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# Effect of Temperature and Gas Velocity on the Dry-Heat Destruction Rate of Bacterial Spores[1]

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Spores of *Bacillus subtilis* were dried in vacuo for use in dry-heat thermal destruction tests. Survivor curve tests were conducted in a specifically designed dry-heat oven. This oven provided accurate temperature control and permitted air or nitrogen to be passed over the spores during the lethal treatment. Experiments were carried out at various flow rates of the two gases (air and nitrogen) and various temperatures, and the data were expressed as survivor curves from which the decimal reduction time (*D* value) was obtained. Linear regression analysis methods were used to compute the slope of the survivor curves. The results indicated that as the flow rate of gas is increased, the effect of temperature on the destruction rate of the spores is lessened, the *z* value becoming very large. It is believed that the higher flow rates of dry gas cause greater dehydration of the spores and that spore moisture loss is one of the major factors in determining the dry-heat thermal destruction rate of bacterial spores.

The dry-heat sterilization of the surfaces of objects such as spacecraft and mechanical components requires knowledge of the factors which affect the resistance of bacterial spores to dry heat. One of the most important applications of this sterilization procedure is in the sterilization of interplanetary space probes (4).

Knowledge of the factors which affect the thermal resistance of bacterial spores in dry heat is limited. Even the obvious parameters necessary for destruction of bacterial spores, such as time and temperature, are not completely understood. This study was undertaken to investigate the effect of open system dry heating on the destruction of bacterial spores.

The term "open system" will be used to describe a system for heating microbial spores where water can be lost or gained without limit during heating. In an infinite time, the spores will be in equilibrium with the water condition of the environment. This definition places no restriction on the rate of water transfer; the spores may lose or gain water either rapidly or slowly. A closed system is a system for heating microbial spores where water movement or availability is restricted. The quantity of water that can be transferred is limited by the quantity of water initially present in the environmental volume.

The destruction of microorganisms by dry heat is different in order and may be different physiologically than wet-heat destruction (K. Fox, B. D. Eder, and I. J. Pflug, *in preparation*). Wet heat is defined as heating in a medium where the relative humidity is 100%. Dry heat is less readily defined, but refers to an environment where the relative humidity is less than 100%. The *D* values of microbial spores are much larger in dry heat than in wet heat (5, 8, 12, 13).

Several factors which affect the dry-heat resistance of bacterial spores are given below.

(i) *Initial number of organisms.* W. A. Sisler (Ph.D. Thesis, Michigan State Univ., 1961) determined survivor curves for different initial populations of spores heated in wet steam at 121 C and in superheated steam at 160 C. He reported that the *D* value (time required to decrease the population by 90%) for a population of $10^6$ spores was greater than the *D* value for $10^7$ spores, when tested in superheated steam. He did not observe these differences with wet steam.

(ii) *Water.* Water has been found in many cases to affect the exhibited resistance of spores to dry heat (9, 10). Recently, Murrell and Scott (11) reported a method whereby they controlled the water activity of the spores during the actual heat treatment. They found maximal resistance at a

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water activity of about 0.3. In all cases, they found a decrease in resistance as the water activity approached either 0 or 1, the resistance being much lower at water activity of 1. These results have been confirmed by Angelotti (1).

(iii) *Menstruum.* Fry and Greaves (7) found that the survival of spores during and after freeze-drying was dependent upon the menstruum from which they were dried. They found that, when bacterial cells were dried from plain distilled water, their survival time in the dried state was shorter than if the cells were dried out of some other solutions, such as glucose or peptone. These workers proposed that the presence of glucose or peptone ensures the retention of a certain amount of moisture in the dry spores which is necessary for survival. Annear (2) found that freeze-dried bacteria could be preserved for long periods of time by suspending them in peptone solutions prior to freeze-drying. He also attributed this increased survival to the fact that the peptone protected the cells from becoming too dry during the freeze-drying process.

