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OBSERVATIONS ON THE THERMAL RESISTANCE OF PUTREFACTIVE ANAEROBE NO. 3679 SPORES IN THE TEMPERATURE RANGE OF 250-300°F.[a]

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The study of the thermal resistance of Putrefactive Anaerobe (P.A.) 3679 reported by Pflug and Esselen *(3)* has been continued to obtain additional data on the behavior of the organism when exposed to moist heat at high temperatures. In the present investigation, consideration has been given to the relationship between spore concentration and D value.[b] An attempt was made to ascertain the reason for the apparent increase in calculated D values with heating times at a given temperature, as has been frequently observed. The effect of the number of samples run at each time and temperature interval on the ultimate results has been assessed on a basis of experimental data and statistical probability.

EXPERIMENTAL AND DISCUSSION

The method of growing the spores, preparation and counting of the spore suspension, apparatus technique, subculture media, and incubation conditions are the same as those described in Pflug and Esselen *(3)*.

A thermal death time curve of 1D for P. A. 3679 suspended in M/15 neutral phosphate buffer, 10,000 spores per 0.01 ml., 0.01 ml. per cup is illustrated in Figure 1. This curve is based on calculated D values as presented by Pflug and Esselen *(3)*. The upper and lower limits of the curve of Figure 1 are for the one per cent level with 92 degrees of freedom. As will be discussed below, the wide range of D values in Figure 1 appear to be due in large part to the tendency for the calculated D values at any particular temperature to increase with heating time (U). This trend has been observed and reported by Reynolds and Lichtenstein *(4)*, and others.

The increase in D value with heating time (U) appeared to be more pronounced when increased numbers of samples were run at a given time interval. To study this effect and at the same time the effect of spore concentration, tests were made at 280°F. using 48 samples at each time-temperature interval with spore concentrations of 120, 10,000 and 1,000,000 per 0.01 ml. The spores were suspended in M/15 neutral phosphate buffer 0.01 ml. per cup and subcultured in liver broth. The resulting data are presented in Table 1.

The data were treated according to the method of Stumbo, Murphy, and Cochran *(6)* as follows:

$$D = \frac{U}{\log a - \log b}$$

U = time of heating (min.)
a = initial number of spores per sample multiplied by the number of replicate samples.
b = probable number of spores surviving at the end of heating time U.
n = number of replicate samples.
p = number of samples showing growth.
q = number of sterile samples as evidenced by lack of growth (1-p).

[a] Contribution No. 902, Massachusetts Agricultural Experiment Station.
[b] D value is the time in minutes required to reduce the number of organisms by 90%.

