APPLIED MICROBIOLOGY, Dec. 1968, p. 1841-1846
Vol. 16, No. 12

Dry Heat Resistance of Spores of *Bacillus subtilis* var. *niger* on Kapton and Teflon Film at High Temperatures

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Received for publication 2 October 1968

To determine parameters that would assure sterility of a sealed seam of film for application in "split-seam entry," spores of *Bacillus subtilis* var. *niger* were sprayed onto pieces of Kapton and Teflon film. Short-time, high-temperature (200 to 270 C) exposures were made with film pieces between aluminum blocks in a hot-air oven, and the *D* and *z* values were determined after subculture of surviving spores. The use of Kapton film allowed the study of high temperatures, since it is not heat sealable and could be used to make thin packages for heat treatment. Spores on Teflon were dry-heat treated in a package designed to simulate an actual seam to be sealed. The *z* values of 29.1 C (52.4 F) for spores on Kapton and 139 C (250.4 F) for spores on Teflon were calculated.

Relatively little attention has been given to the definition of high-temperature—(short-time parameters in the thermal destruction of microorganisms.

With the development of techniques for gnotobiology, means of entry or the transfer of sterile materials without violation of the sterility of the flexible film chamber housing the gnotobiotic animals has resulted. This technique is known as "split-seam" entry (10, 17, 18). With this technique, a sterile sealing tool (hot wire, etc.) and the sterile items to be transferred are placed in an internally sterile plastic bag. This bag is sealed onto a flexible film isolator, and its contents can be inserted into the isolator or film system without violation of sterility. The interior of the passageway formed by cutting or splitting of the sealed seam could contain some film not previously sterilized. Since a minute portion of the seam will be exposed to the interior surface of the system being entered, the time-temperature parameters involved in producing a physically acceptable seam must also kill all microorganisms trapped therein.

This method of entry or insertion has numerous applications; among these are uses in gnotobiology, sterile fill operations, and sterile assembly of spacecraft parts. Of the numerous films available, one which has acceptable properties and could be used in the assembly of spacecraft parts is Teflon FEP film. (Teflon is a proprietary product of the E. I. Dupont De Nemours Co., Inc., Wilmington, Del.)

The existing literature contains only a few references to dry-heat resistance at elevated temperatures or in the sealing temperature range of Teflon film, 500 to 536 F (260 to 280 C). Currently, estimates of thermal kill cycles at the higher temperatures are determined by extrapolation from the existing thermal death time data. Wang et al. (19) in experiments with moist heat showed that this type of extrapolation can be in serious error. They showed a nearly 160% disagreement between experimental results and those found from extrapolation of the thermal death time curve over a range of 20 C to the highest temperature tested (295 F or 143 C).

Some early study in thermal resistance at high temperatures has been reported by Bourdillon et al. (2) in the sterilization of air with different types of furnaces. They concluded that a temperature of 482 to 572 F (250 to 300 C) was required for complete air sterilization. Decker et al. (5), also testing an air sterilizer, worked in a temperature range which more nearly coincides with the temperature range of interest in this investigation. These experiments with spores of *Bacillus subtilis* var. *niger* were designed to test the performance and biological reliability of a commercial electric-grid, hot-air sterilizer. Their results were reported in terms of per cent reduction of the original population and also the retention time at a specified temperature in the range 425 to 625 F. The time for a 6-log reduction was 24 sec at 425 F (218 C) and 3 sec at 575 F (302 C).

Again, in an attempt to determine some pa-

rameters for hot-air sterilization of laboratory material, Francis (6) heated a dried-spore inoculum of 4×10^3 organisms from hay dust in aluminum weighing dishes heated with aluminum blocks and obtained sterility at 428 F (220 C) in 10 sec.

