ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

Eighteenth Quarterly Report of Progress

Research Project R-36-015-001

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Introduction

In our last quarterly report we described a rapid procedure for drying and equilibrating spores prior to thermal inactivation determinations. This technique of preparing spores has continued to be effective in that reproducible D values are obtained by plate count determinations of survivors in the range of 10^6 to 10^2 per test unit.

In this quarter, our efforts have been concentrated upon (a) modification of the heat exposure system (can and contents) to accommodate stainless steel cups in lieu of the tubes that were used in previous studies, (b) heat penetration studies on this system, (c) comparison of D values as obtained from plate-count and most-probable-number data using this system, and (d) study of the rate of cross contamination between cups in our test system.

I. MODIFICATION OF THE HEAT EXPOSURE SYSTEM

To work in the 10^2 to 10^{-2} range of survivors per test unit, a technique of enumeration is needed that will be reasonably precise with this low level of survival. We had been using a most-probable-number technique based on growth or no growth after spores were exposed to heat in 6 x 50 mm tubes contained in a sealed can. These tubes were not satisfactory in that the headspace air in each tube appeared to delay equilibration which in turn gave erratic D values.

To reduce the problem of headspace, stainless steel cups (0.8 cm deep by 1.0 cm diameter) were substituted for the tubes. These cups were placed on shelves (30 per shelf) and four shelves were used in each 206 x 300 tin can (Fig. 1). A stainless steel sanitary fitting cup was placed in the bottom of each can to increase the weight of the filled can so that the can would stay submerged in a silicone oil bath operating at 125°C.

II. HEAT PENETRATION STUDIES

With a 30-cup-per-tray and a 4-tray-per-can array, it is obvious that the cups at the outer periphery of the shelves will heat faster than those in the center. Our need for many replicates to make the most-probable-number system reasonably precise, made it desirable to use the cup-can system since one can contained 120 cups or 120 test units which could be tested for growth or no growth subsequent to heat exposure. We tested heat penetration into this cup-can system to determine if the differences in heating rates experienced by cups in two positions in the can would be significant when the total heat exposure time was considered. Thermocouples were placed to measure the center cup in the bottom shelf and one cup on the periphery of the second shelf. The center of the bottom shelf was selected as representative of the maximum lag because of the lag in heating caused by the stainless steel weight immediately below this cup. A cup on the periphery of the second shelf was selected as representative of the fastest heating because of its proximity to

the side of the can. There was a 3-min difference in heating time to 0.1°C below bath temperature with the cup in the center of the bottom shelf showing the longer heating time. Total heating time for this cup was 24 min (Fig. 2). When the relatively long heating time was considered together with the long exposure times at 125°C, it was judged that this difference in heating rate would not significantly affect the results obtained by the most-probable-number technique.

III. COMPARISON OF D VALUES BY PLATE COUNT AND MOST PROBABLE NUMBER TECHNIQUES

For D-value determinations in the range of 10^6 to 10^{-2} spores per test unit (cup), it would be most effective if plate-count data could be used from the start of the experiment until spore concentration approached approximately 10^2 per test unit. As spore inactivation increased, the most-probable-number technique would be used until the spore concentration was $< 10^{-2}$ per test unit. Before two techniques could be used to generate thermal-death-time data for one experiment, a comparison was run to determine whether the data obtained by the two techniques were different.

B. subtilis var. niger spores that had been suspended in 95% EtOH were diluted in phosphate buffered dilution water to a concentration of 10^8 per ml and these were dispensed with a microburette in 0.01 amounts into sterile stainless steel cups (0.8 mm deep and 1.0 cm in diameter). These cups were placed on the shelves made to fit the thermal-death-time can used in these studies and the spores in cups, open cans, and lids were dried in a vacuum oven for a minimum of 30 min at 46-50°C at 1.5 inch Hg pressure. After initial drying, the spores

together with the stainless-steel-cup-can system, were purged five times with dry nitrogen and cycled with vacuum being used between each purge. After exposure to dry nitrogen the cans were placed in the equilibration hood for about 5 min at 30°C, (the R.H. of this hood is maintained between 2-5%) and the cans were sealed with a commercial can sealer. The equipment used for drying and sealing is shown in Fig. 3.

