Dry-Heat Destruction of *Bacillus subtilis* Spores on Surfaces: Effect of Humidity in an Open System

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Bacillus subtilis var. *niger* spores were tested for dry-heat resistance on stainless-steel strips hung in an oven. Heat resistance was dependent on the relative humidity before and during treatment, which in turn affected the water content of the spores. Higher humidities increased the heat resistance of the spores. D-values ranged from 16.1 min for spores conditioned at <2% relative humidity (RH) and treated at 0.34% RH to 37.6 min for spores conditioned at 89% RH and treated at 1.1% RH. The y-intercept of the regression line ranged from 6.94 × 10⁴ for spores conditioned and treated at the low humidities to 2.00 × 10⁵ for spores conditioned at 89% RH and treated at 0.34% RH. For a constant value of N_0, the y-intercept appears to be lowered by low-humidity conditions. The statistic $\log y_0/\log N_0$ is used to measure the downward displacement of the regression line. Values obtained in this experiment range from 0.90 for spores conditioned at <2% RH and treated at 0.34% RH to 1.04 for spores conditioned at <2% RH and treated at 1.1% RH. A combination of linear regression and analysis of variance methods was used for data analysis. The former estimates D-values and y-intercepts, whereas the latter is sensitive to differences between treatments.

Dry heat has been chosen to sterilize planetary lander spacecraft (12). To preserve reliability and minimize degradation of spacecraft materials, the sterilization treatment should be the minimum which will achieve the desired results (12). Therefore, it is necessary to evaluate carefully the factors which affect dry-heat sterilization.

The important effect of water on the dry-heat destruction of bacterial spores has been reported by a number of authors (1, 6, 7, 9, 10).

Both the relative humidity of the environment prior to treatment (conditioning humidity) and the relative humidity during treatment (treatment humidity) affect the dry-heat destruction of bacterial spores. The present study was designed to separate and measure the effect of conditioning humidity and treatment humidity in an open system for one typical spore suspension.

MATERIAL AND METHODS

Bacillus subtilis var. *niger* spores were deposited on stainless-steel strips and hung in a gravity convection oven with the surfaces parallel to the air stream.

The spores were grown in our laboratory from spores supplied by the Communicable Disease Center Field Station, Phoenix, Ariz. The spores were grown on Difco TAM agar (8) supplemented with 88.0

mg of CaCl₂ per liter and 22.0 mg of MgSO₄ per liter (M. Favero, *personal communication*) at 41 C with a 48-hr incubation time, washed, and stored in ethanol at approximately −10 C. After several months of storage, the spores were rinsed and resuspended in sterile distilled water; the suspension was subdivided into many small bottles and stored at approximately −10 C. A bottle of spores was thawed for each test; unused spores were discarded. Thus all spore samples had the same handling history except for the length of frozen storage.

After thawing, the bottle containing the spore suspension was agitated in an ultrasonic bath and 0.02 ml samples (2.34 × 10⁵ spores) were deposited on the test surfaces with an Eppendorf micropipette. Each test surface was a stainless-steel strip (1 by 2 inch, ca. 2.54 by 5.08 cm) which had been washed; rinsed sequentially in distilled water, isopropyl alcohol, and ether; and then sterilized with dry heat (11).

All preparation and analysis procedures were carried out in a class 100 laminar downflow clean room (5). The contamination rate under these conditions was indistinguishable from zero.

After the spore suspension was deposited on the test surface, it was dried at 23 C for 18 hr in the clean room. The test strips were then placed in plastic glove boxes over night for water conditioning. In the glove box, the relative humidity was maintained at less than 2 or 89% by using wetted silica gel (3).