A recent investigation of the efficiency of a hot-air sterilizer was pursued by Quesnel et al. (14) at 392 F (200 C). At this temperature and with an inoculum of mixed species of *B. subtilis* as an air-dried film, they achieved sterilization of 20 stainless-steel strips weighing 4 g each in an overall cycle of 20 min, whereas six sputum cups with a total weight of 2 kg required a cycle of 38 min. Glass jars of equivalent weight to the steel sputum mugs required a longer cycle in the sterilizer. Their interpretation of the data related the surface to mass ratio of the materials carrying the inoculum to the time to sterilize at this temperature.

MATERIALS AND METHODS

Test organism. The organism used in this study was *B. subtilis* var. *niger* (Detrick strain). The culture was obtained from Charles R. Phillips of the U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.

Sporulation medium. The sporulation medium was prepared from commercial dehydrated material. In this case, the medium was Nutrient Agar (BBL), prepared with tap water with 10 μg of $MnSO_4$ and 80 μg of $CaCl_2$ per liter added. The inoculum buildup medium was the same, without agar added.

Subculture medium (spore viability medium). Dehydrated Trypticase Soy Agar (BBL) was used to determine the viability of heat-treated spores. Medium was dispensed in 25-ml portions into disposable glass test tubes (50 by 150 mm) and covered with polypropylene drop caps. Wide-mouth tubes were used to accommodate the pieces of film. All tubes of medium were incubated for 48 hr at 32 C prior to use, and any positive tubes were discarded.

Assay medium. Trypticase Soy Agar was used in the assay procedure. Sterile, distilled water with 0.05% Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.) was used as a wash solution in the assay of untreated, sprayed pieces of film.

Preparation of spores. A stock culture slant which was the first passage from the original stock culture was washed with 5 ml of sterile distilled water. The suspension was decanted into a sterile, screw-cap tube and heated at 65 C for 15 min to kill any vegetative cells. A 1-ml amount of this suspension was used to inoculate each 500-ml shake flask containing 50 ml of inoculum buildup medium. The flasks were placed on an incubated shaker at 32 C for 16 to 18 hr. A 2-ml amount of this culture was inoculated into 1-liter Erlenmeyer flasks containing 200 ml of inoculum buildup medium and incubated in a shaker at 32 C for 12 to 14 hr. The inoculum was checked for vegetative growth under phase microscopy before use. Glass petri dishes (150 by 25 mm) were prepared with 100 ml each of solidified sporulation agar and preincubated for 24 hr at 32 C. A 2-ml amount of inoculum was spread onto the agar surface of each plate with glass spreaders, and the plates were incubated at 32 C. Sporulation was checked frequently under phase microscopy. Maximal sporulation (99%) was at 48 hr. Plates were refrigerated overnight and then harvested. Each plate was rinsed twice with approximately 10 ml of cold, sterile, distilled water while the plate was revolved on a turntable and the surface was gently agitated with a glass spreader to facilitate the removal of the growth. The accumulated suspension was centrifuged in a refrigerated centrifuge at $10,400 \times g$ for 20 min and decanted. The spores were washed five times in sterile, distilled water, and, after the final wash, the spores were suspended in absolute ethyl alcohol. All procedures of transfer, inoculation, and harvesting in the preparation of the spore suspension were performed under laminar flow conditions.

Preparation of sprayed test pieces. Two types of film were used in these studies, Kapton and Teflon. Teflon was selected as a representative film on which to study time-temperature parameters for heat sealing, utilizing a gauge which would have practical application, whereas the Kapton was selected because of its high electrostatic charge and was chosen more to look at the heat resistance of the spores at heat-sealing temperatures on a film which would withstand the sealing temperatures of Teflon without sealing. Kapton (Dupont registered trademark, E. I. Dupont de Nemours & Co., Inc., Wilmington, Del.) is a polyamide film which maintains its physical, electrical, and mechanical properties over a wide temperature range (from -269 to 400 C). Kapton chars above 800 C, is not heat sealable, and is not soluble in any known organic solvent. Kapton type H film of 1 mil thickness (0.001 inch) was used. At room temperature Kapton and Mylar, a polyester film, are similar. As the temperature varies, the properties of Kapton are less affected than Mylar. Mylar and Kapton are frequently used materials in the space effort. Teflon FEP is a fluorocarbon film. It is a transparent, thermoplastic film which can be heat sealed, thermoformed, laminated, or metalized. It is virtually inert to chemicals and solvents. It can be used continuously at 200 C and has good resistance to impact and tearing. Teflon is widely used in the electronics and electrical industries. Five mil (0.005 inch), type A Teflon was used. The heat-sealing range for this film is 260 to 280 C.