The cans contain cups with spores were heated at 125°C for various times, cooled, and opened with a can opener. The sample cups were placed in peptone water, sonicated, diluted, and plated for spore counts. The same fluid was also used to determine most probable number. For each sample, a series of seven master dilutions was made and 10 - 0.5-ml samples were taken from each. Six samples were assayed for each interval of heating time. The 0.5-ml samples were dispensed into sterile stainless steel cups held in petri dishes; these were incubated for 7 days at 35°C and scored for growth. Drying was prevented by adding water to a filter paper disk placed in the bottom of each petri dish. Most-probable-number values were calculated and compared with plate counts (Table 1), the data plotted on semilog paper (Figs. 4 and 5), and D values calculated. There were 11.7 min for the thermal-death-time curve obtained from plate-count data and 11.6 min obtained from the most-probable-number data.

IV. SOME STATISTICAL APPLICATIONS TO THERMAL DEATH TIME EXPERIMENTS

In the most-probable-number technique, this number is obtained by scoring stainless steel cups containing 0.5 ml of broth as having

growth of B. subtilis var. niger. These cups are placed adjacent to each other with about 20 being placed in a single petri dish. Any cross contamination between the broth in a cup with growth, and an adjacent cup that originally contained no survivors would cause errors in the value obtained as being the most probable number of survivors.

Cross contamination experiments were set up by preparing cups each with approximately 100 viable spores. These were dried and placed adjacent to sterile cups in petri dishes. All cups were filled in the usual manner which is the addition of 0.5 ml of broth with an automatic pipettor in a horizontal laminar flow cabinet. All cups were incubated for 7 days and scored for growth. In no case did growth occur in a cup that was not initially inoculated with spores.

V. EXAMINATION OF CULTURES FROM APOLLO 11

The cultures from Apollo 11 were received in two lots and examinations were begun immediately. The original slants were examined for growth characteristics, colony appearance, and pigment production, and these characteristics were recorded on the record sheets. Gram-stained smears were made and the gram reactions and cellular morphology recorded.

A total of 1,150 cultures were received and on the basis of the above examinations, were divided into appropriate groups for further study. The results of these studies are shown in Table 2.

The predominating type of organism was the catalase positive-gram positive cocci. A total of 874 such strains was identified. The next largest group were non-sporeforming gram-positive bacilli, 80 of which were characterized. Ten sporeforming bacilli and 12 gram-negative bacilli were also characterized.

A large number of strains (174) failed to grow at some stage in the identification procedure and therefore could not be completely identified. This was, however, a marked improvement over the situation with the Apollo 10 strains when a total of 386 could not be identified. A breakdown of this group is shown in Table 3.

The most frequently occurring type (the catalase positive-gram positive cocci) was identified according to schema suggested by Baird-Parker. A breakdown of this group is shown in Table 4. As in the Apollo 10 cultures, the staphylococcal subgroups predominated in these isolations.

Table 1

Comparison of survivors after six heating periods at 125°C when measured by plate-count and most-probable-number techniques

Technique	Heating time (min)	Replicate number 1	2	3	4	5	6
		(count per cup x 10^3)					
Plate Count	4	690	610	730	730	860	--
MPN	4	590	480	510	720	700	--
Plate Count	8.2	180	190	260	350	440	480
MPN	8.2	120	150	190	310	290	410
Plate Count	15.1	220	200	150	160	150	95
MPN	15.1	110	230	120	130	140	100
Plate Count	23.1	12	20	20	19	22	26
MPN	23.1	7.8	17	12	10	26	29
Plate Count	31.1	1.9	1.9	1.8	2.2	4.1	6.7
MPN	31.1	1.1	1.3	1.7	2.2	3.6	6.5
Plate Count	38.1	0.6	0.9	1.2	1.2	1.9	1.6
MPN	38.1	0.6	1.1	1.0	0.9	1.1	0.9

Table 2

Results from studies of cultures from Apollo 11

	Number of strains
Cultures that could not be completely identified because of failure to grow at some stage in the identification procedure.	174
Catalase positive, gram positive cocci	874
Gram positive, non sporeforming bacilli	80
Sporeforming, gram positive bacilli	10
Gram negative bacilli	12
Total number of cultures	1150

Table 3

Analysis of numbers of strains that failed to grow at some step in the identification procedure.

	Number of strains
1. Failed to grow from original slants. These were mostly cocci but a few showed no organisms in gram stained smears.	14
2. Grew on first subculture, were catalase positive cocci, but failed to grow on subsequent subculture.	88
3. Grew on first subculture, were catalase negative, but failed to grow on subsequent subculture.	4
4. Spores seen in gram stain but failed to grown on subculture.	2
5. Non-sporeforming gram positive bacilli in gram stain but failed to grow on subculture.	49
6. Gram negative bacilli seen in gram stain but failed to grow on subculture.	17
TOTAL	174

Table 4

Types of catalase positive cocci identified.

