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

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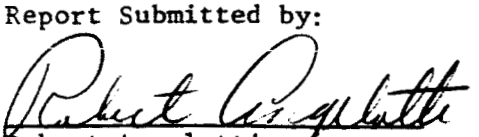

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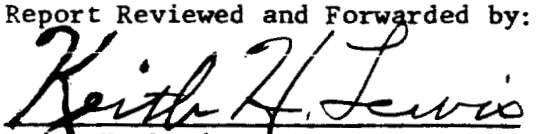

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SUMMARY

Data are presented indicating that Bacillus globigii spores are recoverable from experimentally contaminated blocks of balsa wood by maceration in a Waring Blendor. A technique has been developed whereby spores introduced into acetone soluble plastics may be recovered quantitatively by means of dissolution and entrapment in Seitz filter pads. In connection with the development of a satisfactory model system for insoluble components, a method has been developed which provides quantitative information on the toxicity of plastics. This technique utilizes the concept of the direct surface agar plate (DSAP) which has found wide application in the field of surface contamination[2].

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INTRODUCTION

The general goals of this project have been discussed in the First Quarterly Report of Progress[1]. The immediate problem under investigation is that of attempting to develop a series of comparable methods for the quantitative determination of small numbers of bacterial spores in a wide variety of solid materials used in space craft fabrication.

EXPERIMENTAL

Preliminary Experiment for the Recovery of B. globigii spores from Balsa Wood

In view of the projected use of laminated balsa wood as an impact absorber for unmanned space craft, consideration of techniques for estimating bacterial contamination of this material was appropriate. The experiments presented here were of a range finding variety and simply indicate the feasibility of the procedures described. Additional experimentation as that described under Projected Research for Third Quarter is required.

One gram blocks of balsa wood were cut, wrapped in craft paper, heat-treated in an autoclave for 15 minutes at 121° C and allowed to cool. One milliliter of an aqueous spore suspension was heated at 80° C for 10 minutes, cooled in an ice bath, and serial 10-fold dilutions in acetone were prepared to yield 8×10^6 and 8×10^4 spores per milliliter. One-half milliliter of each spore dilution was injected into separate balsa wood blocks using an 18 gauge, 1-1/2 inch needle.

The blocks were ground immediately after injection in 200 milliliters of cold phosphate buffered dilution water in a Waring Blendor for the time and speeds shown below:

1. One set was ground one minute at low speed (approx. 8,000 rpm), cup agitated by hand and replaced on Waring Blendor and ground for an additional one minute at high speed (approx. 16,000 rpm).
2. One set was ground one minute at low speed, cup agitated by hand, ground an additional 30 seconds at low speed and 30 seconds at high speed.

Plate counts of the ground suspension were made using tryptone glucose extract agar as the plating medium. The plates were incubated at 35° C for 24 hours and counted.

The results of this experiment are summarized in Table I and it is seen that the inoculated spores were not destroyed or prevented from germinating and forming colonies. It was not possible, because of the manner in which the experiment was performed, to determine whether the apparent increase in numbers of spores recovered was due to breaking-up of spore clumps or to residual contamination of the wood blocks.

Recovery of B. globigii Spores from a soluble plastic

Although it is unlikely that lucite will be used in space craft construction, the chemical and physical characteristics of this plastic make it of value as a model system. The more important of these are summarized below:

a. Insoluble in water

b. Can be ground into small particles

c. Can be dissolved easily in several solvents

d. Generally regarded to be biologically inert

The purpose of the following two experiments was to determine if Bacillus globigii spores could be recovered from lucite by dissolution of the plastic surrounding the spores.

1. Recovery of Bacillus globigii spores from a solution of lucite in acetone:

Three flasks were prepared containing 98 milliliters of acetone and 1.0 gram of lucite shavings. Dissolution of the lucite was aided by warming the flasks in a 50° C water bath.

A portion of a B. globigii aqueous suspension spore was heated at 80° C for 10 minutes and cooled in an ice bath. The appropriate serial 10-fold dilutions were made in acetone to give three 10 milliliter aliquots containing 10^7, 10^5, and 10^4 spores per milliliter of acetone. 1.0 milliliter of each dilution was added to a flask of dissolved lucite in acetone to give 10^5, 10^3, and 10^2 spores per milliliter. The flasks were swirled to distribute the spores uniformly. Ten milliliters of each of the three inoculated plastic solutions were added to separate sterile Seitz filters containing 25 milliliters of acetone. The filters were washed with 100 milliliters of acetone in 50 milliliter aliquots. The number of spores inoculated into each filter pad was approximately 1500, 15,000, 1,500,000 based on a plate count of the acetone-spore suspension used to inoculate the lucite solution.

After each filter was washed with acetone, the pad was aseptically placed in a Waring Blendor cup containing 500 milliliters of phosphate buffered dilution water. The pad was ground for 2 minutes at low speed and 1 minute at high speed, and triplicate plate counts were made using tryptone glucose extract agar as the plating medium. The plates were incubated at 35° C for 48 hours and counted.