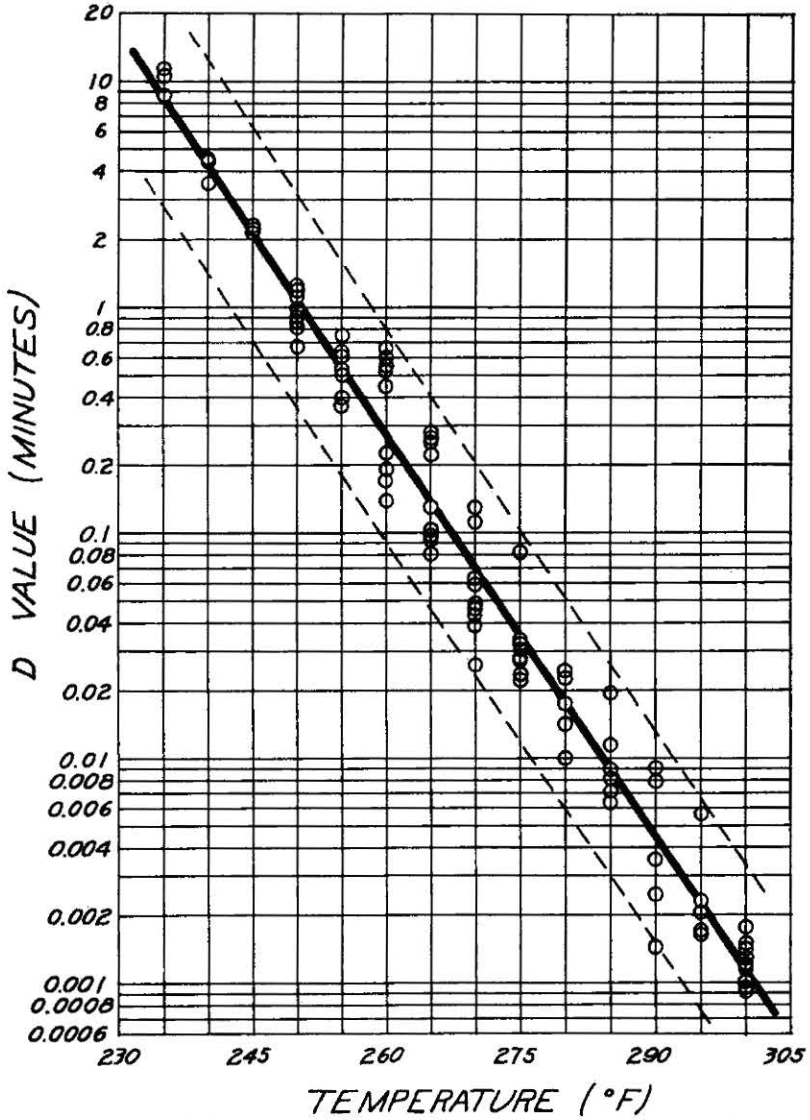

Figure 1. Thermal death time curve for spores of P. A. 3679 suspended in neutral M/15 phosphate buffer with limits for the 1% level with 92 degrees of freedom.

The value of b was obtained by applying the equation of Halvorson and Ziegler *(2)*

$$b = n \ln \frac{n}{q}$$

to data obtained from (n) samples subjected to one time-temperature relationship (U) that sterilized only a portion of (q) of the total number of samples. The D values obtained for the 3 different spore concentrations are in general agreement. It can be observed from Table 1 that the calculated D values increase as the time of heating increases.

The D value is the time in minutes required for the destruction rate curve to traverse one log cycle; the method of calculating D values by the formula

$$D = \frac{U}{\log a - \log b}$$

TABLE 1

Thermal destruction data for P. A. 3679 spores in concentrations of 120, 10,000, 1,000,000 per 0.01 ml. in neutral phosphate buffer at 280°F.

120 Spores per 0.01 ml.				10,000 Spores per 0.01 ml.				1,000,000 Spores per 0.01 ml.			
Time in minutes	Number of tubes +	b	D minutes	Time in minutes	Number of tubes +	b	D minutes	Time in minutes	Number of tubes +	b	D minutes
0.0200	48			0.0690	47	186	0.0202	0.0600	48		
0.0244	48			0.0740	46	152	0.0212	0.0730	48		
0.0282	48			0.0780	46	152	0.0223	0.0880	48		
0.0321	48			0.0784	47	186	0.0230	0.0990	48		
0.0350	47	186	0.0235	0.0900	45	133	0.0253	0.1120	47	186	0.0207
0.0402	47	186	0.0269	0.0900	42	99.8	0.0244	0.1250	47	186	0.0231
0.0440	42	100	0.0250	0.0940	43	109	0.0258	0.1380	44	119	0.0246
0.0481	41	92.4	0.0268	0.0950	28	42.1	0.0234	0.1520	20	25.9	0.0242
0.0520	45	133	0.0318	0.1000	36	66.5	0.0259	0.1640	18	22.5	0.0269
0.0562	48			0.1020	18	22.5	0.0236	0.1770	0		
0.0599	3	3.09	0.0183	0.1067	18	22.5	0.0247	0.1905	1	1.01	0.0248
0.0638	10	11.2	0.0235	0.1100	13	15.2	0.0244	0.2035	1	1.01	0.0265
0.0685	12	13.8	0.0261	0.1140	16	19.5	0.0260	0.2170	0		
0.0724	15	18.0	0.0289	0.1150	10	11.2	0.0248	0.2290	0		
0.0760	9	9.97	0.0275	0.1200	15	18.0	0.0271	0.2430	1	1.01	0.0317
0.0800	17	21.0	0.0328	0.1200	7	7.56	0.0250	0.2560	0		
0.0848	15	18.0	0.0338	0.1270	6	6.41	0.0260	0.2680	0		
0.0881	13	15.2	0.0342	0.1300	14	16.5	0.0291	0.2810	0		
0.0921	8	8.75	0.0327	0.1330	4	4.17	0.0262	0.2940	0		
0.0958	11	12.5	0.0359	0.1340	4	4.17	0.0265	0.3070	0		
0.1000	3	3.09	0.0306	0.1395	4	4.19	0.0275				
0.1040	0			0.1400	7	7.56	0.0291				
0.1077	0			0.1460	0						
0.1123	0			0.1510	0						
0.1162	1	1.01	0.0310								
Average			0.0288	Average			0.0252	Average			0.0253

assumes that the destruction rate curve is a straight line when plotted on semi-logarithmic paper as determined by 2 points (a, O) and (b, U). Thus, each test that sterilizes only a portion of the samples yields a D value.

Since the initial concentration is a constant during any test, all destruction rate curves will pass through the point (a, O). It has been observed that when large numbers of samples are tested and the calculated probable numbers of surviving spores (b) are plotted on a destruction rate curve, they align themselves around a line that requires more time to traverse one log cycle than the lines connecting any of these points (b, U) with the initial point (a, O).

