Dry Heat Inactivation of *Bacillus subtilis* var. *niger* Spores as a Function of Relative Humidity

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Dry heat sterilization of *Bacillus subtilis* var. *niger* spores at 105 C is enhanced in the relative humidity range 0.03 to 0.2%. D-values of 115 and 125 C are predicted by a kinetic model with parameters set from 105 C data. These predictions are compared to observations.

Dry heat is the principal process being considered for spacecraft sterilization by the National Aeronautics and Space Administration. It is known that, at a given temperature, D-value (time to sterilize 90% of a population) varies with air moisture content. However, there is a paucity of data available for setting spacecraft sterilization cycles as a function of relative humidity.

This is a report on dry heat sterilization of *Bacillus subtilis* var. *niger* spores at relative humidities (RH) from 0.0033 to 1.67% at oven temperature. 105 C D-values were determined for 20 RH values in this range. These values were used to set parameters in a kinetic model which incorporates relative humidity as an environmental parameter. The model is then used to predict D-values at 115 C and 125 C and compare them to experiments at these temperatures.

**MATERIALS AND METHODS**

**Humidity determination.** The standard formula for RH is (5):

\[
\text{RH} = \left( \frac{e}{e_{sat}} \right) \times 100
\]

where \(e\) is the pressure of water vapor present and \(e_{sat}\) is the pressure of saturated water vapor at the same temperature. Since, for air of a given moisture content, saturation temperature and dewpoint are the same, the RH inside an oven during dry heat sterilization \((\text{RH}_o)\) is given by

\[
\text{RH}_o = \left( \frac{e_o}{e_d} \right) \times 100
\]

where \(e_o\) is the saturated vapor pressure at dewpoint and \(e_d\) is the saturated vapor pressure at oven temperature. The saturated vapor pressures are found in handbook tables.

If the RH is known at a given temperature \((T)\), the relative humidity of the same air at oven temperature is given by

\[
\text{RH}_o = \frac{(\text{RH}_T e_T)}{e_o}
\]

where RH\(_T\) is the relative humidity at T and e\(_T\) is the pressure of saturated water vapor at T.

**Accuracy of RH determinations.** RH\(_o\) was determined either by use of equation 2 and direct dewpoint readings or by use of equation 3 and RH\(_T\) readings from LiCl sensor-strip chart recordings.

It was assumed that the direct dewpoint readings were accurate to ±0.5 C. If \(e_o\) is the saturated vapor pressure at dewpoint, \(e_o < e_d < e_{sat}\). For this reason we took the accuracy range for dewpoint RH\(_o\) determinations to be \(\left( \frac{e_d}{e_o} \right) \pm \frac{1}{2} \left( \frac{e_{sat} - e_o}{e_o} \right)\) × 100.

The LiCl sensor-strip recorder units were calibrated as a system by the Primary Standards Laboratory of Sandia Laboratories and gave % RH ± 1% at ambient temperature. The temperature at which these readings were taken was 26 C.

It can be seen from equation 3 that the error in RH\(_o\) which results from a 1% error in RH\(_T\) is the ratio \(\frac{e_{sat}}{e_o}\). The accuracy range for LiCl RH\(_o\) determinations were calculated as % RH\(_o\) ± \(\frac{e_{sat}}{e_o}\).

**Humidity control systems.** The humidity control used for a given experiment depended on the desired air moisture content. This determined the dewpoint for the oven input air.

The initial step in the humidity control system assured an excess air moisture content. The ambient RH at our location is frequently as low as 10%. Thus for dewpoints above −10 C the input air was first passed through fritted glass tubes submerged in deionized water at 26 C. For dewpoints below −10 C, the moisture content of the incoming air was adequate.

After assurance of a sufficient moisture content, the air was then either cooled to the desired dewpoint and the excess moisture was removed by condensation, or, if the dewpoint was below 2 C, the air was cooled to 2 C and excess moisture was removed by condensation under pressure. Condensation under pressure enabled us to attain dewpoints as low as −18 C. For dewpoints below −18 C, air having this dewpoint was passed through a desiccant bed having a bypass arrangement for dewpoint regulations.

For dewpoints above 2 C, control was maintained by regulation of condensation temperature. Condensation occurred in cooling coils submerged in a tem-
temperature bath controlled to ± 1 C.

For dewpoints in the range −18 to 2 C, dewpoint was controlled by regulation of the pressure under which condensation took place.

If condensation takes place under the pressure \( P_\text{a} \) absolute,

\[
\text{RH \%} = \frac{|(\epsilon P_\text{a}/P_\text{d})|/\epsilon_{\text{sat}}|} \times 100
\]

where \( P_\text{d} \) is ambient pressure absolute. Thus the dewpoints covered by this system configuration were easily controlled by pressure regulation. This pressurized air was expanded to ambient pressure prior to entry to the subsequent phase.