The results of this experiment are summarized in Table II. It is seen that the recovery of spores compares favorably to the number initially added to the solution. These data indicate that the lucite-acetone system does not appreciably inhibit recovery of the spores and that the filter pad technique is satisfactory over a wide range of spore concentrations.

2. Recovery of Bacillus globigii spores from lucite by dissolution in acetone:

A portion of an aqueous suspension of B. globigii was heated at 80° C for 10 minutes and cooled in an ice bath. Appropriate serial 10-fold dilutions in acetone were prepared to yield 1×10^5 and 1×10^7 spores per milliliter. Fifty milliliters of acetone were placed in two sterile beakers and to one beaker was added 1×10^5 spores and to the other 1×10^7 spores. The inocula were stirred with separate glass rods and 5.0 grams of lucite shavings were added to each beaker and stirred until the plastic was dissolved. The suspensions were poured into separate petri dishes and allowed to dry 24 hours with the lid of the dishes partly open.

The dried plastic was removed from the petri dishes and placed in 500 milliliters of acetone and allowed to dissolve for 24 hours.

The dissolved plastic was passed through a Seitz filter and the beaker was washed with 500 milliliters of acetone in 100 milliliter aliquots. The filter was washed with 100 milliliters of acetone in 50 milliliter aliquots. The filter pad was ground in 500 milliliters of phosphate buffered dilution water on a Waring Blendor for two minutes at low speed and one minute at high speed. Plate counts in triplicate were made using tryptone glucose extract agar, and the plates were incubated at 35° C for 24 hours.

The results of this experiment, shown in Table III, reveal that Bacillus globigii spores incorporated into lucite can be quantitatively recovered by dissolution of the plastic and entrapment of the spores in a Seitz filter pad.

Determination of the Toxicity of Plastics

Prior to undertaking an evaluation of various techniques such as wet grinding, air abrasion, and cryogenic conditions for recovering spores from insoluble solids, it is necessary to know the degree of survival of the organism in the materials being studied. A system has accordingly been developed for this purpose utilizing the principle of the direct surface agar plate (DSAP). This technique was developed in connection with quantitative evaluation of procedures for measuring surface contamination.

Three stainless steel sheets (12" x 14") having a 4 mill finish were cleaned with soap and water, rubbed with cotton soaked in a 1:1 solution of alcohol and ether, washed again with soap and water, rinsed with distilled water, allowed to dry, wrapped in craft paper,

and autoclaved at 121° C for 15 minutes. Just prior to use, the stainless sheets were again cleaned with alcohol-ether and lightly flame seared.

A portion of an aqueous suspension of B. globigii spores was heated at 80°C for 10 minutes and cooled in an ice bath. Serial 10-fold dilutions were made in phosphate buffered dilution water, and the final dilution of 5×10^3 spores per milliliter was made in reconstituted, sterile, non-fat dry milk.

Two milliliters of the milk dilution of spores were pipetted onto each sheet and brushed evenly over the surface with a one-inch paint brush; the final strokes were made with the grain of the metal. This volume of inoculated milk was calculated to yield approximately 60 spores per square inch of stainless steel surface. The milk was allowed to dry and DSAP circles and sterile stainless steel rings were placed in the positions shown below.

Day 1

Sheet 1		
B_1	B_2	A_3
A_1	A_2	B_3

Sheet 2		
A_3	B_1	B_2
B_3	A_1	A_2

Sheet 3		
B_2	A_3	A_1
A_2	B_3	B_1

Day 2

Sheet 1		
B_2	B_3	A_1
A_2	A_3	B_1

Sheet 2		
A_1	B_2	B_3
B_1	A_2	A_3

Sheet 3		
B_3	B_1	A_2
A_3	A_1	B_2

Day 3

Sheet 1		
A_3	A_1	B_2
B_3	B_1	A_2

Sheet 2		
B_2	A_3	B_1
A_2	B_3	A_1

Sheet 3		
B_1	A_2	B_3
A_1	B_2	A_3

A_1 Metallurgical plastic; A_2 Castolyte; A_3 Conventional Polyester; B_1, B_2, B_3 DSAP circles

Tryptone glucose extract agar was pipetted into the DSAP circles at the same time as the plastics were poured into the stainless steel rings. The agar was covered with Rodac plate lids, and the sheets were incubated for 24 hours at 35° C in a high humidity incubator. After 24 hours the DSAP circles were counted, and the plastics were pulled off the steel. DSAP circles were drawn on the same area of the stainless steel sheet that was formerly covered with plastic and also on that surface of the plastic that had been in contact with the steel. Tryptone glucose extract agar was pipetted into the circles, and the circles were covered with sterile Rodac plate lids and incubated as before.

The colonies that developed in the DSAP agar circles were stained with 0.1% 2, 3, 5 triphenyl tetrazolium chloride before being counted.

The experiment was repeated as above on two succeeding days. These data were then subjected to statistical analysis after the square roots of the data were taken. It was assumed that the square root transformation would achieve approximate normality of the data.

Firstly, the DSAP values were analyzed to determine that the mean of the square roots were equal from sheet to sheet and day to day. The results of this analysis are shown in Table IV and indicate that the mean from day to day is significantly different at $\alpha = 0.01$ where α is the probability of rejecting the hypothesis that the means are equal when in fact they are equal. The means from sheet to sheet averaged over days could not be shown to be different.