In Figure 2 this relationship is illustrated for the 120, 10,000 and 1,000,000 spore levels. The data from which these curves are derived are given in Table 1. In Figure 2 the slopes (D value) of the regression lines through the b values for the 120, 10,000 and 1,000,000 spore levels are 0.043, 0.041 and 0.046 min., respectively, as compared to the average calculated D values of 0.0288, 0.0252 and 0.0253 min. Although the D values derived by one method or the other are in general agreement, the values derived by the 2 methods are not in good agreement.

The curves illustrated in Figure 2 suggest that the calculated number of surviving spores (b), for a high percentage of positive tubes, is somewhat low, especially when

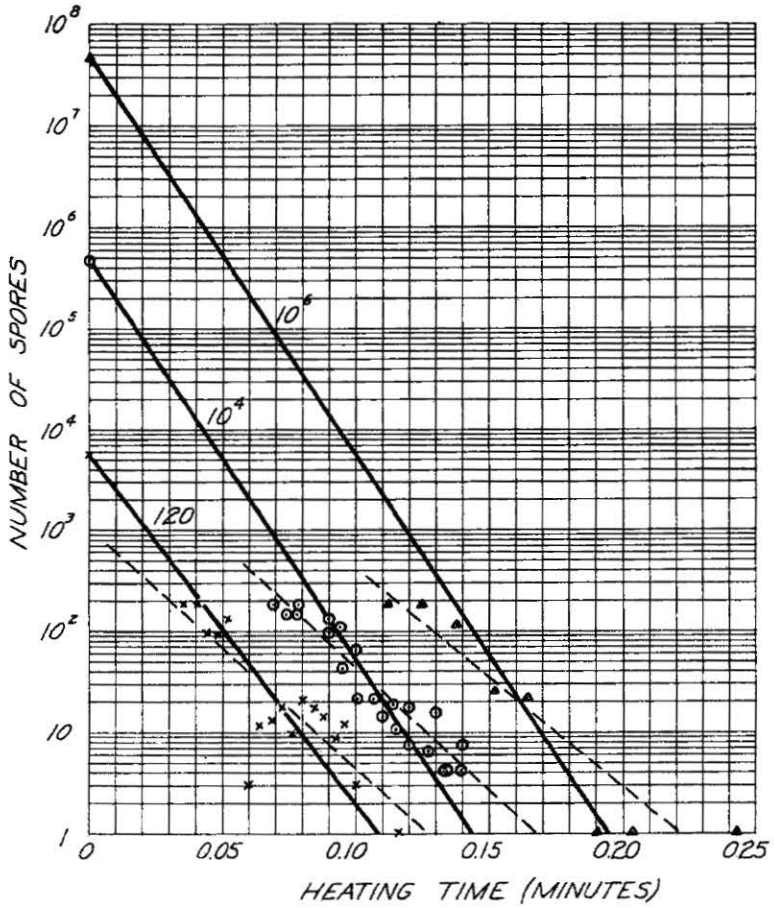

Figure 2. Destruction rate curve for concentrations of 120, 10,000 and 1,000,000 spores of P. A. 3679 per 0.01 ml. at 280.0°F.(137.8°C.) suspended in neutral phosphate buffer. Solid lines represent destruction rate curves for average calculated D values, broken curves represent regression lines calculated from probable number of spores (b).

they are compared to a destruction rate curve having an average calculated D value. Preliminary data in making quantitative counts of surviving spores indicate that this might well be the case. The number of surviving spores at comparable time intervals determined by quantitative counting are considerably greater than the number of spores calculated from the number of positive tubes.

From further inspection of Figure 2 it is obvious that if the calculated number of spores (b) were in agreement with the straight line destruction rate curve based on the average D value a uniform calculated D value would be obtained. Owing to the difference in slope of the calculated destruction rate curve as compared to the curve through the number of spores (b) calculated from the number of positive tubes there cannot help but be an increasing D value with heating time (U).

Another factor to be considered in the evaluation of spore destruction data is the reliability of the initial data. If the assumption is made that there is a binomial distribution, the number of tubes showing growth under identical conditions would be expected to vary. The expected variation depends upon the number of samples.