A small electric fan in each box prevented humidity stratification. The boxes were maintained at the ambient room temperature of about 76 F. The relative
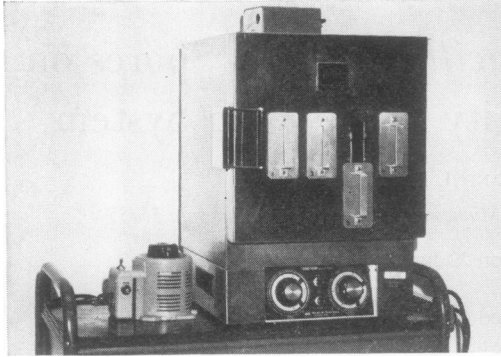
FIG. 1. *Treatment oven. Racks with strips are inserted through the small openings.*
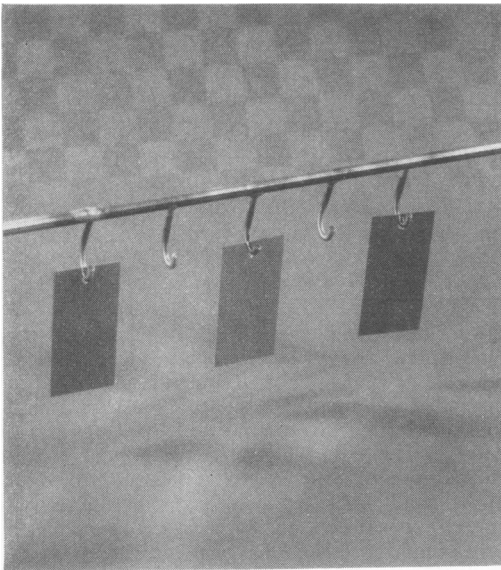

FIG. 2. *Treatment rack with strips.*

humidity of the boxes was measured with a Honeywell model W611A relative humidity indicator.

The treatment system used a modified gravity convection oven (Blue M model #OV-12A) shown in Fig. 1. The strips (1 by 2 inch) were hung on racks and inserted through the small magnetically held doors to avoid disturbing the temperature of the oven. A rack with strips is shown in Fig. 2. A diffuser plate inside the oven helped to minimize temperature variation across the oven. A variable transformer and thermistor controller (Honeywell model 3679) were added to the oven to decrease "overshoot" and to promote more accurate temperature control.

To maintain the relative humidity of the treatment system, the oven was placed in a refrigerator or in a humidified incubator where the dew point was maintained at 5 or 21 C. This produced treatment relative humidities of 0.34 and 1.1%, respectively, at the treatment temperature of 125 C.

To measure temperatures in the oven, 12 thermo-

couples were located in a grid above the location of the samples. The temperature of each strip during treatment was measured by a thermocouple directly above it.

All data presented here have been corrected for slight temperature variation by using the NASA-adopted z-value of 21 C. (2).

Four treatment times (10, 30, 50, 70 min) and three strips per treatment time were used. The data presented here were from two replicate experiments. Recovery of spores from the strips followed the NASA Standard Methods (11). Strips were placed in 125-ml flasks with the contaminated surface facing downward; 50 ml of phosphate buffer was added, and the sample was sonically treated for 2 min in the center of an ultrasonic bath filled with a 0.3% solution of Tween 80 in distilled water (11). Aliquots of 0.05, 0.1, 1.0, or 10 ml, or combinations of these amounts of fluid, were plated in duplicate on Trypticase Soy Agar. Plates were incubated at 32 C for 48 hr.

To avoid systematic errors, samples were randomly assigned to treatments and the processing and counting of petri plates was carried out in random order. To the extent possible, consistent with the volume of processing, the number of a plate in the experimental scheme was concealed from the person who counted it.

The data were analyzed by a combination of linear regression and analysis of variance. Linear regression gave estimates of the slopes and intercepts of the various survival curves. The analysis of variance distinguished more precisely between the effects of various treatments and indicated whether the effects of the treatments and their interactions were significant.

The analysis of variance was a combination of factorial and split-plot approaches. Each whole plot was a rack, carrying three strips, which was exposed to a particular treatment time and process. The whole plots were analyzed by a factorial scheme which allowed the effects of conditioning and treatment humidity to be separated.

For both analyses the data were transformed according to the formula $x = \log_{10}$ (datum $+ 1.0$). The transformation put the data in the standard semilogarithmic format for the linear regression. It also made the variance of the data independent of the mean which is a requirement for analysis of variance. The number 1.0 was added to all data values so that data values of zero could be included in the analysis. The error produced by the addition of 1.0 was not significant.

RESULTS AND DISCUSSION

Table 1 shows the data from which the analyses were performed.

Figure 3 summarizes the results in graphical form. Each point represents the geometric mean (mean of logs) of six strips, three processed in each of two replicate experiments. The lines are the least squares regression lines.

Table 2 gives the death parameters for the experiments. The statistic $\log y_0/\log N_0$ measures the displacement of the regression line intercept from the control (N_0) value.

