RECOVERY OF VIABLE MICROORGANISMS FROM SOLIDS.

I. MODEL SYSTEMS

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ABSTRACT

Test solids used in this study were dental inlay material, plaster of Paris, and paraplast. Solid pellets were made by mixing an appropriate amount of these materials with a spore suspension of Bacillus subtilis var. niger. Pulverization was accomplished by the use of a blender-mill or mortar and pestle. Rates of recovery were low in preliminary tests when the mortar and pestle were used but modifications resulted in a significant increase in the percentage of viable microorganisms recovered. The physical state of the spore inoculum influenced the recovery rates. Significantly higher recoveries were obtained when spore suspensions which were cleaned either chemically with lysozyme or physically by differential centrifugation were used instead of conventional spore suspensions. Similar results were obtained with paraplast. Use of the blender-mill resulted in recovery of fewer viable spores from test solids than use of the mortar and pestle. However, the recoveries were consistent and significant enough to warrant routine use of the blender-mill. More microorganisms were recovered consistently with the membrane filter than by standard plate counts. Die-away of microorganisms occurred within all of the test solids in storage.

The use of ethylenediamine-tetraacetic acid (EDTA) for dissolving seeded pellets of plaster of Paris was found to be an excellent control method whereby die-away of the test organism could be taken into account in calculating efficiency of recovery.

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INTRODUCTION

Valid procedures for the sterilization of spacecraft are contingent upon the ability to assess efficiently and precisely the levels of microbial contamination on the surfaces and within the interior of space hardware. The most complex problem is freeing viable microorganisms embedded within solids so that they can use nutrients and subsequently produce visible growth. The solid must be reduced to a particle size small enough to insure the release of entrapped microorganisms without destroying them. Consequently the ideal technique is one that destroys the least number of microorganisms during assay, and destroys essentially the same number each time, so that suitable and valid extrapolations can be made.

Methods for recovering viable microorganisms from solids have evolved slowly. In addition, sterility tests in use today do not satisfy completely the requirements of the ideal test, nor does it appear that any one method will recover all the viable microorganisms that may be trapped in the interior of space hardware.

Solid materials of various types have been studied by several investigators in an effort to enumerate embedded microorganisms. Various pulverization techniques were investigated in an attempt to free viable microorganisms from their encasement without causing a detrimental effect on the microorganisms (1,4,5,6,7,9,10,11).

The main techniques tested were pulverization by mortar and pestle, ball mills, and blenders. In addition drilling, sawing, and abrasion were tested. However, most of these investigations were hampered by the

lack of a valid model system. The usual procedure was to add a known number of microorganisms to either powdered or liquid material, induce solidification, subject the sample to the particular assay procedure, and then calculate the percentage of microorganisms recovered. It was assumed that the severe environmental conditions caused by solidification, storage, and the presence of different types of chemical compounds did not affect the test microorganism. This, however, usually is not the case. Ideally, such a model system should consist of a solid which can be dissolved without harming the test microorganism, so that one portion can be assayed for the actual number of viable microorganisms present, irrespective of pulverization technique. The results of this assay rather than assay of the original inoculum should be used to calculate percentage of recovery.

The main objectives of this study were: 1) to develop an experimental model system which could be used to test various methods for recovering viable microorganisms from solid materials; 2) to evaluate the effect of the solidification process and storage on the survival of microorganisms within test solids.

MATERIALS AND METHODS

Solid pellets, approximately 1 cm in length by 1/2 cm in diameter, of dental inlay material (silicon dioxide, Cristobalite Investment, Kerr Manufacturing Company, Detroit, Michigan) and plaster of Paris (calcium sulfate, The Synkoloid Company) were made by mixing 0.25 to 0.30 g of the sterile dry powder (dry heat sterilized at 170 C for 3 hr) with 0.1 ml of an

aqueous spore suspension in plastic molds. The test microorganism used was Bacillus subtilis var. niger. Prior to seeding the different types of material, the spore suspension was heat-shocked at 80 C for 15 min. The pellets were allowed to dry in a 37 C incubator having a relative humidity of 38 to 42 percent and were stored in this environment until assay. The pellets were removed from the mold by dissolving the plastic coat with acetone prior to assay. Preliminary tests showed that the acetone treatment did not affect the spore content of the pellet. The assay method consisted of grinding the pellets in 10 ml of sterile Eugon broth (BBL) by mortar and pestle for one minute or in a "Pica" blender-mill (Cat. #3800, Pitchford Manufacturing Corporation, Pittsburgh, Pa.) for 15 seconds. Techniques employing standard plate counts (pour plates) and in some cases membrane filters were used to determine the number of viable spores recovered. The culture medium was trypticase soy agar (BBL). Incubation time was 24 and 48 hours at 32 C for the membrane filter and standard plate counts, respectively. More time was required in the latter method due to the slower development of subsurface colonies.