Large sheets of sterile film of either type were suspended in a clothesline fashion. The receptacle bottle of a chromatography sprayer was filled with spore suspension in ethyl alcohol. Sheets of film were sprayed at a distance of approximately 3 feet. The spore film dried instantly. Kapton was sprayed on both sides and Teflon on one. The level of inoculation was 10^6 on each 1-inch (2.54 cm) square.

Assay of nonheated, sprayed film. Squares (1 inch) of film were used for assay. Because of difficulty in removing spores from the Kapton for assay, the pieces of Kapton film were soaked overnight in the wash solution before assay. For both Kapton and Teflon, a square of sprayed film was placed in a screw-cap, polypropylene bottle with 50 ml of wash solution and

exposed to ultrasonic treatment at 35,000 kc for 30 min. The wash solution was routinely, serially diluted and plated with Trypticase Soy Agar and incubated for 48 hr at 32 C. Each piece of film was washed twice, and the counts from each wash were added. Initially each piece of film was also plated to detect any spores remaining on the surface, but this was discontinued since nothing significant remained.

Preparation of inoculated film packets. The Kapton packet (Fig. 1) contained 25 squares (1 inch) of Kapton film spray-inoculated on both sides. These squares were evenly spaced and maintained between two 8 by 8 inch sheets (20.3 by 20.3 cm) of Kapton. This 8 by 8 inch lamination was then placed between two 10 by 12 inch (25.4 by 30.5 cm) sheets of Kapton, and the edge was taped. The total thickness of the Kapton packet was 5 mil (0.005 inch). The high, natural electrostatic charge of Kapton film effectively held the inoculated film squares firmly in place. The packets containing the inoculated Teflon were similar (Fig. 2). They consisted of two layers of 5-mil Teflon with the inoculated sides facing together (simulating a seam to be sealed). The inoculated sheets of Teflon were heat sealed on one edge and cut to the seal, forming five 1-inch fingers of film. A Kapton separator (1 mil) was placed between the inoculated surfaces so that the heat treatment would not fuse the contaminated surfaces together. Fusion would have occluded the test organisms and prevented recovery of survivors.

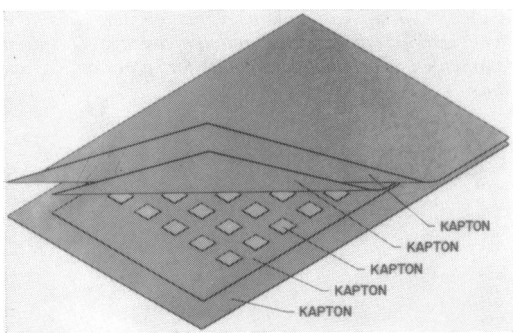

FIG. 1. *Diagrammatic representation showing the method of preparation of the Kapton test pieces.*

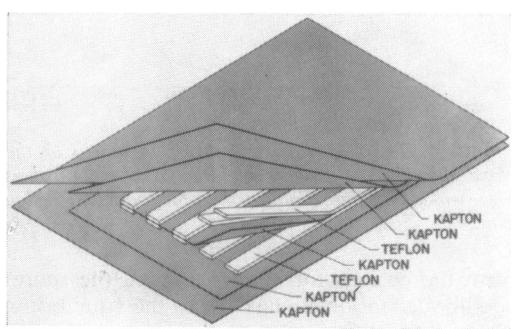

FIG. 2. *Diagrammatic representation showing the method of preparation of the Teflon test pieces.*