Type of organism	Number of strains	Approximate %
Staphylococcus, Subgroup I	22	3
Staphylococcus, Subgroup II	221	25
Staphylococcus, Subgroup III	6	< 1
Staphylococcus, Subgroup IV	194	22
Staphylococcus, Subgroup V	192	22
Staphylococcus, Subgroup VI	142	16
Micrococcus, Subgroup 1	31	4
Micrococcus, Subgroup 2	10	1
Micrococcus, Subgroup 3	6	< 1
Micrococcus, Subgroup 7	34	4
Micrococcus, Subgroup 8	1	< 1

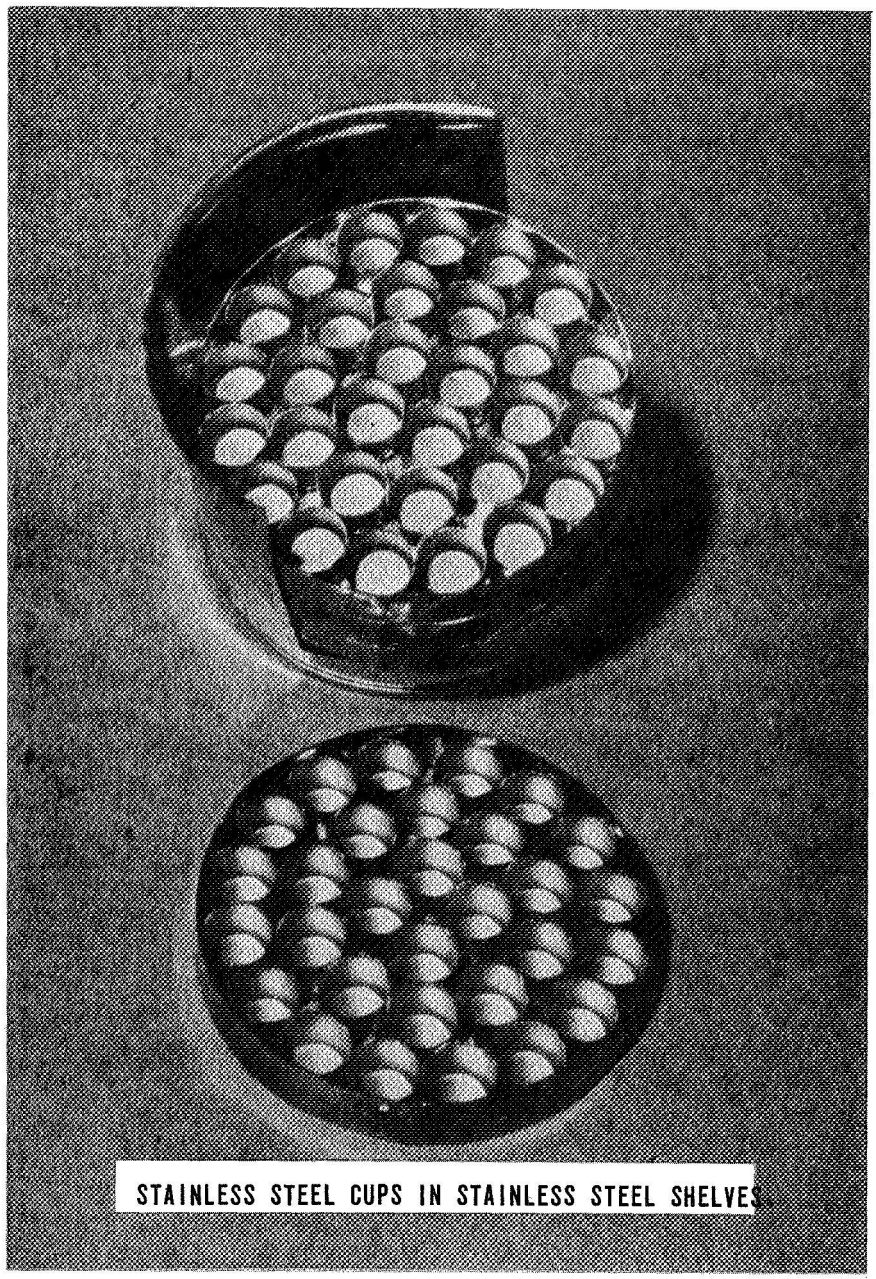

Fig. 1. Cup-can system used for destruction rate studies.