One ml of liquid paraplast, which had been dry-heat sterilized at 170 C for 3 hr, was seeded with a benzene spore suspension of B. subtilis var. niger. The paraplast was allowed to harden and was stored at room temperature until assayed. At the time of assay, the paraplast was dissolved in sterile benzene and viable spores were enumerated by use of membrane filters. Membrane filters (Millipore HA 0.45 micron pore size) were placed on trypticase soy agar and incubated at 32 C for 24 hours. Initial tests showed that benzene did not harm the spores or

alter the efficiency of the membrane filters.

Pellets of plaster of Paris were dissolved in a 10 percent solution of sterile ethylenediamine-tetraacetic acid (EDTA, Harleco, Scientific Products) and assayed by procedures using both the standard plate count and membrane filters.

Preparation of the different spore suspensions.

Three suspensions of spores of B. subtilis var. niger were employed throughout this study. Each suspension was produced in a slightly different manner.

Suspension A was prepared by inoculating the surface of TAM agar (Difco) with 0.2 ml of a heat-shocked (80 C, 15 min) spore suspension and incubated for 5 days at 32 C. Colonial growth was washed from the agar surface with cold sterile distilled water. The suspension was subjected to ultrasonic energy for 30 minutes in an ice bath to break up the large matted fragments and then washed 5 times in cold sterile distilled water, distributed into tubes, frozen, and stored until used. Most of the cellular debris was removed and approximately 75 to 80 percent of the suspension were spores. The rest were vegetative cells. This suspension was the "dirtiest" of the three.

Suspension C was produced by inoculating 50 ml of tryptose broth with a suspension of heat-shocked spores and incubating at 32 C on a rotary shaker for 18 hr. A 10 percent inoculum was used to seed a flask of modified "G" medium (8) which was incubated as above for 4 hours. A similar transfer was made after 4 hours. This latter culture was incubated until sporulation occurred. The suspension was washed 3 times and

then subjected to a lysozyme treatment (1 mg/L at pH 9.4 for 2 hr at 20 C). After this treatment the suspension was washed 10 times, diluted, distributed in sterile tubes, frozen, and stored until used.

Suspension F was prepared by inoculating the surface of a plate of TAM agar supplemented with 0.02 percent magnesium sulfate and 0.08 percent calcium chloride with a heat-shocked spore suspension. The culture was incubated at 42 C for 24 hours. Approximately 99 percent sporulation occurred at this higher incubation temperature. A small portion of the colonial growth was suspended in sterile distilled water and the suspension was heat-shocked and another plate of TAM inoculated. This process was done successively 3 times. Finally the suspension was subjected to ultrasonic energy for 30 minutes in an ice bath and centrifuged at 9,000 rpm. The spores formed a gummy pellet and the cellular debris occurred as an orange layer which was removed easily by gentle washing. The suspension was washed 10 times with cold sterile distilled water, distributed in tubes, frozen, and stored until used. It consisted of 99 percent spores and was the "cleanest" of the three suspensions.

RESULTS

A relatively low percentage of recovery was obtained in preliminary tests using seeded dental inlay pellets. Modification of these techniques resulted in an increased recovery rate. Recovery methods investigated initially were: 1) dry grinding, 2) wet grinding, 3) use of various liquid menstrua, and 4) different grinding times. Greatest recovery was obtained consistently by wet grinding with mortar and pestle for 1 minute, using Eugon broth as the liquid menstruum.

Figure 1 shows the results obtained in preliminary tests using pellets seeded with a conventional spore suspension (suspension "A"). Maximum recovery was 17.9 percent after 7 days, with a slight reduction after 15 days of storage. A higher rate of recovery of viable spores was obtained using pellets inoculated with spore suspension "C" (lysozyme-treated) as shown in Figure 2. After 1 day of storage a reduction in the number of viable spores was observed. This die-away appeared to stabilize after 15 days. The effect produced by heat-shocking the ground pellet at 80 C for 20 minutes is also presented. It resulted in a lower spore recovery, indicating that part of the population may have germinated while in the solid.

The effect of storage on spores embedded in paraplast and dental inlay materials is presented in Figure 3. Recovery of viable spores from the two different materials appeared to follow the same pattern. A sharp decrease in recovery of spores was observed at 7 days, with more reduction and a stabilization after 15 days of storage.