(iv) *Support medium.* J. A. L. Augustin (Ph.D. Thesis, Michigan State Univ., 1964) found that the dry-heat destruction rate of putrefactive anaerobe 3679 was higher on tin than on aluminum. Fox and Pflug (6) determined the dry-heat destruction rate of *Bacillus subtilis* spores on four test surfaces and found the largest *D* values when the spores were heated on tin. The *D* values decreased in the order: tin, aluminum, glass, filter paper. Other workers (1, 3) have observed a similar phenomenon.

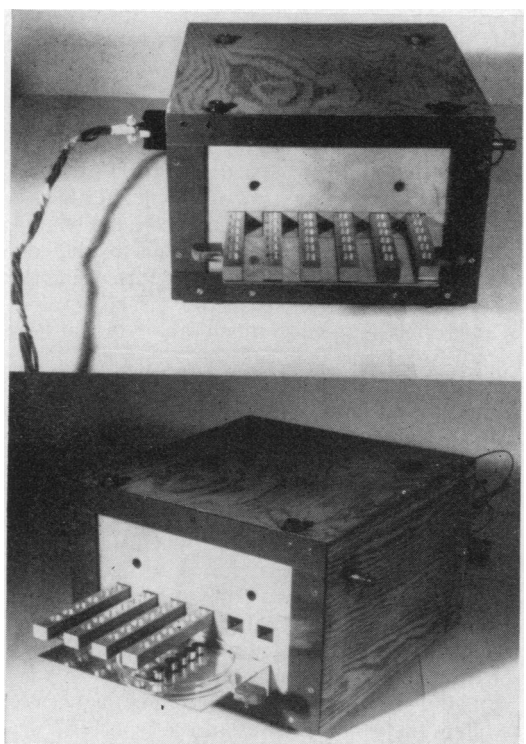

FIG. 1. *(top) Front view of dry-heat oven showing sample bars pushed out and shelf in upper position. (bottom) Front view of dry-heat oven showing shelf in lower position to receive sample cups.*

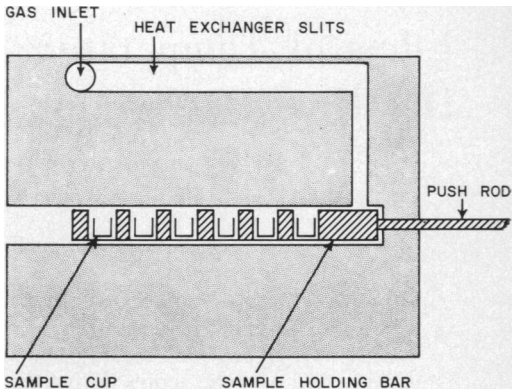


FIG. 2. *Diagrammatic pattern of gas flow through dry-heat oven.*

(v) *Effect of test atmosphere.* Pheil et al. (15) studied the effect of various gas atmospheres. They found that the destruction rates of *B. subtilis* in oxygen, air, and carbon dioxide were essentially the same, but were higher in helium and nitrogen. Silverman (16) found that the dry-heat resistance was greatly reduced when dry air flowed over the heated spores at the rate of 3 liters/min, compared to spores heated in still air. He also found that the resistance increased when the moisture content of the air was increased.

## MATERIALS AND METHODS

A simple oven was designed and constructed to meet the following requirements: (i) maintain constant temperature of the spores throughout the test period; (ii) hold several sets of samples with each set consisting of several replicates and each set of replicates removable from the oven without disturbing the other samples; (iii) reproducible heating and cooling times and minimal cooling times; (iv) permit aspetic removal of heated samples; and (v) provision for preheating air or other gases used in the gas-flow tests.

