor curves, and decimal reduction time (DRT) curves. The temperature-survivor curves emphasized that inactivation is accelerated more by increases in the treatment temperature than by increases in the exposure time. Time-survivor curves and DRT curves were not linear. Generally, exceedingly concave time-survivor curves were observed with the standard plating medium; however, only slightly concave curves were observed when $CaCl_2$ and sodium dipicolinate were added to the medium. For a given UHT sample, larger D values were obtained by use of the medium with the added $CaCl_2$ and sodium dipicolinate. The DRT curves of all data were concave and appeared to have two discrete slopes (z_D values). The z_D values observed in the upper UHT range (above 260 F; 127 C) were twice those observed at lower test temperatures.

The primary purpose of large scale ultrahigh temperature (UHT) processing of milk is the attainment of "practical sterility" in a palatable product. Inactivation of spores formed by certain thermophiles requires the most severe heat treatment. However, the less heat-resistant spores of the mesophilic aerobes may be the most troublesome because of possible large populations in milk and the ability of the spores to germinate and grow under the usual conditions of storage of sterile milk (Ridgway, 1958; Burton, 1959).

From the bacteriological standpoint, relatively little information has been presented on large-scale UHT thermal-inactivation studies. Williams et al. (1955), using UHT preheating (135 C) of raw milk before in-bottle sterilization, reported that the number of survivors after the UHT preheating was too low to be detected by the method used. Arph and Hallström (1962) stated that good-quality raw milk contained less than one spore per 30 liters after an UHT treatment of 140 to 142 C for 3 to 4 sec. Williams et al. (1957) observed that 135 C for 2 to 4 sec was sufficient to inactivate 99.99999% of *Bacillus subtilis* (786) spores suspended in water. Franklin et al. (1958*a*) found that 132 C for 2 to 4 sec resulted in 99.99999% inactivation of *B. subtilis* (786) spores suspended in milk.

Studies of large-scale UHT treatments of bacterial spores are needed for precise calculation of thermal processes, which not only prevent spoilage but also insure inactivation of all spores of public health significance. The present investigation was undertaken to study the thermal-inactivation characteristics of spores of *B. subtilis* A with the use of large-scale UHT processing equipment.

MATERIALS AND METHODS

Test organism. B. subtilis strain A was originally isolated by J. D. Ridgway (Cheshire Sterilized Milk Co., Ltd., Stockport, England), and was obtained for this study from Z. John Ordal (University of Illinois, Urbana). Stock cultures were maintained on nutrient agar (Difco) slants. The slants were incubated for 24 hr at 45 C and were stored at 3 C until needed.

Media. All media were prepared from the dehydrated forms (Difco) and were fortified with additional constituents. The media were dispensed in 100- or 135-ml quantities into milk-dilution bottles, autoclaved for 15 min at 121 C, and stored

[1] Contribution from the Departments of Botany and Bacteriology and Food Science, North Carolina Agricultural Experiment Station, Raleigh. Published with the approval of the Director of Research as paper no. 1986 of the Journal Series.

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at room temperature until needed. The composition of fortified nutrient agar (FNA) was: beef extract, 3 g; peptone, 5 g; NaCl, 8 g; agar, 20 g; glucose, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.08 g; $MnCl_2$ solution (5,000 ppm), 1 ml; modified G_b mineral solution, 100 ml; and distilled water, 900 ml. The G_b mineral solution of Pelcher, Fleming, and Ordal (1963) was modified by eliminating K_2HPO_4. FNA was modified (CNA) by incorporating $CaCl_2$ and sodium dipicolinate according to the method of Busta and Ordal (1964).

Preparation of spore suspensions. Because of the requirement for large numbers of spores, several suspensions prepared under the same conditions were combined into one composite which was used in the large scale UHT trials.