Dry-heat treatment of packets. The Kapton and Teflon packets were heated by placing them between two aluminum blocks. The blocks [8 inches (20.3 cm) wide by 12 inches (30.4 cm) long by 1 inch (2.5 cm) deep] were hinged on one end so that they could be rapidly opened and closed. The packet containing the inoculated film was pressed firmly between the blocks for the time interval of the heat treatment. The packets were cooled instantaneously by placing them between an identical set of cold (0 C) aluminum blocks. The heated blocks were maintained at the desired temperature by holding them in a forced-draft, hot-air oven (Hotpack Corp., Philadelphia, Pa.). The temperature of the blocks was continuously monitored by copper-constantan thermocouples attached to the blocks and to a multipoint recorder (Minneapolis-Honeywell, Philadelphia, Pa.). Each heat treatment was timed from the closing to the opening of the heated blocks. The come-up time for the Kapton packets was less than 1 sec, whereas the come-up for the Teflon packet due to its thickness was approximately 3 sec.

Subculture of heat-treated squares of film. Samples (100) were heated for each time-temperature designation. All subculturing techniques were performed in a vertical laminar flow room [20 feet (616 cm) wide by 20 feet (616 cm) long by 10 feet (308 cm) high] with personnel gowned in sterile garments completely covering their bodies including a face mask for the nose. All materials were wiped with disinfectant before being admitted to the clean room. Each heat-treated piece of film was transferred with sterile forceps (using a new pair for each transfer) into the tubes containing the subculture medium and incubated at 32 C for 3 weeks.

Analysis of data. D values (in this case, seconds required at a given heating temperature to reduce the spore population 10-fold) were calculated by the method of Stumbo as described by Schmidt (15) and are given by the formula: $D = (U \text{ or } t)/(\log A - \log B)$, where A is the total number of samples heated multiplied by the number of spores per sample; B is calculated assuming one surviving spore per container when less than the total number of containers showed survival; U or t is exposure time at a given temperature. In addition, the calculations were also done by the method of Stumbo, Murphy, and Cochrane (16) as described by Pflug and Schmidt (13) which used basically the same formula described above; but the number of survivors described by B is calculated by the most probable number of Halvorson and Ziegler (8) by using the formula $U/(N_o - N_u)$. The z value was determined from the plot of the phantom thermal death curve or thermal resistance curve and from the regression coefficient derived from the statistical analysis. The z value can be defined as the number of degrees fahrenheit for the death time curve to pass over 1-log cycle. The temperature coefficient for the change in rate of destruction with temperature can also be expressed by the Q_{10} value with the relationship $z = 18/\log Q_{10}$ for degrees F and $z = (0.555 \times 18)/\log Q_{10}$ for degrees C. F value (F_o equals sterilization

time in minutes with respect to a given organism and has been traditionally given at 250 F) can be interconverted from D values by using the following relationship: $F = D (\log A + 2)$. The value of $\log A$ is from Stumbo's equation. The initial population then is reduced to 1 during an exposure to heat at a given temperature for the time period of $D \times$ number of logs of the initial population; after which the area of probability of a survivor is entered. Activation energy can be calculated from the simplified Arrhenius equation shown by Schmidt (15) as follows:

$$E = 4.58 (T_2 \times T_1)/(T_2 - T_1) \times k_2/k_1$$

where T is absolute temperature, k is the reaction rate constant and was calculated from D by $k = 2.3/D$, and 4.58 is a constant. Pflug and Schmidt (13) point out that calculations of activation energies perhaps have more applicability with dry heat than with wet heat.

Statistical analysis. A regression analysis of the data and the least-squares regression line were calculated by using a program on an IBM 360 computer.

RESULTS AND DISCUSSION

Table 1 presents the D values obtained for *B. subtilis* var. *niger* spores heated on both Kapton and Teflon film at various temperatures. The regression lines indicating the z values for spores on both films are shown in Fig. 3–4.