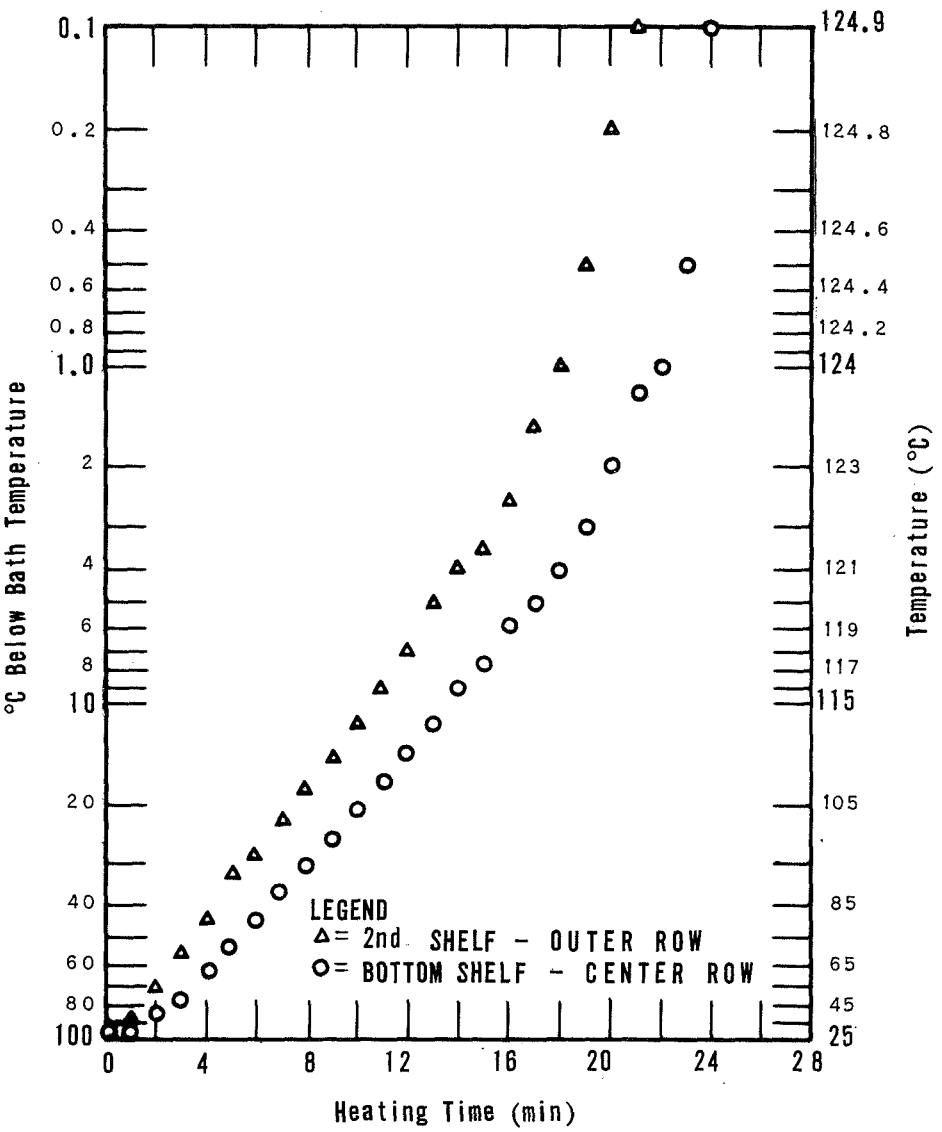

Fig. 2. Heat penetration rate for stainless steel cups.