The physical state of the spore inoculum appeared to influence the recovery of viable spores from solid materials. Typical results are shown in Figure 4. When the spores used as inocula were "cleaned" and h l cellular debris removed, higher numbers of viable spores were recovered from the seeded solids. Highest recoveries were obtained with suspension "F" which was the cleanest of the three suspensions. The method employing the mortar and pestle was better than the blender-mill for recovering viable spores from the solid materials tested, as shown in Figures 5 and 6.

The results of studies employing plaster of Paris as a test solid are presented in Tables 1 and 2 and Figures 6 and 8. From these data several observations can be made. First, the blender-mill recovered fewer viable spores from the plaster of Paris than the mortar and pestle but the recoveries were high enough to warrant its use as an assay technique.

Second, it is obvious that using the original inoculum to calculate percentage of recovery is not valid because there is a definite die-away of microorganisms within the solid during its formation and storage which is not attributable to the assay procedures. Also, it appears that some spores may have been able to germinate within the solid and were killed by subsequent pulverization.

Third, more spores were recovered consistently with the membrane filter than with pour plates. Although no explanation can be given at the present time, higher recovery with the membrane filter may have been due to the washing away of slightly toxic chemicals in the test solids which did not manifest toxicity on normal spores but did so with injured or environmentally stressed spores. Also, since more oxygen was accessible, more spores, especially those which were injured, may have germinated and produced colonies.

A reduction in the number of viable spores recovered was observed when all of the test solids were stored (Figures 1, 2, 3, 7, and 8).

Pulverization of the test solids by the two methods revealed two different types of particles. A sphere-shaped particle was formed when the dental inlay material was pulverized by either method. The plaster

of Paris material produced a rod or spindle-shaped particle which had a tendency to aggregate. A small portion of pulverized material was placed on a glass slide, mixed with an equal volume of nigrosin, and examined microscopically. Measurements were made with a calibrated micrometer. For each technique and material used, 200 particles were counted. The results are shown in Tables 3 and 4. Particle sizes of 1 micron and smaller were obtained by both methods, but the blender-mill usually produced a greater number of particles smaller than 1 micron.

DISCUSSION

An experimental model system was designed which may be used to test various methods for recovering viable microorganisms from solid materials. Two types of pulverizing equipment were employed to reduce the test solid materials: mortar and pestle, and a blender-mill. These methods offer two different types of pulverizing action. The mortar and pestle provides a slow shearing force on solid materials, and the blender-mill employs a hand-pestle type of motion that achieves particle size reduction by a combination impact and mulling action. The best recovery of viable spores was obtained using mortar and pestle. Comparing the percentage recovery of viable spores from dental inlay material during a storage period of 50 days, the mean recovery rates were 70.7 percent with mortar and pestle and 44.5 percent with the blender-mill. When plaster of Paris was the test material, the mean recovery rate was 63.0 percent with the mortar and pestle and 35.0 percent with the blender-mill. Although the results show that less viable spores were recovered when the blender-mill was employed, the recovery was consistent and significant

enough to warrant its use with solids which do not lend themselves to pulverization by mortar and pestle. In addition, the blender-mill eliminates human variables and is a more uniform technique.

The use of conventional spore suspensions to seed test solids resulted in low recovery of viable spores. As the spore suspensions were "cleaned" and had cellular debris removed, recoveries were significantly increased. Best recovery was obtained when spore suspension F was used to inoculate the two test materials. This suspension was the cleanest of the three.

The maximum die-away of microorganisms in stored solids occurred within 15 days. The reason for this die-away is not clear. Effects of hardening of the test solids undoubtedly exposed the spores to physical pressures. Also some of the spores may have germinated within the test solids and the resultant vegetative cells destroyed by dehydration or lack of nutrients.

These results emphasize the importance of the preparation and ultimately the physical condition of the spore suspensions to be used as test inocula in model systems. Even in the absence of gross cellular debris, a large number of vegetative cells in a spore suspension may provide enough trace nutrients to allow a portion of the spore population to germinate (3). This factor, coupled with calculating percentage of recovery based on the number of viable spores in the inocula, could account for the extremely low recoveries reported earlier (6,9). In all probability, if die-away during solidification and storage had been taken into consideration, the recovery rates would have been significantly higher than reported. When one recalculates much of the data in these

reports, using the values obtained from dissolved test solids rather than the original inocula, the recovery rates are indeed significantly higher.

Using EDTA to dissolve plaster of Paris pellets proved to be an excellent method for determining the actual number of viable microorganisms within the test solid when the efficiency of the various assay techniques was compared. In some cases higher recovery was obtained by pulverization with mortar and pestle than by dissolving with EDTA. This difference was probably due to the inhibitory effect of EDTA in high concentration. Studies are being conducted at present to determine the extent of this factor.