Much recent study (1, 9) on dry heat and particularly that of Murrell and Scott (11) have shown that the water activity, a_w (equilibrium relative humidity), of the spores themselves and of the environment in which the spores are heated affects the heat resistance. In our investigation, the spores were sprayed onto the films from an ethyl alcohol suspension and were essentially dry. It is probable, however, that in the time required to form the packets for heat treatment, the spores equilibrated to something close to the equilibrium relative humidity of the environment. However, as has been shown by Gilbert et al. (7), spores equilibrated after desiccation will not equilibrate

TABLE 1. *Comparison of D values with data in the literature*[a]

Temp F (temp C)	D value in seconds		
	Teflon	Kapton	Decker et al.
392 (200)		2.15	
410 (210)	2	0.99	
419 (215)		0.54	
425 (218.3)			3.4
428 (220)		0.46	
464 (240)	1.18		
475 (246.1)			1.4
482 (250)	0.99		
500 (260)	0.89		
518 (270)	0.71		
525 (273.9)			0.7
575 (301.6)			0.4

[a] Average D, end point calculation.

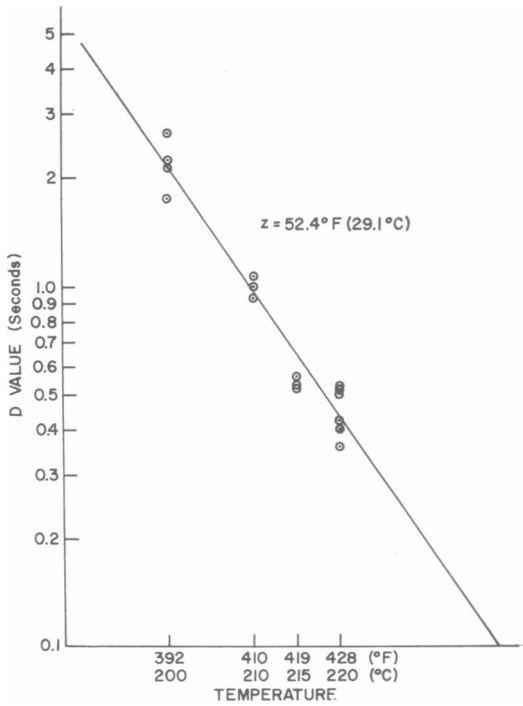

FIG. 3. *The least-square regression line with the slope showing the z value and the average D values represented as original data points for spores on Kapton film.*

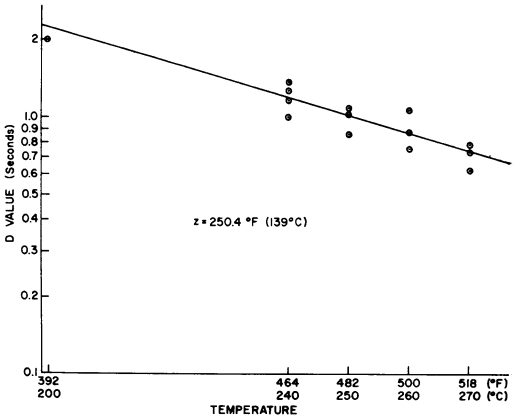

FIG. 4. *The least-squares regression line with the slope showing the z value and the average D values represented as original data points for spores on Teflon film.*

the same final a_w as spores that have never been desiccated. This results in a final, equilibrated a_w which is lower than for spores never desiccated. Our investigation was carried out essentially so that the results could be practically applied in the heat sealing of Teflon film. In actual experience, the spores on a given piece of film would have an undetermined water content but would probably be in a relatively desiccated state. The environment in which these packets were prepared was at approximately 50% relative humidity. The equilibrated a_w of spores showing the greatest heat resistance is in the range 0.2 to 0.6. Considering the fact that the spores were previously desiccated, it can be assumed that they were probably not at an a_w range of minimal heat resistance.