The oven was a large aluminum block 5 × 8.5 × 9 inches (12.7 × 21.59 × 22.86 cm) with six slots for the six sets of samples. The large mass of metal with the accompanying thermal capacity simplifies temperature control. Five sides of the block were insulated, leaving only the face exposed. The temperature was controlled by four electrical heaters located within the aluminum block. The temperature was sensed by a Honeywell thermistor, also located in the

aluminum block. This thermistor, when used with the Honeywell thermistor controller, gives a temperature sensitivity of ±0.2 C. The dry-heat oven is shown in Fig. 1, which also shows the dual-purpose shelf located below the heating chambers. The upper position directly under the heating chambers enables all 36 sample cups to be loaded into the oven simultaneously, and the lower position (about 1 inch below the previous position) permits support of a sterile petri dish for removal of the sample cups from the oven.

The top of the block contains slits 0.0625 inch in width, 5 inches in length, and 1 inch deep (0.16 × 12.7 × 2.54 cm). Their purpose is to heat the incoming gas to oven temperature before the gas is discharged over the spores. The gas enters through an opening on the side of the oven and goes into a manifold that feeds the six sets of slits. The gas flows to the rear of the sample heating chambers, over the spore samples, and out the front of the oven. Figure 2 shows a schematic diagram of the gas-flow pattern through the oven.

Commercial compressed nitrogen and compressed air were used in these tests. The compressed gas tanks were fitted with standard two-stage pressure-reducing valves. The gas flowed from the pressure-reducing valve to a rotameter and then to the oven.

The organism used in this study was *B. subtilis* 5230. To prepare the spores for heat treatment, 0.01 ml of a suspension containing approximately $10^7$ spores/ml was dispensed with a micrometer syringe into small tin cups, 11 mm outside diameter × 8 mm deep (14). Prior to inoculation, the washed cups were placed in petri dishes, sterilized in the autoclave at 121 C for 30 min, dried at 20 C for 24 hr in a vacuum (at 0.74 mm of mercury), and stored in a desiccator over calcium chloride until used.

Five inoculated cups were dropped into each of five screw-cap test tubes containing 10 ml of sterile distilled water to determine the initial number of organisms for each test. These tubes were placed in a boiling-water bath for 10 min to heat-shock the spores. The caps were tightened after the heat-shock, and the tubes were shaken vigorously for 10 min to remove spores from the cup surfaces and to distribute the spores throughout the 10-ml suspension. A 1:100 dilution was made of each tube, and 1 ml of the dilution was plated with dextrose-tryptone-starch-agar (DTS). A thin DTS overlay was poured over the solidified surface to prevent spreading of surface colonies. The plates were inverted and incubated for 48 hr at 37 C. The average count of five plates was taken as the initial number of organisms. The initial number remained fairly constant throughout the entire testing period.

The spores were prepared for the heating studies as outlined above, and the dried inoculated cups were placed in the dry-heat oven for the appropriate heat treatment. At the conclusion of the heating test, the cups were dropped into a sterile petri dish and were transferred to screw-cap test tubes containing 10 ml of sterile distilled water. To remove the spores from the surface of the cups, the tubes were shaken vigorously for 10 min. Appropriate dilutions were made according to the particular heat treatment which the samples had received, and 1 ml of the dilution was plated onto DTS agar as outlined above.

The method of analysis used involved plotting the number of viable spores found at various time intervals against time on semilogarithmic coordinates. Lag correction factors were calculated, but the heating time was not corrected since the come-up time was very short and the cool-down time of the samples very rapid. The data were analyzed by linear regression from which the *D* value was calculated. Since the survivor curves were not single-slope straight lines, the *D* value was computed from the second portion of the curve. Confidence limits of 95% were calculated for each *D* value and are shown along with the *D* values in Table 1. Figure 3 shows a sample survivor curve obtained by using the previously described techniques.