Paraplast was an ideal solid to work with since it could be dissolved by benzene which has no detrimental effect on the spores or membrane filter. Although it does not lend itself to pulverization by either of the two methods tested, it can be used to study the survival of spores in solids.

From the results obtained in this study the following conclusions can be made: 1) The blender-mill can be used effectively to recover viable microorganisms from solids which can be pulverized, and 2) in designing a model system to test the efficiency of recovery techniques care must be taken not only to use valid controls but also to employ clean spore preparations. Both of these factors are critical.

In the next phase of this study, attempts will be made to develop a model system using a plastic material. Although several plastic systems have been described earlier, most are either toxic or soluble only

in solvents which kill the test spores. The latter factor eliminates the possibility of performing a valid control. The only valid system reported to date appears to be the one designed by Angelotti and Lewis (2) using methyl methacrylate as the solid and acetone as the solvent.

Our preliminary work concentrated on polystyrene as a model plastic because of its solubility in carbon tetrachloride. Carbon tetrachloride is not toxic to spores of B. subtilis var. niger and can be passed through standard membrane filters with no deleterious effect on the filter. However, polymerization by both injection molding and peroxide catalyst was found to be lethal to test spores.

However, spores were successfully incorporated into pieces of plastic formed by laminating thin sheets of polystyrene together with carbon tetrachloride or by compressing powdered polystyrene into pellets using carbon tetrachloride as an adhesive. Spores have been isolated from these plastics after more than three months at room temperature.

Our efforts are currently being directed toward the methyl methacrylate system described by Angelotti and Lewis (2). Spores of B. subtilis var. niger have been isolated from polymerized methyl methacrylate after dissolution in acetone. In this process it has been found that the rate of dissolution of polymerized methyl methacrylate in acetone is markedly increased by ultrasonic energy. After a model system of this sort is perfected, recovery rates of the blender-mill, sawing, and drilling will be compared.

The use of solvent resistant membrane filters (Gelman, alpha-6, 0.45 micron pore size) provided a precise and quantitative method for enumerating spores in acetone-plastic solutions. Although acetone had no

effect on the filter, water caused it to distort. Consequently when the filter was placed on the surface of an agar medium it swelled and wrinkled.

The effect of plating technique on the development of visible colonies of B. subtilis var. niger on these filters was determined. Maximum recovery was obtained when the filter was placed directly on the surface of the agar medium while it was still molten. Although the filter swelled, it did not wrinkle and remained on the surface of the medium. When the filter was overlayed with the agar medium colonies developed slowly or not at all and counting was difficult. Poor recovery also was obtained when the filters were placed directly on the solidified surface of the agar medium.

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TABLE 1. COMPARISON OF PULVERIZING BY MORTAR AND PESTLE AND DISSOLVING WITH ETHYLENEDIAMINE-TETRAACETIC ACID (EDTA) FOR EFFICIENCY IN RECOVERING VIABLE SPORES FROM PLASTER OF PARIS PELLETS.

Age of pellet** (days)	Pour plate		Percentage recovery	
	Dissolved by EDTA	Pulverized by mortar and pestle	Based on EDTA recovery as 100%	Based on inoculum* as 100%
	Colony count x 10^5	Colony count x 10^5		
1	2.90	3.68	126.9	92.7
7	2.07	2.14	103.4	53.9
14	2.63	2.34	89.0	58.9
21	2.53	1.60	63.2	40.3
29	2.53	2.60	102.8	65.5
50	2.45	2.64	107.8	66.5
Age of pellet** (days)	Membrane filter		Percentage recovery	
	Dissolved by EDTA	Pulverized by mortar and pestle	Based on EDTA recovery as 100%	Based on inoculum* as 100%
	Colony count x 10^5	Colony count x 10^5		
1	3.00	3.97	132.3	100.0
7	3.07	3.47	113.0	87.4
14	2.65	2.95	111.3	74.3
21	1.90	2.01	105.8	50.6
29	2.51	3.09	123.1	77.8
50	2.57	3.00	116.7	75.6

* Pellets inoculated with 3.97 x 10^5 spores.

** Stored at 37 C.

TABLE 2. COMPARISON OF PULVERIZING BY BLENDER-MILL AND DISSOLVING WITH ETHYLENEDIAMINE-TETRAACETIC ACID (EDTA) FOR EFFICIENCY IN RECOVERING VIABLE SPORES FROM PLASTER OF PARIS PELLETS.