The results of the experiment are shown in Tables V and VI. Means and 95 per cent confidence intervals are shown for each day for the DSAP counts and for the counts on the three plastics. In all cases, the counts on the bottom of the plastic and under the plastic on the steel surface have been added together and treated as one value. Table V shows the counts per circle and 95 per cent confidence limits of the data after the results had been reconverted from square roots. The per cent recoveries and the 95 per cent confidence intervals of the recoveries are approximated by dividing the value for the original inoculum per circle into all the values for each day and multiplying by 100. The per cent recoveries expressed in this way are shown in Table VI.

In conclusion, we can state that:

a. The DSAP recovers over 90 per cent on the average of the spore contaminations present per unit area on the stainless steel surface.

b. Castolyte plastic is extremely toxic and, in fact, only one spore survived.

c. Polyester plastic is non-toxic. This plastic may be expected to permit 90 per cent survival of spores, about $\pm$ 12 per cent, 95 trials out of 100.

d. Metallurgical plastic is moderately toxic and may be expected to permit 60 per cent survival of spores, $\pm$ about 30 per cent, 95 trials out of 100.

PROJECTED RESEARCH FOR THIRD QUARTER

Activities during the third quarter will be directed toward:

a. Further exploration of techniques for the sterilization of balsa wood and the recovery of experimentally introduced spore contamination.

b. The development of a stable, dried, spore inoculum for incorporation into plastics.

c. Evaluation of a wet grinding system for recovery of spores from insoluble plastics.

REFERENCES

1. Previous report (First Quarter).
2. Angelotti, R. and Foter, M. J., 1958 A direct surface agar plate laboratory method for quantitatively detecting bacterial contamination on non-porous surfaces, Food Research, 23, 170-174.

Table I

Recovery of Bacillus globigii spores from Balsa Wood

No. of spores inoculated into each block*	No. of spores recovered by grinding 1 min. low and 1 min high speed (No. spores/block)	No. of spores recovered by grinding 1 min low, 30 sec low, 30 sec high speed (No. spores/block)
4×10^6	7×10^6	7×10^6
4×10^6	6×10^6	7×10^6
4×10^4	5×10^4	7×10^4
4×10^4	5×10^4	6×10^4

*Based on a plate count of the spore suspension used to inoculate the block.

TABLE II

Recovery of Bacillus globigii spores from an acetone solution of lucite.

No. of spores in Solution	No. of spores recovered from the filter pad	% of spores recovered from the filter pad
1500	1450	96.7
1500	1350	90.0
15,000	12,200	81.3
15,000	11,900	80.4
1,500,000	800,000	53.3
1,500,000	925,000	61.6

*Number based on the plate count of the acetone-spore suspension used to inoculate the lucite solution.

TABLE III

Recovery of Bacillus globigii spores from Lucite

No. of spores inoculated into 5 grams of Lucite*	No. of spores recovered from 5 grams of Lucite	% of spores recovered
12,800,000	14,100,000	110
128,000	111,000	87

*Based on a plate count of the spore suspension used to inoculate the plastic.

TABLE IV

Analysis of variance for square roots of DSAP counts

SOURCE	SS	DF	MS	F-RATIO
Days	9.31201	2	4.65600	21.28651**
Sheets	1.23062	2	0.61531	2.81310
Sheets X Days Interaction	4.03907	4	1.00976	4.61646**
Error	3.93715	18	0.21873	---
TOTAL	18.51885	26	---	---

SS = Sum of Squares

DF = Degrees of Freedom

MS = Mean Square

F-Ratio = SS/MS

**Significant at $\alpha = 0.01$

TABLE V

Results of plastic toxicity experiment - Expressed as mean and 95% confidence interval of Bacillus globigii counts per DSAP circle.

Test	Day	Mean Value	95% Confidence Interval	
			Minimum	Maximum
DSAP	1	254.0	243.8	265.5
	2	244.7	236.9	252.5
	3	212.3	198.1	227.1
Metallurgical Acrilic	1	176.3	170.1	182.6
	2	146.2	64.3	261.2
	3	137.6	67.1	233.3
Polyester	1	250.9	216.0	288.3
	2	229.6	204.9	255.7
	3	188.2	164.4	213.7
Castolyte	1	0	-	-
	2	0.3	-	-
	3	0	-	-
Plate count of original inoculum	1	268	-	-
	2	244	-	-
	3	232	-	-

TABLE VI

Results of plastic toxicity experiment - Expressed as a per cent of the original inoculum.

Test	Day	Mean Value	95% Confidence Interval	
			Minimum	Maximum
DSAP	1	94.8	91.0	99.1
	2	100.3	97.1	103.5
	3	91.5	85.4	97.9
Metallurgical Acrilic	1	65.8	63.4	68.1
	2	59.9	26.4	107.1
	3	59.3	28.9	100.6
Polyester	1	93.6	80.6	107.6
	2	94.1	84.0	104.8
	3	81.1	70.9	92.1
Castolyte	1	0	-	-
	2	0	-	-
	3	0	-	-