A dewpoint of −52 C was attainable by passage of −18 C dewpoint air through a desiccant bed. Control was achieved through a bypass arrangement which allowed the mixing of the desiccated and −18 C dewpoint air.

Air having the proper moisture content was warmed to 26 C, the RH determination was made either by dewpoint or LiCl sensor measurement, and the air was then fed into the oven. Air flow was metered at a level which maintained an oven overpressure of 0.03 ± 0.001 inch H₂O and provided a 1-min replacement of oven air.

The equipment configuration shown in Fig. 1 is for a test using input air with a dewpoint above −18 C. The desiccant bed, the only item not shown, was constructed from a sealable cylinder of approximately 3 ft³ volume. A 6-inch layer of the desiccant, CaSO₄, was supported at the center of the chamber by a porous mesh. Air entered at the bottom and exited at the top.

**Spores.** B. subtilis var. niger spores acquired from Fort Detrick were cleaned of vegetative material by multiple centrifugation and suspended in 95% ethanol at a concentration of approximately 10⁷ spores/ml. The suspension was stored at 4 C.

**Sample preparation.** The spore suspension was insonated for 2 min to distribute the spores uniformly. For each sample, 0.1 ml of the insonated spore suspension was pipetted onto the surface of an aluminum disc 1.25 inches in diameter. These discs were cut from 0.0015-inch biological grade aluminum foil. After the ethanol had evaporated, four of the inoculated discs were placed on an aluminum strip 1.5 by 7 by 0.020 inch. A single clean foil disc was placed over each inoculated disc, and the entire unit was covered by another aluminum strip. The assembly was held together by wire clamps. One such assembly was prepared for each sampling period.

The sample strips were then placed in an evacuated, 23-inch Hg vacuum, dessicator over CaSO₄ for approximately 16 hr before exposure to the dry heat environment.

**Exposure method.** The spores were exposed to the RH-controlled dry heat environment in the temperature chamber shown in Fig. 1. The strips were placed on a perforated metal cage within the chamber. The chamber door was modified by the addition of small plugs at its center. Thus, individual strip assemblies could be inserted or removed.

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**Fig. 1.** Humidity control assembly, temperature chamber, and strip recorders in operation.
quickly with negligible effect on temperature and RH.

The slight overpressure, 0.03 inch H2O, in the temperature chamber was necessary for the maintenance of RH stability. This overpressure prevented the diffusion of outside air into the temperature chamber.

**Recovery methods.** Each sample strip of four inoculated discs represented a single sampling period. After exposure, the spores were removed from the foil discs by 2-min insonation at an energy level of 11 watts/inch² in 10 ml of sterile 0.1% Tween 80-water. Tenfold serial dilutions were made as required and plated out in duplicate on Trypticase Soy Agar. Plate counts were made after 72 hr of incubation at 35 C.

All inoculation, assembly, and recovery operations were carried out in a class 100 vertical laminar airflow clean room.

**Data analysis.** Each experiment covered from four to six sampling periods. Data for each sample time came from eight plate counts for each of the serial dilutions. All data for a given experiment were input to a computer program which (i) computed the coefficient of variation for the eight plate counts for each dilution; (ii) selected from the data for a given sampling time that dilution which had the smallest coefficient of variation; (iii) computed a best linear least squares fit in the semilog plane to those data points from the dilution values with smallest coefficient of variation; (iv) computed the D-value from this least squares fit; (v) computed a 95% confidence interval about the D-value as follows: 0.95 Cl = D ± 1.96 * Error, where D is the D-value, E is the standard error in the estimate of the slope of the least squares linear fit, and S is the slope of that line; and (vi) computed the average coefficient of variation for the data used for the least squares fit.

The coefficient of variation provides a measure of the tightness of the data for a given dilution. The 95% confidence would be 0 if the standard error in the estimate of the slope were 0. This provides a measure of the linearity of the data and indicates the extremes of the D-value range for that experiment.

Since there are inherent errors in both the D-value and RH determination, the data were smoothed by taking a moving average of each three consecutive points in the D-value-RH plane, consecutive in the sense of increasing RH.

**RESULTS**

The test series consisted of 27 experiments. Twenty-two were carried out over an RH range of 0.0033 to 1.6% at a sterilization temperature of 105 C. Twenty of these experiments were for determining 105 C D-values as a function of RH and two were designed for checking possible diffusion effects resulting from our use of covered discs.