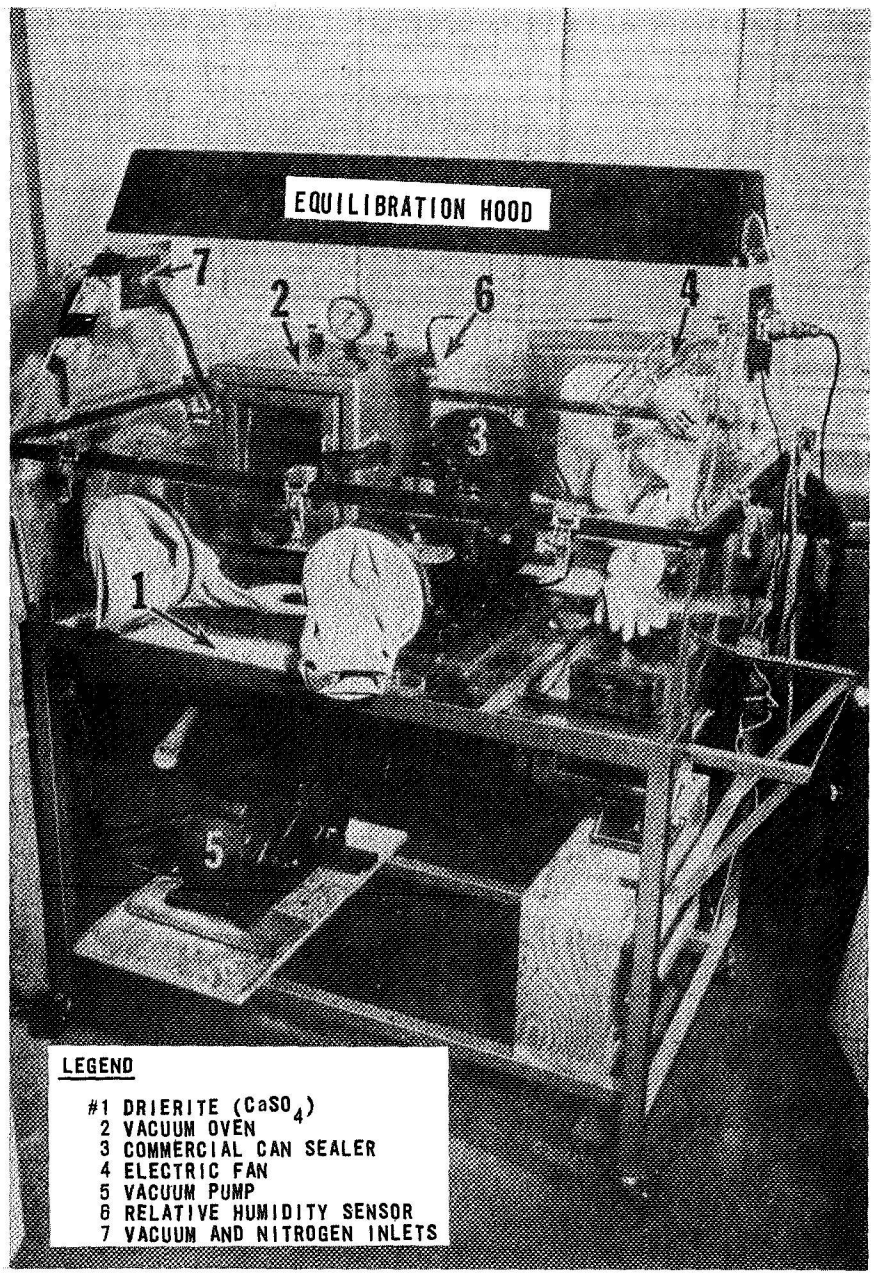

Fig. 3. Equipment used for drying and equilibration of spores.