Age of pellet** (days)	Pour plate		Percentage recovery	
	Dissolved by EDTA	Pulverized by blender-mill	Based on EDTA recovery as 100%	Based on inoculum* as 100%
	Colony count x 10^5	Colony count x 10^5		
1	2.90	2.77	95.5	69.8
7	2.07	1.33	64.3	33.5
14	2.63	1.60	60.8	40.3
21	2.53	1.08	42.7	27.2
29	2.53	0.82	32.4	20.7
50	2.45	0.73	29.8	18.4

Age of pellet** (days)	Membrane filter		Percentage recovery	
	Dissolved by EDTA	Pulverized by blender-mill	Based on EDTA recovery as 100%	Based on inoculum* as 100%
	Colony count x 10^5	Colony count x 10^5		
1	3.00	2.95	98.3	74.3
7	3.07	1.66	54.1	41.8
14	2.65	1.90	71.7	47.9
21	1.90	1.45	76.3	36.5
29	2.51	1.29	51.4	32.5
50	2.57	1.23	47.9	31.0

* Pellets inoculated with 3.97 x 10^5 spores.

** Stored at 37 C.

TABLE 3. PARTICLE SIZE DISTRIBUTION OF PLASTER OF PARIS PULVERIZED BY MORTAR AND PESTLE AND BY BLENDER-MILL.

Size (microns)	Mortar and pestle		Blender-mill	
	Percentage		Percentage	
	Length	Width	Length	Width
0.00 - 1.00	1.0	8.5	8.0	32.5
1.00 - 2.00	5.5	27.0	15.0	37.5
2.00 - 3.00	9.00	20.0	19.0	15.0
3.00 - 4.00	9.0	13.5	16.0	5.5
4.00 - 5.00	6.0	10.5	7.0	2.5
5.00 - 10.00	34.0	19.0	24.5	6.5
10.00 - 20.00	29.0	1.5	7.5	0.5
20.00 - 50.00	6.5	0.0	3.0	0.0

TABLE 4. PARTICLE SIZE DISTRIBUTION OF DENTAL INLAY PULVERIZED BY MORTAR AND PESTLE AND BY BLENDER-MILL

Size (microns)	Mortar and Pestle Percentage	Blender-mill Percentage
0.00 - 1.00	21.0	24.7
1.00 - 2.00	41.5	40.7
2.00 - 3.00	21.0	18.3
3.00 - 4.00	7.0	8.3
4.00 - 5.00	2.5	4.3
5.00 - 10.00	4.5	3.0
10.00 - 20.00	2.5	0.7