## RESULTS AND DISCUSSION

Survivor curves were determined at four temperatures (124, 129, 135, and 140 C), and the *D* values were obtained by linear regression on the second portion of the curve. Figure 3 shows the typical shape of the survivor curves obtained in this study. Table 1 shows the linear regression *D*

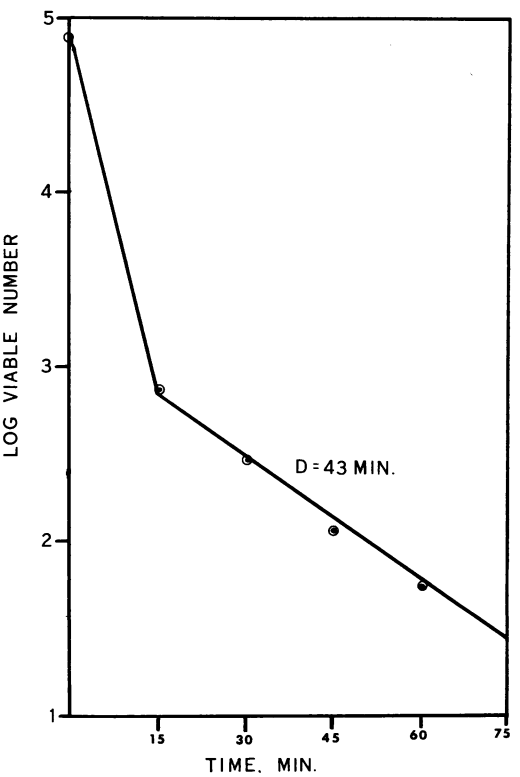


FIG. 3. *Sample survivor curve for Bacillus subtilis in dry-heat oven (still air) at 140 C.*

TABLE 1. *D values and their 95% confidence limits (in minutes) for individual thermal destruction tests*

| Temperature | Air flow (ft³/min) | | | Nitrogen flow (ft³/min) | |
|---|---|---|---|---|---|
| | 0 | 1.4 | 4.0 | 1.4 | 3.0 |
| *C* | | | | | |
| 124 | 255 (137-1713)<br>110 (72-234)<br>511 (254-—) | 216 (112-3079) | 169 (99-582) | 151 (102-293) | 117 (83-195) |
| 129 | 144 (90-351)<br>149 (70-—)<br>203 (80-—) | 107 (73-203) | 108 (60-487)<br>116 (78-233) | 93 (64-168) | 97 (67-175) |
| 135 | 106 (56-830)<br>94 (62-194) | 62 (42-123)<br>95 (42-206) | 141 (83-446)<br>71 (50-121) | 48 (36-71) | 57 (44-80) |
| 140 | 43 (34-57)<br>50 (36-78) | 109 (56-2781)<br>74 (47-177) | 128 (62-—)<br>61 (39-144) | 64 (39-190) | 107 (68-255)<br>77 (54-135) |

TABLE 2. *Average D values (in minutes) determined by linear regression*

| Temperature | Air flow (ft³/min) | | | Nitrogen flow (ft³/min) | |
|---|---|---|---|---|---|
| | 0 | 1.4 | 4.0 | 1.4 | 3.0 |
| *C* | | | | | |
| 124 | 383 | 216 | 169 | 151 | 117 |
| 129 | 165 | 107 | 112 | 93 | 97 |
| 135 | 100 | 95 | 106 | 48 | 57 |
| 140 | 47 | 74 | 95 | 64 | 77 |

TABLE 3. *z-Values for various gas-flow conditions*

| Flow condition | z-Value | $D_{121\,C}$ |
|---|---|---|
| | *C* | *min* |
| Dry heat closed system-TDT can[a] | 17 | 600 |
| Wet heat closed system-TDT can[a] | 14 | 0.50 |
| Still air-dry oven | 17.5 | 800 |
| 1.4 ft³/min air flow-dry heat oven | 28 | 340 |
| 4.0 ft³/min air flow-dry heat oven | 55.5 | 120 |
| 1.4 ft³/min nitrogen flow-dry heat oven | 23 | 270 |
| 3.0 ft³/min nitrogen flow-dry heat oven | 40.5 | 150 |

[a] These data from K. Fox et al. (*in preparation*).

values obtained for the individual experiments with their 95% confidence limits. Table 2 shows the average linear regression *D* values obtained from Table 1 after discarding the following *D* values, which showed opposition to the theory that as the temperature increases the *D* value decreases: $D_{124}$ = 110 min (still air); $D_{129}$ = 62 min (1.4 ft³/min of air); $D_{140}$ = 109 min (1.4 ft³/min of air).