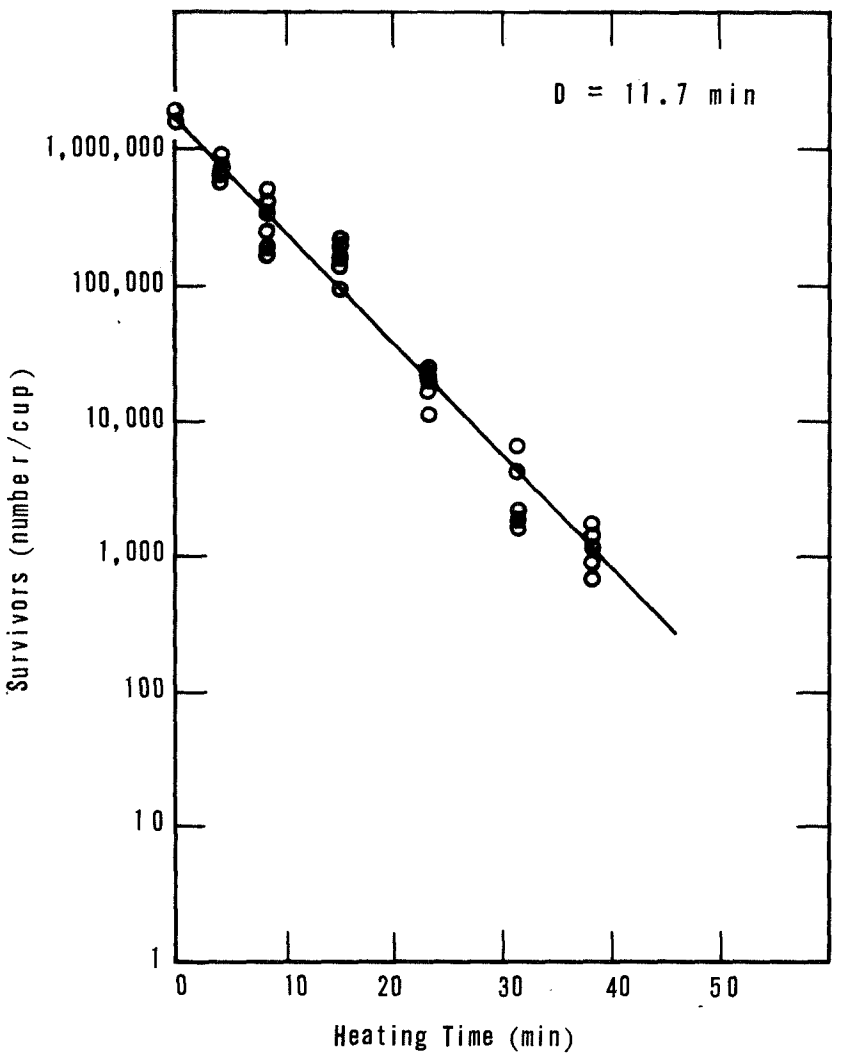

Fig. 4. Thermal inactivation of B. subtilis var niger at 125°C. Survivors measured by Plate Count.

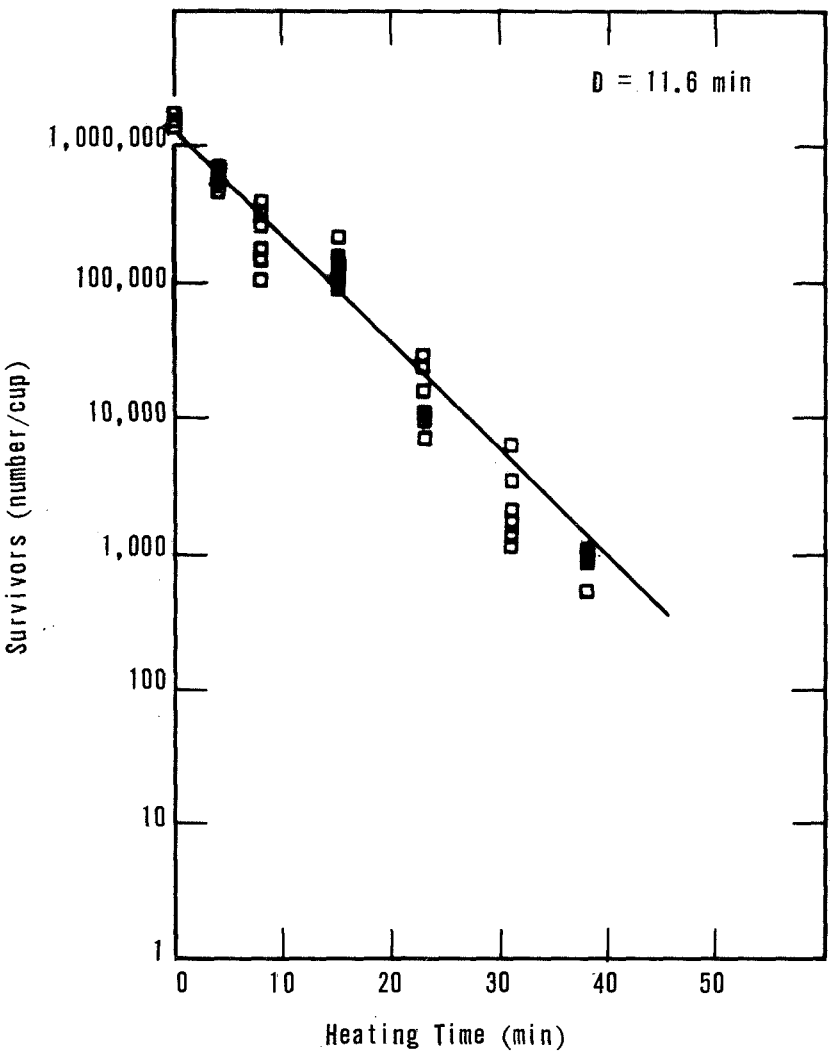

Fig. 5. Thermal inactivation of B. subtilis var niger at 125°C. Survivors measured by most probable number technique.