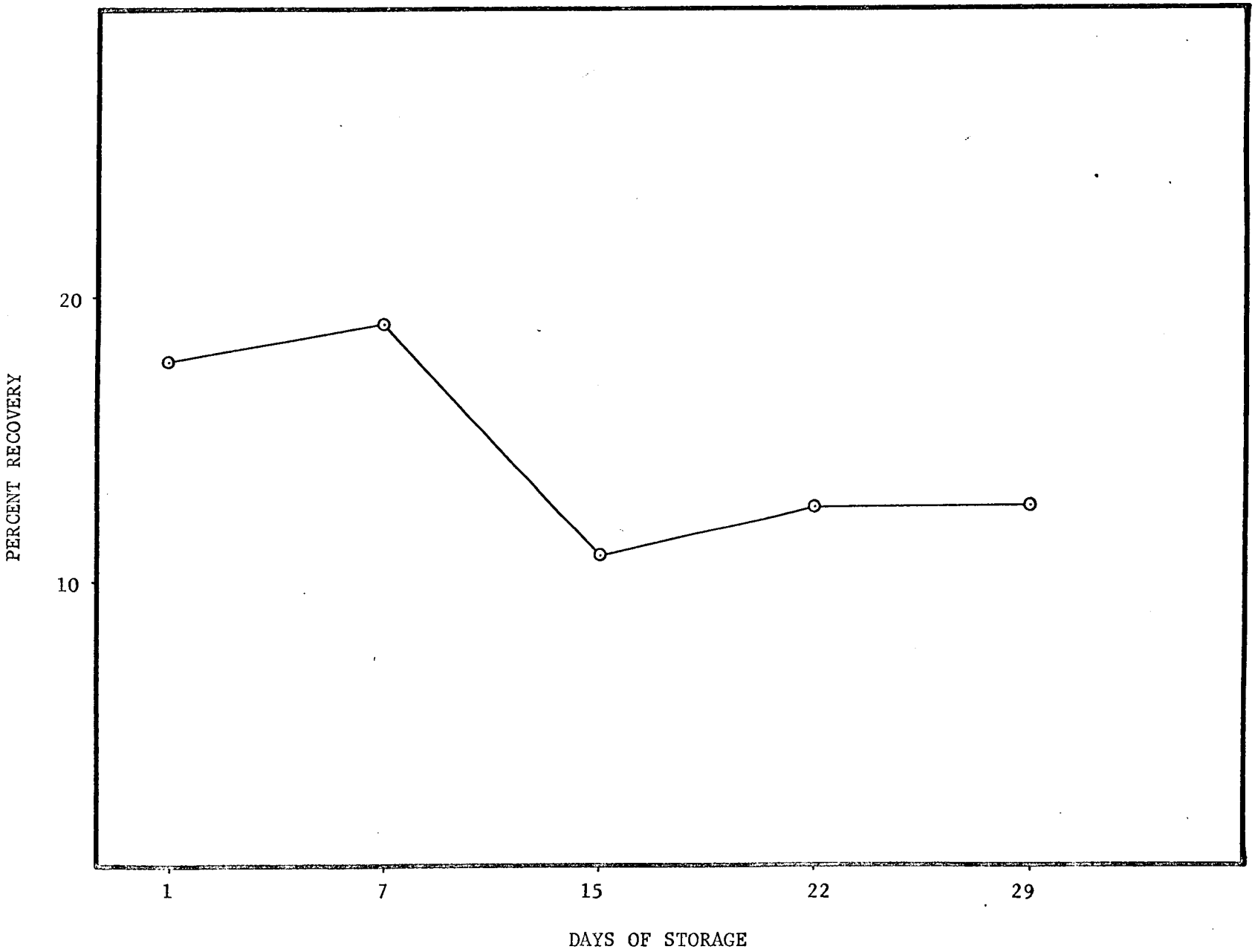

Fig. 1. Effect of storage on the survival of spores of B. subtilis var. niger embedded in dental inlay material.