The inoculum, a 16-hr shake culture in nutrient broth (Difco) grown at 45 C, was dispensed in 1-ml quantities into petri dishes (150 by 25 mm) containing 100 ml of FNA, and was distributed over the entire surface of the agar. After 48 hr of incubation at 45 C, followed by 24 hr at 3 C, the growth was harvested in cold sterile distilled water. The subsequent suspension was passed through sterile gauze to remove any large particles of agar. The spores were collected and washed by centrifugation for 20 min (Servall, model RC-2; SS3-GS, 5.75-inch head) at 650 × *g* for the initial separation and at 1,465, 2,520, and 4,080 × *g* for the three subsequent separations. After each centrifugation, the spore pellet was suspended in about 130 ml of water by vigorous shaking. After the final washing, the spore pellet was suspended in 20 ml of water. The resulting spore suspension was stored at 3 C until needed.

Determination of apparent viable-spore populations. Platings were made according to the method given in *Standard Methods for the Examination of Dairy Products* (American Public Health Association, 1960), except that distilled water dilution blanks were used, and all platings employed FNA and CNA with incubation at 45 C for 18 hr.

Heating menstruum. Fresh skim milk, prepared by cold milk separation of mixed herd milk of the Raleigh milkshed, was used as the heating menstruum. Skim milk from this source contained approximately 9% solids as determined periodically by the Dietert method (Milk Industry Foundation, 1959). Approximately 150 gal of skim milk were required for each UHT trial. The milk was separated the day before the UHT trial, and was stored overnight in a refrigerated vat (about 3 C). During this period, the number of spores in uninoculated control samples was determined by plating after pasteurization at 80 C for 10 min. Usually, plate counts of fewer than 30 spores per milliliter were obtained, and no colonies resembling those characteristic of *B. subtilis* A were noted. Inoculated control samples were treated in the same manner as the uninoculated controls. The disc assay method, as described in *Standard Methods for the Examination of Dairy Products* (American Public Health Association, 1960), was used to determine the presence or absence of inhibitors in raw and pasteurized samples of the skim milk prior to each trial. Spores of *B. subtilis*, either strain A or ATCC 6633, were used in the inhibitor assays. None of the skim milk samples showed zones of inhibition.

TABLE 1. *Average holding times of the three holding tubes at various temperatures*

Test temp		Time (sec) for holding tube no.		
		1	2	3
F	(*C*)			
215	(102)	2.83	4.43	8.63
235	(113)	2.72	4.34	8.38
255	(124)	2.67	4.27	8.08
275	(135)	2.57	4.17	7.83
295	(146)	2.43	4.11	7.50

Ultrahigh temperature system. The direct steam-injection heating system used in the UHT inactivation trials was described by Roberts and Dill (1962). Slight modifications of the product flow along with additional means of recording the product temperatures were made. Before each trial, the system was sanitized and then sterilized by maintaining steam under pressure (15 psi gauge for 30 min) from the steam-injector heater past the sampling valve to the product discharge valve. The inoculated skim milk from the refrigerated storage vat was preheated to about 135 F (57 C) by continuous flow through the regenerator and heating sections of a high-temperature short-time (HTST) pasteurizer. The skim milk then was heated directly to the test temperature by injection and, at the end of the holding tube, was discharged into a vacuum chamber and cooled to about 135 F (57 C) by flash evaporation. Samples (about 150 ml) were collected manually in sterile bottles immediately after the vacuum treatment. The samples were further cooled in a cold-water bath (about 3 C).

Timing the product flow. The UHT system was operated with water (about 57 C) and the HTST unit was bypassed during the timing procedure. The holding tubes were fabricated from 1.5-inch (3.8-cm) stainless-steel sanitary pipeline along with a short section of 1.5-inch Pyrex glass pipeline. A Solu-Bridge automatic timer (model RT-3; Industrial Instruments, Inc., Cedar Grove, N.J.) was used to measure the holding time. Average holding times of the three holding tubes at the temperatures used are shown in Table 1. Since 255 F (124 C) was at the mid-range of the test temperatures, holding times for tubes 1, 2, and 3 were referred to (for convenience only) as 2.7, 4.3, and 8.1, respectively. However, the actual measured times or interpolated times were used in calculating the *D* values and constructing the time-survivor curves.