The environmental conditions, D-values, average coefficients of variation, and 95% confidence intervals on the D-values for the 20 D-value determination experiments are shown in Table 1. Figure 2 shows the D-values and 95% confidence intervals as a function of RH at sterilizing temperature. The moving averages discussed above are also shown in Fig. 2.

Figure 3 shows a comparison of survivors from covered foil disc assemblies and open planchetts. The solid line is the least squares fit to the covered disc data, and the dotted line is that for the planchetts. The simultaneous exposure to 105 C of the covered discs and planchetts was at an RH of 0.0033 ± 0.00014%.

To set sterilization parameters, it was necessary to have D-values for a fixed RH at two temperatures. D-values other than 105 C were desired for predictive checks. To this end, three experiments were carried out at 125 C and two at 115 C. The results of these experiments are shown in Table 2.

**Kinetic analysis.** A model for dry heat sterilization which gives D-value as a function of temperature and relative humidity is useful for spacecraft sterilization applications. It was suggested in (1) that if D ≡ const were constant over the RH range of interest, D-value could be modeled by expressing ΔS* as a function of RH. Here ΔH* and ΔS* are the thermodynamic parameters.

**Table 1. Statistical analysis of 105 C D-value: relative humidity (RH) experiments and error analysis of RH determinations.**

| Date     | RH at 105 C (%) | D-value (hr) | Avg coefficient of variation |
|----------|----------------|--------------|-----------------------------|
|          | Calculated     | Error ±      | Mean                        |
| 0.95 CI  | α*             |              |                             |
| 12/29/70 | 0.0033         | 0.00014      | 1.40                        |
| 1/17/71  | 0.00473        | 0.00028      | 1.55                        |
| 12/23/70 | 0.0107         | 0.00065      | 1.35                        |
| 12/22/70 | 0.0166         | 0.0010       | 1.41                        |
| 9/15/70  | 0.026          | 0.0025       | 0.99                        |
| 9/11/70  | 0.050          | 0.0035       | 1.06                        |
| 9/15/70  | 0.071          | 0.0035       | 1.10                        |
| 8/11/70  | 0.090          | 0.027        | 0.99                        |
| 9/19/70  | 0.125          | 0.0059       | 1.11                        |
| 9/9/70   | 0.140          | 0.0275       | 1.30                        |
| 7/31/70  | 0.180          | 0.0275       | 1.39                        |
| 8/5/70   | 0.283          | 0.0275       | 1.61                        |
| 8/4/70   | 0.286          | 0.0275       | 1.48                        |
| 7/28/70  | 0.31           | 0.0275       | 1.53                        |
| 9/17/70  | 0.50           | 0.065        | 1.82                        |
| 7/13/70  | 0.64           | 0.0275       | 2.40                        |
| 7/14/70  | 0.76           | 0.0275       | 2.50                        |
| 7/15/70  | 0.98           | 0.0275       | 2.50                        |
| 7/16/70  | 1.2            | 0.0275       | 2.93                        |
| 7/21/70  | 1.67           | 0.0275       | 2.50                        |

* Confidence interval.
namic parameters of Eyring kinetics in which reaction rate \( r \) is expressed by (2)

\[
r = (kT/h) \exp (-\Delta F^*/RT)
\]

where \( \Delta H^* \) is the enthalpy of activation and \( \Delta S^* \) is the entropy of activation.

The assumption that sterilization can be expressed in terms of D-values is equivalent to the assumption that sterilization is logarithmic. Analogously, first-order kinetics is logarithmic. We will assume that both temperature and RH can be modeled as environmental sterilization parameters by first-order kinetics.

The relationship between D-value and the reaction rate constant of equation 5 is given by

\[
r = (\log e 10)/D
\]

where \( D \) is D-value. From equations 5 and 6 we see that

\[
T\Delta S^* - \Delta H^* = RT \log e (r_1h/kT_1)
\]

Thus, given \( D_i \) and \( D_j \) at temperatures \( T_i \) and \( T_j \) for a fixed RH, equation 7 can be used to find reaction rate constants \( r_1 \) and \( r_2 \). Then from equation 8, one can solve the two linear equations in two unknowns.

\[
\frac{T_i\Delta S^* - \Delta H^*}{T_j\Delta S^* - \Delta H^*} = \frac{\log e r_1}{\log e r_2 + \log e T_j/T_i}.
\]

The sensitivity of a computed \( \Delta H^* \) to observed \( D \) is indicated by the fact that if one takes the extreme 0.95 confidence interval D-values for 105 and 125 C at about 0.25% RH, \( \Delta H^* \) is computed by equation 9 to be between 22.5 and 29.4 kcal/mole. However, for a given D-value, the right side of equation 8 is constant so that if \( \Delta H^*_i, i = 1, 2 \), are two \( \Delta H^* \) values, the relationship between \( \Delta S^*_i \) and \( \Delta S^*_j \) found from equation 8 by these \( \Delta H^* \) values is

\[
\Delta S^*_i = \frac{T_i}{T_j} \Delta S^*_j
\]

where \( \Delta S^*_j \) is computed by equation 9.