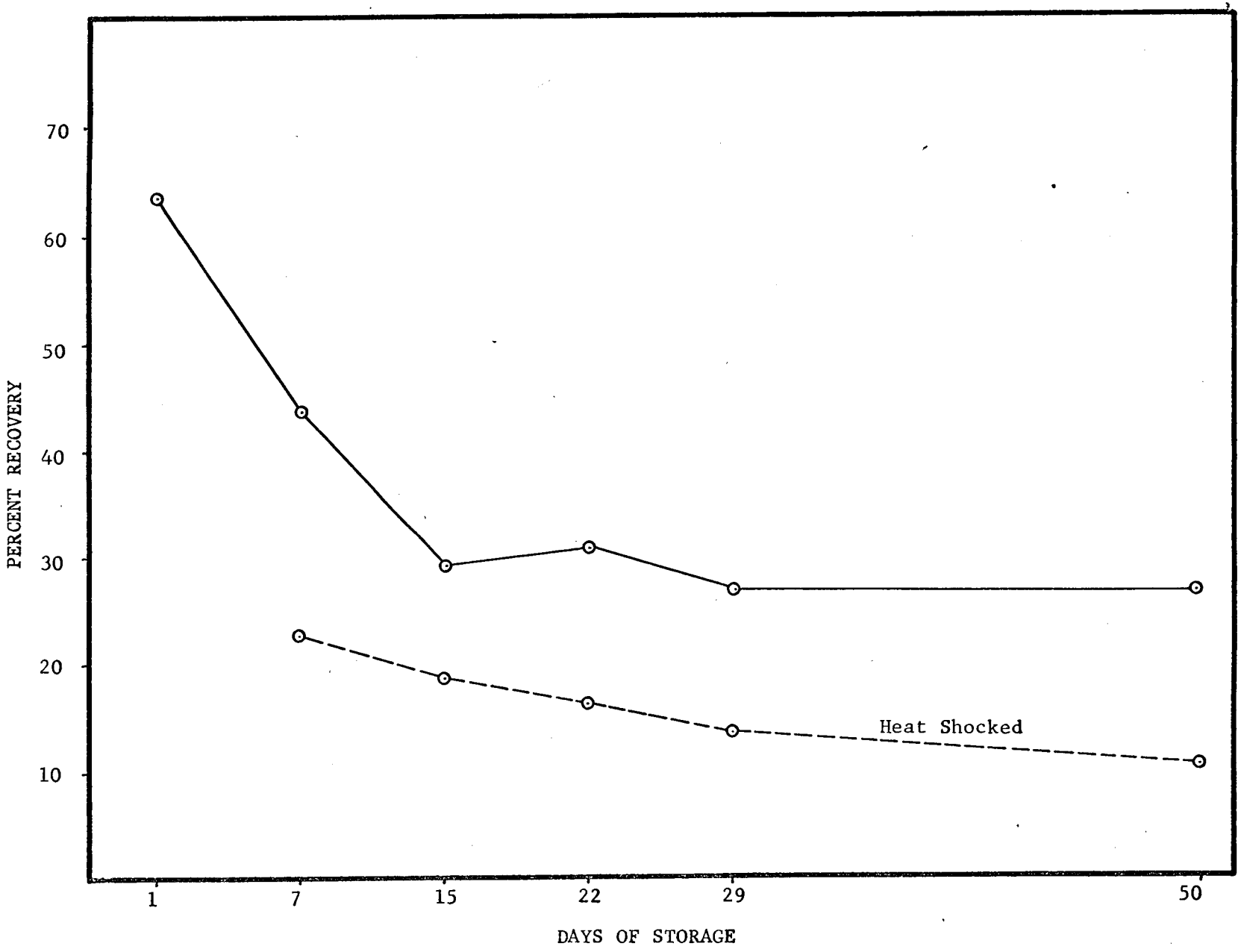

Fig. 2. Effect of heat shocking pulverized dental inlay pellets inoculated with spores of B. subtilis var. niger.

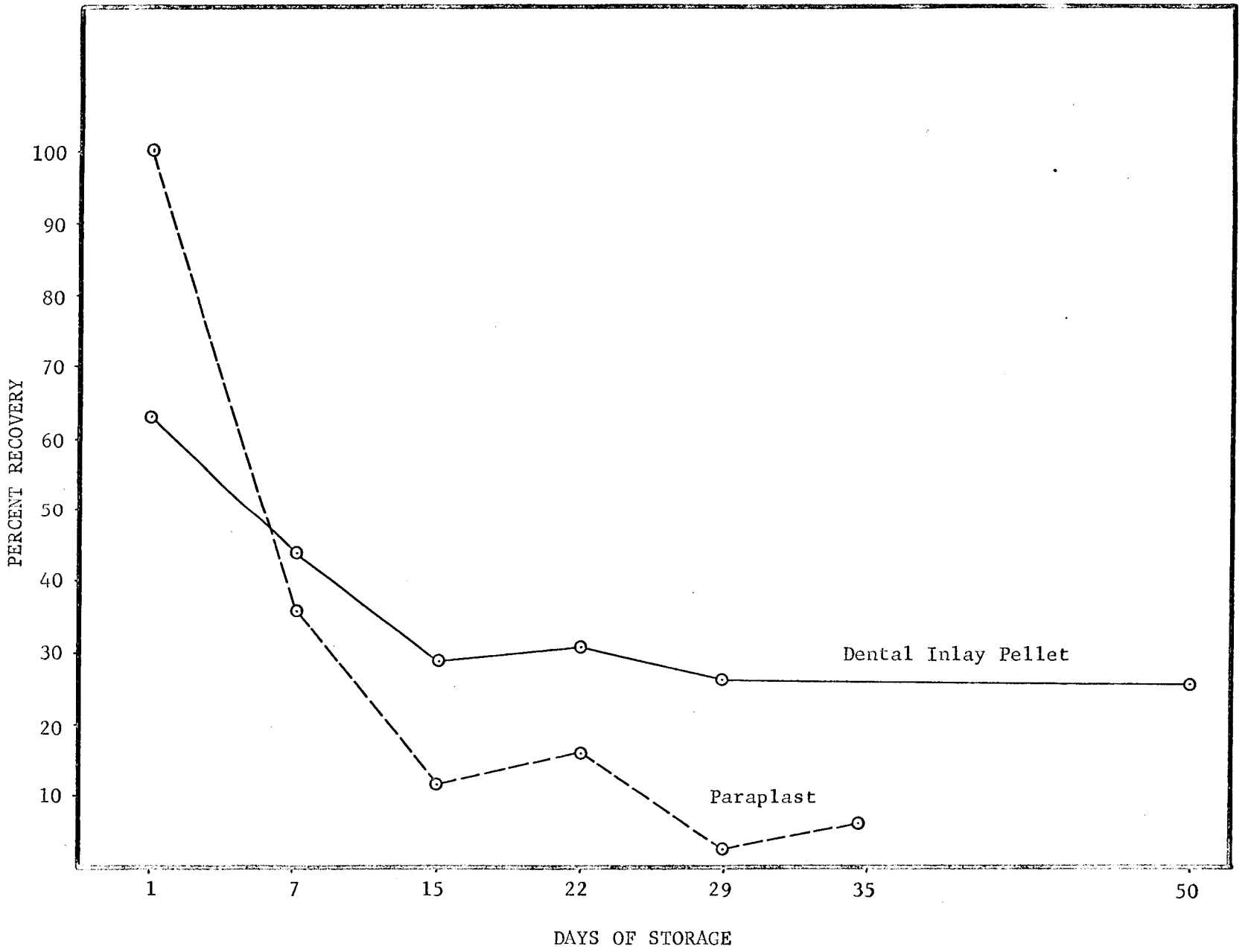

Fig. 3. Effect of storage on the survival of spores of B. subtilis var. niger embedded in paraplast and dental inlay material.