About 3 sec were required for the front of the salt solution to pass from the end of the holding tube through the vacuum chamber to the sam-

pling valve, as measured manually with a stop watch and a galvanometric flow timer (General Electric model AAM 319). Approximately 5 sec were required for all of the salt solution to pass through the vacuum chamber. Thus, in the UHT inactivation trials, a 2-min flush before sampling after a temperature change was sufficient to remove residual milk of the previous exposure temperature.

Heating procedure. Prior to a large-scale UHT trial, the skim milk was inoculated and allowed to mix for at least 5 min; an inoculated control sample was then taken. On completion of sterilization of the equipment, the vacuum chamber was switched from pressure to vacuum; simultaneously, the inoculated skim milk was started through the system. The treated samples were taken at various temperature intervals, starting at 295 F (146 C) and reducing to 215 F (102 C) to minimize contaminating the milk by survivors from preceding treatments. Usually, two samples were taken at each temperature interval. The first samples (146 C) were taken about 5 min after regaining smooth operation of the system, to ensure a representative sample after flushing out of this initial product. At least 2 min were allowed before sampling after changing the treatment temperature. After sampling the lowest treatment temperature (91 C), the steam supply to the injector was shut off, and an additional control sample was taken. This portion passed through the entire UHT system but was not heated by steam injection.

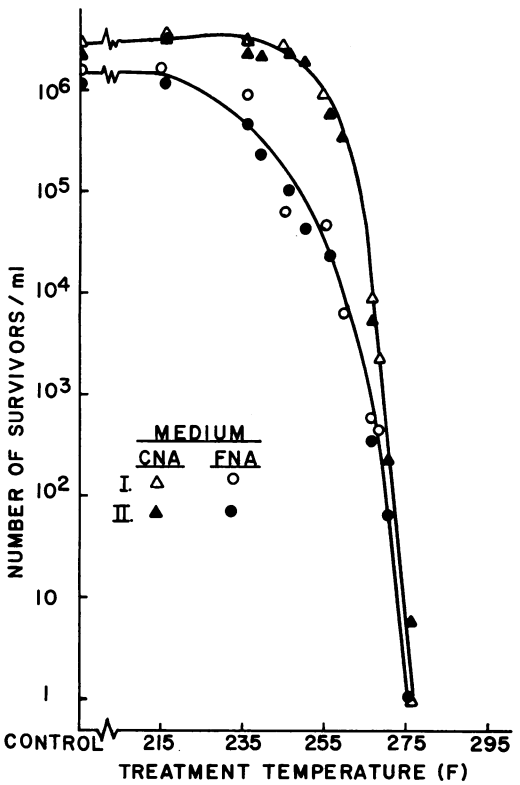

FIG. 1. *Temperature-survivor curve showing apparent survival after 2.7 sec of UHT treatment of Bacillus subtilis A spores suspended in skim milk. Each point represents the average of duplicate plates. Survival was measured with CNA and FNA in both trials.*

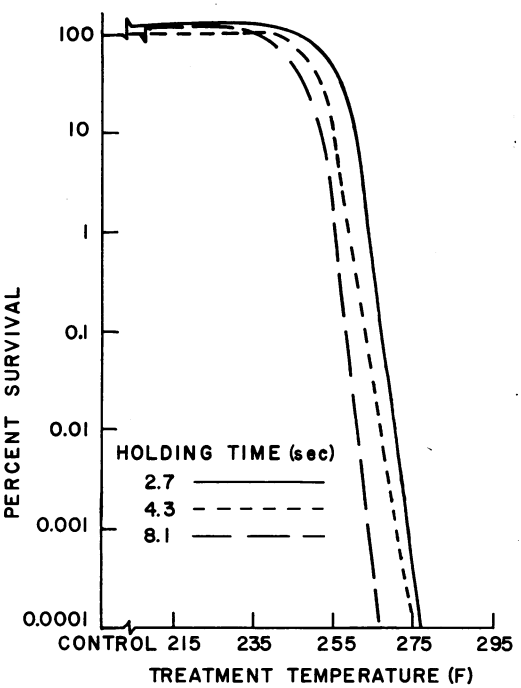

FIG. 2. *Comparison of the temperature-survivor curves observed with three holding times for UHT treatment of Bacillus subtilis A spores suspended in skim milk. Each curve represents averages of duplicate trials. Survival was measured with CNA.*