Table 3 summarizes the *z* values (temperature change required to produce a ten-fold change in *D* value) obtained for the various experiments.

The data presented in Table 2 indicate that at the low temperatures (124 and 129 C) the *D* value decreased as the flow rate of the air or nitrogen increased. At 135 C, the *D* value did not appear to change with changing flow rate of either air or nitrogen. At 140 C, there appeared to be a reversal in effect, since the *D* value increased as the flow rate of air or nitrogen increased.

The survivor curve has a different meaning in the open system than in the closed system. The moisture content of the spores in the open system is not constant throughout the heating period; the spores become drier as they are heated, because of loss of moisture from the open system. If the water activity of the spores were held constant during the heating period, we could be more

certain that the rate of death obtained was a true *D* value. However, it is not possible at the present time to hold the water activity of the spores constant and at the same time maintain an open system. Murrell and Scott (11) reported that they held the water activity constant throughout the heating schedule, but to do this it was necessary to use sealed test tubes containing certain salts.

From Table 3, we can see that for both the nitrogen and the air tests the largest *z* value was obtained at the highest flow rate, thus indicating that as the flow rate of the gas increased, the thermal resistance became less temperature-dependent. We have observed essentially the same *z* values in the dry-heat closed system with thermal death time cans, the open system with zero air flow, and the open system having the low flow rate. The low flow rate of nitrogen has given a slightly higher *z* value. Therefore, in systems where the rate of dehydration (rate of flow) of the spores is relatively low (closed systems and low flow-rate systems), the main factor influencing the thermal resistance is the temperature.

The use of nitrogen has produced resistances slightly lower than those obtained in air. It was originally thought that if oxidation of dry bacterial spores was contributing some lethal effect, the use of nitrogen should increase the resistance since there would be little if any oxidation during heating in nitrogen. The results show, however, that the resistance in nitrogen as compared to air was higher only at 140 C. Since at this temperature the *D* value of the spores heated in air generally increased as the air flow rate increased, we cannot infer anything concerning oxidation as a cause of death. If oxidation were occurring during dry heating in air, we might expect the *D* value to decrease as we increased the air flow, but we would expect the *D* values under nitrogen to be very high. A possible cause for the lower nitrogen *D* values is the relative moisture content of the two gases. The commercial nitrogen gas contained 0.00025% water, whereas the compressed air contained about 0.1% water. The drier nitrogen may have caused more rapid desiccation of the spores with a corresponding decrease in heat resistance. Pheil et al. (15) suggested that if the oxygen content of the gas is responsible for the major destructive effect in dry-heat sterilization, then the dry-heat resistance should be lowest in oxygen. However, these workers found that the resistance was not measurably different between oxygen and air systems.

There are several possible reasons for the variability among replicate plate counts. The most plausible explanation appears to be the recovery of the spores from the sample cups. The plate-count enumeration method presupposes good removal of the spores from the test surface and requires that the same proportion of spores are removed from all cups. It is possible that during the heating process spores become "baked' onto surfaces, and, when the cups are soaked for 10 min to remove the spores, not all spores are removed from the cups. Other possible sources of error include variations in the original number of spores per sample, small convection currents set up by the flowing air in each cup, and variations in the rate of air flow among the sample chambers. To check the last possibility, the six replicate cups for each time interval were removed one from each bar instead of all six from the same bar. Thus, if one sample chamber was different from the other sample chambers, it could be spotted; however, when the data were examined, no difference was found. It was thought that, as the spores were stored in the stock suspension, their resistance might have changed; however, when the *D* values were examined in relation to the data obtained, no correlation was evident.

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