Partial explanation of the differences in z and D values of the spores heated on the two types of film can be found in the examination of the packet and the permeability of the films to water vapor. The thinner Kapton packet (total of 5 mil) is made from film permeable to water vapor [5.4 g/(100 inches^2) (24 hr)/mil] and has measurable moisture absorption capacity (1.3 at 50% relative humidity at 23.5 C). The water vapor in the spore and in the environment can be easily lost during heating, thus heating spores which are dry and which should show lower heat resistance. On the other hand, the Teflon packet is thicker (total of 15 mil) and contains a piece of Kapton inserted between two pieces of Teflon. Teflon is impermeable to water vapor, and thus the water vapor content of the spores and that released from the Kapton film are retained in the immediate environment of the spore. The spores are then heated at an intermediate a_w giving higher heat resistance. This factor of penetration of water vapor combined with the longer come-up time for the thicker packet and consideration of the surface area to mass ratio for the Teflon packets may provide some reasons for the divergence in z values.

The come-up time for the Kapton film package was less than 1 sec, so that the calculated z probably represents a more accurate value for the spores than the thicker Teflon package with its longer come-up time. The Kapton system was chosen so that spore destruction at elevated temperatures could be studied without the film sealing. The thinner Kapton packet (total 5 mil) also offered a spore-carrier system with less mass.

The D values reported show a general agreement with the data of Decker et al. (5) with which they are compared in Table 1. Analysis of the data of Decker et al. (5) showed a straight-line Arrhenius plot. Arrhenius plots with our data were drawn and also produced straight lines. The results of calculations of activation energy are given in Table 2 and are compared to the data of Decker et al. (5) and some other values in the literature. The lower activation energies obtained at these high temperatures compared with values obtained for dry-heat resistance at lower temperatures (3) might indicate some different lethal reaction. Calculated activation energy from the investigation of Decker et al. (5) associated with the death reaction would be approximately 12,600 cal/g-mole.

TABLE 2. *Activation energies and Q_{10} values compared with values in the literature*

Source	Activation energy (cal/g-mole)	Q_{10} value
Kapton (this study)	35,500	2.1
Teflon (this study)	9,500	1.2
Wax[a] dry heat spores of *B. subtilis*	40,200	
C-N bond energy[a]	49,000	
Decker et al. (5), spores of *B. subtilis*	12,600	

[a] R. G. Wax, Ph.D. Thesis, Pennsylvania State University, 1963.

As has been pointed out by Quesnel et al. (14), the ratio of surface area to mass could be a significant factor in dry-heat sterilization. Since the value for specific heat and the coefficient of thermal conductivity are essentially the same for Kapton and Teflon, the difference in z and D values between the two films may reflect the greater surface area to mass ratio with the Teflon packets.

It would seem that regardless of the explanations given, the common practice of extrapolating the z curve to higher temperatures cannot be defended. The lower z values for dry heat (30 to 40 F range; 1, 4, 12) obtained at lower temperatures (135 C) would show considerable error if arbitrarily extrapolated to the 200 to 275 C range.

In the application of the data derived from this study to actual heat-sealing practice, it is necessary to determine the time required at a given sealing temperature (using average D values) with a hypothetical load plus a safety factor which is the probability of one viable spore in any given number.

A total F calculation (heat-sealing time at given temperature to assure sterilization) from the data presented would indicate a time for F_{250C} = 7.9 sec, F_{260C} = 7.1 sec, F_{270C} = 5.68 sec, assuming a maximal load on the film of 10^3 spores plus a probability of one viable

spore in 10^3. Our investigation has shown that a normal load on unsterilized Teflon is about 10 to 20 organisms per square inch.

ACKNOWLEDGMENTS

The valuable technical assistance of Jackie Anderson, Gerard Glaser, Laurie Hodges, Herman Hunt, and Wiley Martin is gratefully acknowledged.

This investigation was supported under NASA contract NAS 5-9245.

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