**Table 2. Statistical analysis of 115, 125 C D-value: relative humidity (RH) experiments and error analysis of RH determinations**

| Date      | Temp (C) | RH at temp | D-value (hr) | Avg coefficient of variation |
|-----------|----------|------------|--------------|-----------------------------|
| 2/26/71   | 125      | 0.40       | 0.34         | 0.90                        | 0.38 |
| 3/26/71   | 125      | 0.26       | 0.25         | 0.035                       | 0.56 |
| 4/6/71    | 125      | 0.645      | 0.33         | 0.04                       | 0.38 |
| 1/4/71    | 115      | 0.269      | 0.69         | 0.087                       | 0.20 |
| 3/3/71    | 115      | 0.70       | 0.99         | 0.03                        | 0.20 |

*Confidence interval.
\[ \Delta S^*_t = \Delta S^*_1 + \left[ \left( \Delta H^*_t - \Delta H^*_1 \right) / T \right]. \] (10)

If one assumes \( \Delta H^* \) constant over the RH range of interest and computes \( \Delta S^*_t \) as a function of RH by using the moving average D-values and RH values of Fig. 2, then \( \Delta S^*_t \) can be altered for other \( \Delta H^* \) assumptions by use of equation 10. Table 3 shows the moving averages of Fig. 2 and \( \Delta S^*_t \) as a function of RH under the assumption that \( \Delta H^* = 25 \) kcal/mole. Figure 4 shows these \( \Delta S^*_t \) values as a function of RH.

Finally, the utility of a modeling technique depends on its predictive qualities. Figure 5 shows D-values with 95% confidence intervals for the 115° and 125°C sterilization experiments given in Table 2. The mean D-values are indicated by dots and the confidence intervals by double arrows. The horizontal bars indicate the predicted D-values for those RH values by using \( \Delta H^* = 25 \) kcal/mole, \( \Delta S^*_t \) from Fig. 4 and the D-value from equations 6, 5 and 7, i.e., \( \Delta F^* \) is found from equation 6, \( r \) from equation 5 and from 7: \( D = [\log_{10} q] / r \).

**DISCUSSION**

The technique of holding \( \Delta H^* \) constant and expressing \( \Delta S^*_t \) as a function of RH appears to be a useful supplement to the kinetic modeling of dry heat sterilization. This allows for the inclusion of both RH and temperature as envi-

| Moving avg | \( \Delta S^*_t \) in e. u. given that \( \Delta H^* = 25 \) kcal/mole | D-value in hours |
|------------|-------------------------------------------------|-----------------|
| RH         | \( \Delta S^*_t \)                           | D-value         |
| 0.0062     | 1.429                                           | -8.198          |
| 0.0106     | 1.433                                           | -8.204          |
| 0.0177     | 1.248                                           | -7.930          |
| 0.0309     | 1.153                                           | -7.773          |
| 0.0489     | 1.051                                           | -7.587          |
| 0.0702     | 1.050                                           | -7.587          |
| 0.0952     | 1.066                                           | -7.617          |
| 0.118      | 1.132                                           | -7.735          |
| 0.148      | 1.263                                           | -7.954          |
| 0.201      | 1.430                                           | -8.200          |
| 0.250      | 1.491                                           | -8.282          |
| 0.293      | 1.540                                           | -8.347          |
| 0.365      | 1.611                                           | -8.437          |
| 0.483      | 1.920                                           | -8.785          |
| 0.633      | 2.242                                           | -8.890          |
| 0.793      | 2.467                                           | -9.284          |
| 0.98       | 2.645                                           | -9.422          |
| 1.28       | 2.645                                           | -9.422          |

**FIG. 4.** \( \Delta S^*_t \) as a function of relative humidity under the assumption that \( \Delta H^* = 25 \) kcal/mole.

**FIG. 5.** D-values for 115° and 125°C as a function of RH at oven temperature. The dots indicate mean D-values, and the double arrows indicate 95% confidence intervals. The horizontal bars represent predictions from the kinetic model.
Environmental parameters. Equation 3 shows how one can convert the RH scale of Fig. 4 to a temperature other than 105 °C and equation 10 shows how to construct a $\Delta S^\ddagger$ curve for a different $\Delta H^\ddagger$ base.

Considering the number of experiments and lengths of confidence intervals, the drop in D-value through the oven temperature RH range of 0.02 to 0.2% must be real. An analysis which predicted this drop was presented in (1).

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