The glass section at the beginning of the holding tube allowed visual verification that it remained full throughout the trial. The treatment temperatures were recorded by two means: (i) a Taylor recording controller (series no. 122 R), and (ii) a recording potentiometer (Speedomax G; Leeds & Northrup Co., Philadelphia, Pa.). There was no measurable difference between the product temperature at the beginning and at the end of the holding tube; also, close agreement of the product temperature before the steam-injector heater and after the vacuum chamber was noted.

RESULTS

Preliminary studies comparing several complex nutrient media indicated that the highest plate counts were obtained with FNA and its com-

panion medium (CNA) containing $CaCl_2$ and sodium dipicolinate. These media, therefore, were selected for the recovery of viable spores in UHT-treated samples.

Since temperature rather than time was varied in each UHT trial, temperature-survivor curves were constructed by plotting the logarithm of the number of surviving organisms against temperature. Data of duplicate trials of the 2.7-sec holding time, which are representative of all trials, are presented in Fig. 1. The CNA counts of the control are about twice those of FNA. After UHT treatment, this difference in plate counts increased to greater than 10-fold in the 245 F (118 C) to 255 F (124 C) treatment range.

A plateau, characteristic of temperature-survivor curves, was obtained whenever survival was plotted against temperature. With FNA as the plating medium, the lowest temperature which caused a reduction in apparent surviving spores was about 223 F (106 C) for each holding time. The region of increasing death (portion of sharp curvature) extended over a temperature interval of 10 to 20 F (5 to 10 C), depending upon the holding time. At increased temperatures, the curves approached a straight line. Similar results were obtained at all holding times.

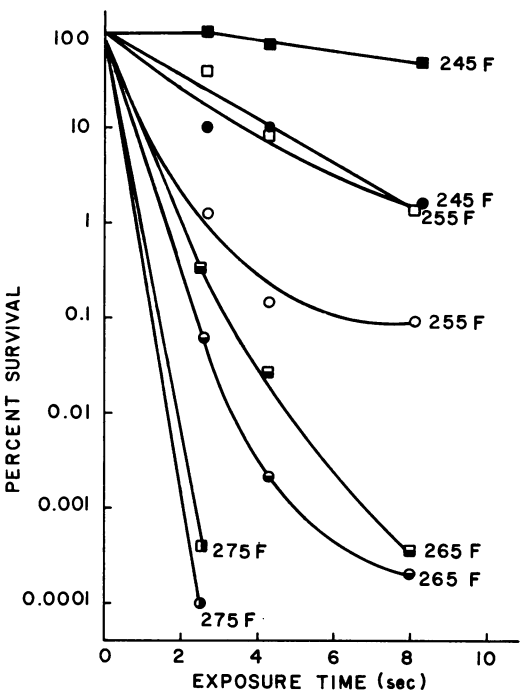

FIG. 3. *Time-survivor curves showing the relationship of D values to media used to determine survivors after UHT treatment of Bacillus subtilis A spores suspended in skim milk. Each point represents the average of duplicate trials at the given holding time. Numbers indicate treatment temperatures. Symbols:* □, *survival was measured with CNA;* ○, *survival was measured with FNA. Note: CNA points are based on CNA initial counts as 100%; FNA points are based on FNA initial counts as 100%.*

With CNA as the plating medium, the lowest temperature which caused a reduction in viable population decreased from 245 to 240 to 237 F (118 to 116 to 114 C) as the holding time increased from 2.7 to 4.3 to 8.1 sec. A range of 15 F (8 C) encompassed the region of increasing death for each holding time. The change in temperature required at a constant holding time for the survivor curve to traverse one logarithmic cycle in the straight-line region was used to compare the temperature-survivor curves at various holding times. These values for the CNA curves were 3.5, 4, and 3 F (1.9, 2.2, and 1.7 C) for the 2.7-, 4.3-, and 8.1-sec holding times, respectively. The temperature-survivor curves of the CNA data are shown in Fig. 2.