TABLE 1. *Raw data table*

| Conditioning RH	Treatment RH	Time (min)	Day 1			Day 2		
			Front	Center	Rear	Front	Center	Rear
%	%							
2	0.34	10.0	4.23E 4[a]	2.46E 4	1.50E 4	4.21E 4	3.09E 4	3.42E 4
		30.0	1.65E 3	1.84E 2	3.23E 1	3.76E 2	3.48E 3	3.17E 3
		50.0	2.33E 0	1.37E 0	1.23E 1	1.00E 2	1.57E 2	8.65E 1
		70.0	1.00E 0	0	0	1.34E 1	7.08E 1	2.53E 1
2	1.1	10.0	1.15E 5	1.02E 5	1.41E 5	7.61E 4	9.31E 4	1.00E 5
		30.0	1.26E 4	1.16E 4	1.88E 4	2.37E 4	3.28E 4	2.10E 4
		50.0	5.00E 2	5.12E 2	5.94E 2	2.55E 3	2.40E 3	2.70E 3
		70.0	5.00E 1	6.13E 1	1.07E 2	3.16E 2	2.68E 2	3.64E 2
89	0.34	10.0	6.65E 4	8.05E 4	7.05E 3	1.20E 5	1.46E 5	8.53E 4
		30.0	4.24E 3	1.74E 3	9.16E 2	1.65E 4	1.75E 4	1.46E 4
		50.0	1.51E 1	4.74E 1	5.11E 1	3.84E 2	3.51E 3	2.04E 3
		70.0	1.66E 1	3.19E 0	5.55E 0	2.50E 2	3.49E 2	1.58E 2
89	1.1	10.0	1.02E 5	1.14E 5	1.31E 5	1.01E 5	1.54E 5	1.03E 5
		30.0	5.62E 3	7.87E 3	1.51E 4	2.23E 4	4.93E 4	5.83E 4
		50.0	9.40E 3	6.55E 3	7.89E 3	3.93E 4	3.57E 4	1.61E 4
		70.0	2.47E 2	3.38E 3	1.44E 3	3.10E 3	5.32E 3	4.41E 3

[a] a.bc Ed = a.bc × 10^d.
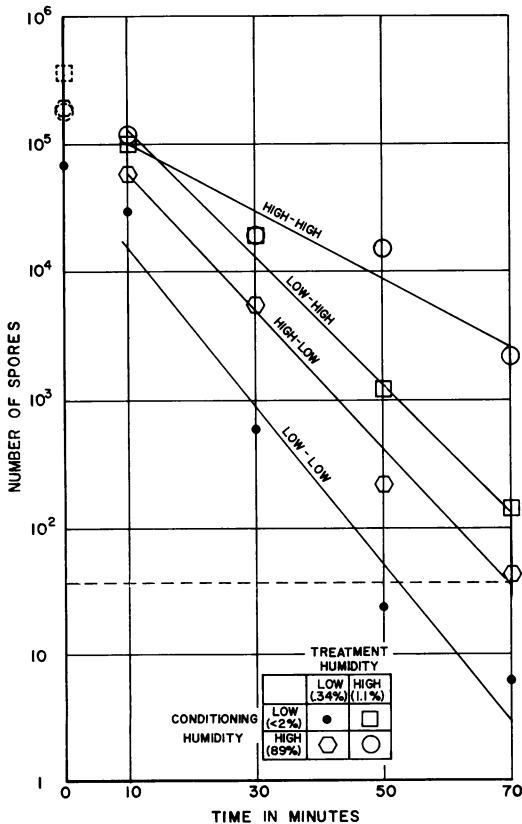
FIG. 3. *Means and regression lines. Each point is the geometric mean of six strips (three strips treated on each of 2 days). The points at t = 0, except N_0, represent extrapolation of the regression lines. Data points below the horizontal dotted line are based on mean recoveries of less than 30 colonies per strip.*

When examining Fig. 3 and Tables 2 and 3, note that the initial numbers were ignored in the data analysis because, as has been found by others (4, 13), the behavior of the thermal destruction curve between zero time and the time of removing the first treatment sample varied depending on the treatment.

Both conditioning and treatment humidity are shown by the analysis of variance to have statistically significant effects on spore survival. The *D*-value under low-low humidity conditions is less by a factor of 2.3 than the *D*-value under high-high conditions—16.1 min versus 37.6 min. In addition, under low-low conditions the survivor curve is shifted downward by 8%.