In Figure 3 the expected variation at the 95% confidence level is illustrated. Data for Figure 3 were obtained from Hald *(1)* and Snedecor *(5)*. The curves of Figure 3 are arranged so that the expected variation can rapidly be obtained for 6, 12, 24 and 48 replicate samples. It can be observed from Figure 3 that the expected variation with a small number of samples is very great and that the accuracy of any test may be

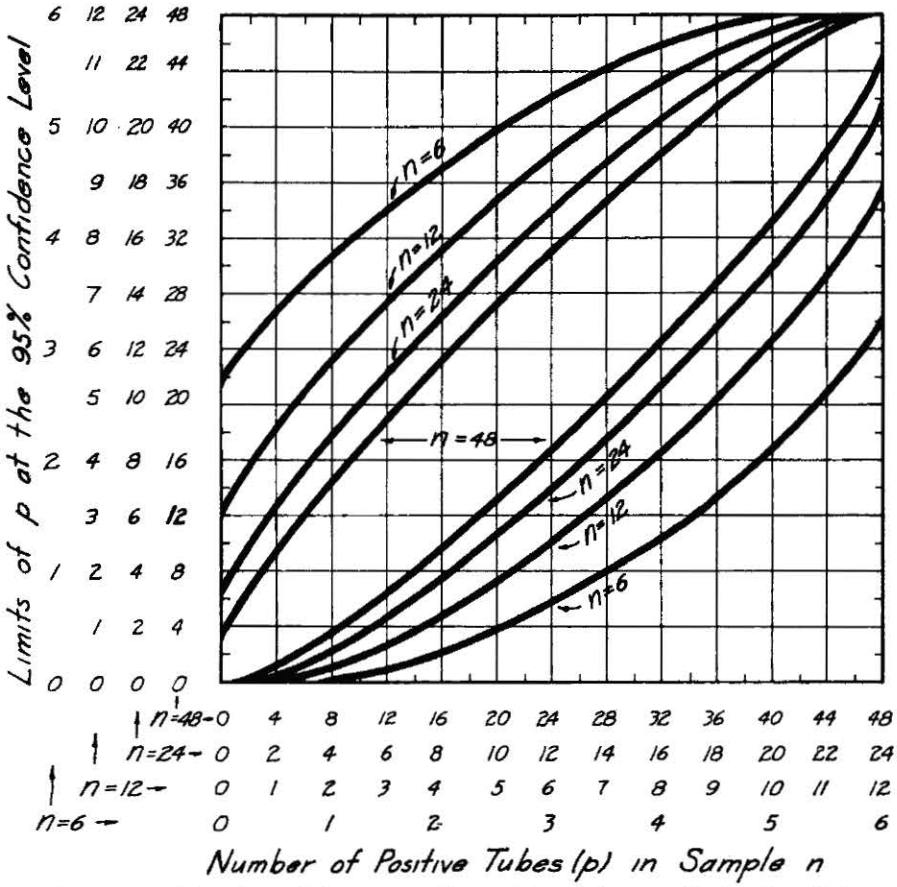

Figure 3. Relationship of the 95% confidence interval for sample size (n) of 6, 12, 24 and 48 for binomial distribution.

increased considerably by using a larger number of samples for each time-temperature interval.

The number of samples to be run at each time-temperature interval may then be based on the accuracy desired, as obtained from Figure 3. The use of 24 replicate samples for each time-temperature interval has been employed as a routine practice in this laboratory, although larger numbers of replicates are preferred in some cases.

A particular test of 16 runs of 6 tubes each, at 14 different time intervals, making a total of 1,344 samples were checked at the 6, 12, 24 and 48 level by random grouping. In all cases more than 95% of the samples fell within the limits of Figure 3. This result is significant in that it indicates that results are consistent within the limits of experimental error.

SUMMARY

A thermal death time curve for P. A. 3679 spores in neutral phosphate buffer in the temperature range 235 through 300°F. with regression limits has been presented. Thermal destruction data have been presented for 120, 10,000 and 1,000,000 P. A. 3679 spores per 0.01 ml. in neutral phosphate at 280°F. The D values for these 3 spore concentrations were in general agreement. The trend of D values to increase with heating time (U) has been discussed. The normal variation expected within the 95% confidence level has been discussed in relationship to accuracy and number of samples to be tested at each time-temperature interval.

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LITERATURE CITED

1. HALD, A. *Statistical Tables and Formulas.* 1952, John Wiley and Sons, Inc., New York.
2. HALVORSON, H. O., AND ZIEGLER, N. R. Application of statistics in bacteriology. *J. Bact.*, 25, 101 (1932).
3. PFLUG, I. J., AND ESSELEN, W. B. Development and application of an apparatus for study of thermal resistance of bacterial spores and thiamine at temperatures above 250°F. *Food Technol.*, 7, 237 (1953).
4. REYNOLDS, H., AND LICHTENSTEIN, H. Evaluation of heat resistance data for bacterial spores. *Bact. Rev.*, 16, 126 (1952).
5. SNEDECOR, G. W. *Statistical Methods.* Fourth ed., 1946, Iowa State College Press, Ames, Iowa.
6. STUMBO, C. R., MURPHY, J. R., AND COCHRAN, J. Nature of thermal death time curves for P. A. 3679 and *Clostridium botulinum. Food Technol.*, 4, 321 (1950).