Exact replication of the sampling temperatures in the different trials was not possible owing to inherent limitations of the temperature control device on the UHT unit. Thus, to construct classical time-survivor curves from these data, survival at a given temperature was read from the temperature-survival curves. Representative time-survivor curves for the data obtained with FNA and CNA are shown in Fig. 3. Generally, the curves of the FNA data were exceedingly concave, whereas those of the CNA data were slightly concave. At a given treatment temperature, more survivors were observed with CNA than with FNA.

Decimal reduction time (DRT) curves were constructed from D values by use of the following formula which was adapted from Schmidt (1957):

$$D_{(n)} = \frac{U}{\log a - \log b}$$

where U is the holding time, log a is the initial per cent survival (100%), and log b is the per cent survival after exposure to temperature n. The per cent survival for b was read from the temperature-survivor curves at each exposure temperature. The DRT curves are shown in Fig. 4. These curves were not linear throughout the temperature range; however, linear portions were noted within certain temperature ranges. At least two z_D values were observed: a smaller one in the lower temperature range and a larger one in the higher temperature range. These changes in the z_D values occurred in the 240 F (116 C) to 250 F (121 C) range when inactivation was measured with FNA and in the 255 F (124

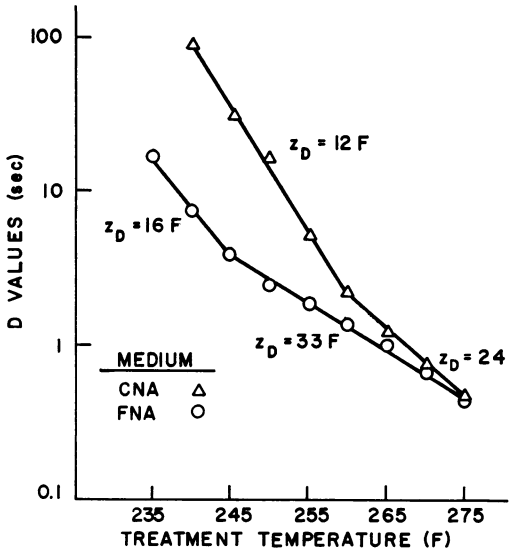

FIG. 4. *Decimal reduction time curves for UHT treatment of Bacillus subtilis A spores suspended in skim milk. Curves represent averages of six trials. Numbers indicate z_D values in Fahrenheit. Survival was measured with CNA and FNA.*

C) to 265 F (129 C) range with CNA. Both curves were concave and converged at the higher treatment temperatures. The DRT curve constructed from averages of all FNA data appeared to have z_D values which ranged from 16 F (8.9 C) to 33 F (18 C) in a temperature range from 235 F (113 C) to 275 F (135 C). The z_D values of the curve using the averages of all CNA data were 12 F (6.7 C) in the 235 F (113 C) to 260 F (127 C) temperature range and 24 F (13 C) in the 260 F (127 C) to 275 F (135 C) temperature range. These changes in z_D values are shown in Fig. 4.

DISCUSSION

Food processing at UHT requires extensive information on thermal inactivation under the conditions used in large-scale operations. Obviously, the detection of surviving spores is essential for the precise measurement of thermal-inactivation data. In the present study, the medium (FNA) which was representative of numerous standard nutrient media often used to detect survivors of heat treatments enumerated maximal populations when compared with other standard media. However, this representative medium indicated 90% inactivation in the same UHT-treated samples that exhibited essentially no inactivation when analyzed with the companion medium, CNA. The only difference in these two media was that CNA contained the germinating agents $CaCl_2$ and sodium dipicolinate. These data indicated that FNA was inferior to CNA for the detection of survivors; however, since past evaluations of spore heat resistance often were made with media resembling FNA, it is necessary to emphasize the influence of plating media. The implications in these data of apparent heat injury of the spores are examined extensively by Edwards, Busta, and Speck (1965).