The data show that a reduction in either conditioning or treatment humidity from the high-high values will cause a considerable lowering of the *D*-value of *B. subtilis* spores on stainless-steel surfaces. The data suggest that, if objects to be dry-heat sterilized are conditioned in a low-humidity environment prior to treatment, the probability of achieving sterilization is increased and that further increase can be obtained by lowering the relative humidity of the treatment environment.

A comparison of the low-high and high-low curves shows the desirability of applying analysis of variance techniques to data of this type. Although the *D*-values and intercepts appear to be close together, the analysis shows otherwise. If it is hypothesized that there is no difference between the results of the low-high and high-low treatments, the probability of the data being separated by this amount or more is less than 0.025.

The data also show the importance of consider-

TABLE 2. D_{125}-values, y-intercepts (y_0) of the regression lines and their 0.95 confidence intervals, and the ratio log y_0/log N_0 [N_0 = 2.34E5 (1.74E5–3.15E5)]

Conditioning RH	Treatment RH	Day 1			Day 2			Pooled		
		D_{125} values	y_0	Log y_0/log N_0	D_{125} values	y_0	Log y_0/log N_0	D_{125} values	y_0	Log y_0/log N_0
Low <2%	Low 0.34%	13.7 (11.1, 18.1)	6.13E4 (9.79E3, 3.85E5)	0.89	19.3 (16.0, 24.3)	7.85E4 (2.56E4, 2.41E5)	0.91	16.1 (13.1, 20.1)	6.94E4 (1.62E4, 2.97E5)	0.90
Low <2%	High 1.1%	17.5 (14.2, 22.6)	4.83E5 (1.23E5, 1.90E6)	1.06	23.9 (21.9, 26.4)	3.06E5 (2.03E5, 4.60E5)	1.02	20.6 (18.5, 23.1)	3.78E5 (2.13E5, 6.69E5)	1.04
High 89%	Low 0.34%	15.8 (13.2, 19.8)	1.22E5 (3.20E4, 4.65E5)	0.95	22.0 (19.0, 26.2)	3.29E5 (1.53E5, 7.07E5)	1.03	18.4 (14.7, 24.5)	2.00E5 (4.79E4, 8.36E5)	0.99
High 89%	High 1.1%	32.5 (24.2, 49.8)	1.62E5 (5.28E4, 5.00E5)	0.97	44.5 (34.7, 62.1)	2.16E5 (1.10E5, 4.22E5)	0.99	37.6 (29.5, 51.9)	1.87E5 (8.65E4, 4.05E5)	0.98

TABLE 3. Analysis of variance

Source	SS[a]	df	MS	F
Mainplot				
Between subjects	185.9220	31	5.9975	
Days	12.6575	1	12.6575	30.48 ($P < 0.001$)
Conditioning RH	11.0480	1	11.0480	26.60 ($P < 0.001$)
Treatment RH	33.4900	1	33.4900	80.64 ($P < 0.001$)
C × T	0.2465	1	0.2465	0.59[b]
Treatment time	111.1031	3	37.0344	89.18 ($P < 0.001$)
Other interactions	11.1478	9	1.2386	2.98 ($P < 0.05$)
Mainplot residual	6.2291	15	0.4153	
Subplot				
Within subject	5.8389	64	0.0898	
Position	0.1632	2	0.0816	0.82[b]
Position × process	2.4000	30	0.0800	0.81[b]
Subplot residual	3.2757	32	0.0993	
Total	191.7607	95	2.0185	2.0185

[a] Abbreviations: SS, sum of squares; df, degrees of freedom; MS, mean square; F, frequency.
[b] $P > 0.1$, not significant.

ing the intercept as well as the slope of thermal death curves. The difference between the low-low and low-high D-values is rather small, but, when combined with the difference in intercept, there is a more than 10-fold difference in survival after 100 min of treatment. Variation was attributed to the day on which the experiment was carried out. The reason for this is not known at this time, but the problem is under study. Despite the day-to-day variation, it has been repeatedly confirmed for this and other experimental systems that within a given day the relationships between the data are preserved.

The humidity both before and during treatment affected the survival of *B. subtilis* var. *niger* spores in the experiments reported herein. The effects should be considered separately.

To describe the results of a thermal kill treat-

ment, both the slope (D-value) and the intercept must be specified. Small changes in treatment humidity caused rather large changes in the killing effectiveness of dry heat in the reported experiments. A combination of regression and analysis of variance approaches to data analysis increases the amount of information which can be obtained from a given experiment.

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