The operation of large-scale UHT equipment does not facilitate the use of extended holding times; therefore, the temperature-survivor curve, a plot of the number of surviving spores against temperature, best illustrated the thermal-inactivation characteristics of the system. Although little precedent for this graphical presentation could be found in the literature (Burton et al., 1958; Franklin, Williams, and Clegg, 1958*b*; Franklin et al., 1959), temperature-survivor curves may become more meaningful and useful with the present trend toward UHT processing of milk (Burton, 1959; Speck, 1961; Read, 1964).

One characteristic of thermal inactivation clearly expressed in the temperature-survivor curves is the great influence of small changes in temperature in the lethal range (straight-line region); i.e., small changes in temperature resulted in large changes in the number of survivors. Consequently, in UHT processing of foods, precise temperature control at all times is imperative. Also, a slight increase in treatment temperatures of specific thermal processes would impart a significant safety factor and would appear to be more satisfactory than an extension of holding time.

In much of the previous thermal-inactivation work, the holding time was varied at a constant exposure temperature and time-survivor curves were constructed from the data. In most instances, these curves are linear or approximately linear (Schmidt, 1957). Since time-survivor curves have been used in determining *D* values and in calculating thermal processes, this type of curve was constructed from the data of this investigation. Differences in numbers of apparent survivors measured with FNA and CNA also were reflected in the classical time-survivor curves. A striking feature was the great concavity of the curves of data obtained with FNA. Concave time-survivor curves may result from heterogeneity in heat resistance (Hansen and Riemann, 1963) of the spores or of germination systems within spores. Recently, Alderton, Thompson, and Snell (1964) proposed another explanation for this decreasing death-rate curve by showing that heating slowly evokes a protective response in the spores; i.e., a greatly increased heat resistance gradually develops during the heating. No explanation is readily apparent for the con-

cavity observed in curves obtained with FNA but not observed in those obtained with CNA.

A useful and descriptive graphical presentation of thermal-inactivation data is the DRT curve which is constructed by plotting the logarithm of the D values against temperature. Usually the DRT curves are linear, but they often cover only a relatively narrow temperature range when used in calculation of thermal processes. Hansen and Riemann (1963) pointed out that "the z value cannot be constant over a wide temperature range because at some borderline temperature the D value must be infinite and the corresponding z value must therefore be zero." The data obtained over the extended temperature range used in the present study indicated a smaller z_D value in the lower temperature range and a larger one in the higher temperature range. There may be a question as to whether this change is discrete (two z_D values) or continuous. Nevertheless, within the limits of present investigation, there was a change to a higher z_D value in the upper temperature range.

Other workers have reported an apparent change in slope of DRT curves in the UHT range. Esselen and Pflug (1956) noted a change for *Clostridium sporogenes* (P.A. 3679) spores in fresh carrots, and this change also was from a smaller z_D value at lower temperatures to a large z_D value. They postulated that the change in slope could be due to changes in spore destruction rates as a result of chemical changes occurring in the foods during heating or to an increased z_D value due to superheated steam at the higher temperatures, or both. These factors did not appear to influence the present study.

The UHT system using steam injection provided conditions which were particularly well suited to promote the efficiency of killing spores with heat according to the criteria proposed by Alderton et al. (1964), i.e., low storage temperature before instantaneous equilibration to a high treatment temperature for a short holding time. The concave time-survivor and DRT curves, in addition to the influence of the plating media, emphasize the importance of conducting thermal-inactivation experiments under the actual processing conditions; also, optimal methods for the enumeration of survivors are essential if the data are to be used in calculating thermal processes. Failure to do so could result in significant errors in terms of survivors if higher temperature processes were calculated by extrapolation of the DRT curve obtained from lower temperature-processing data, especially if extrapolated over a wide temperature range.

ACKNOWLEDGMENTS

The senior author was a National Defense Education Act Graduate Fellow during 1962–64.

We are grateful to V. A. Jones for assistance on engineering aspects of the study and to K. M. V Mathur, J. D. Moore, Jr., and J. I. Middleton for technical assistance.

This investigation was supported by Public Health Service grant EF-00378 from the National Institutes of Health.

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