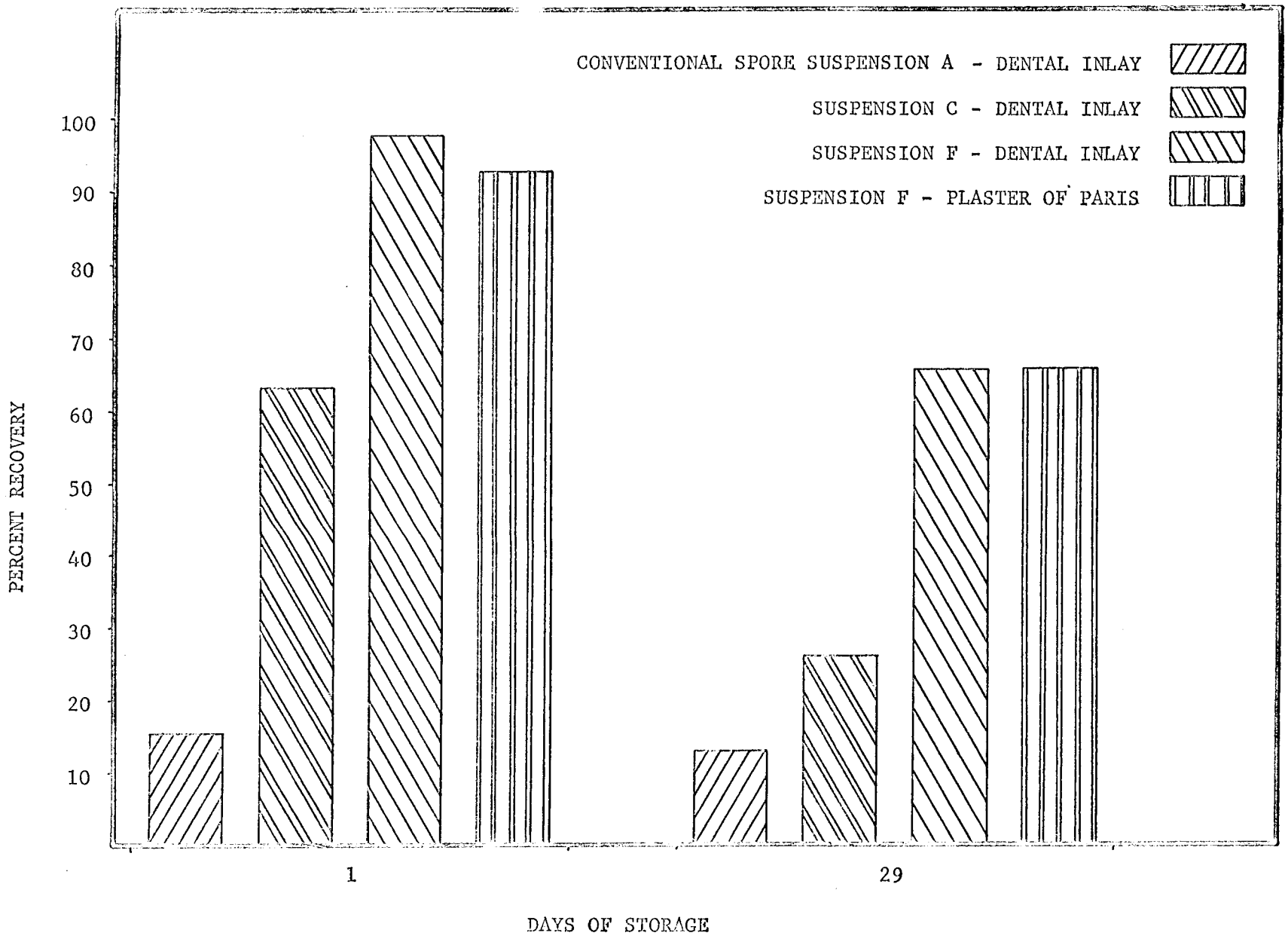

Fig. 4. Effect of physical state of spore inoculum on the survival of spores of B. subtilis var. niger embedded in solid materials.

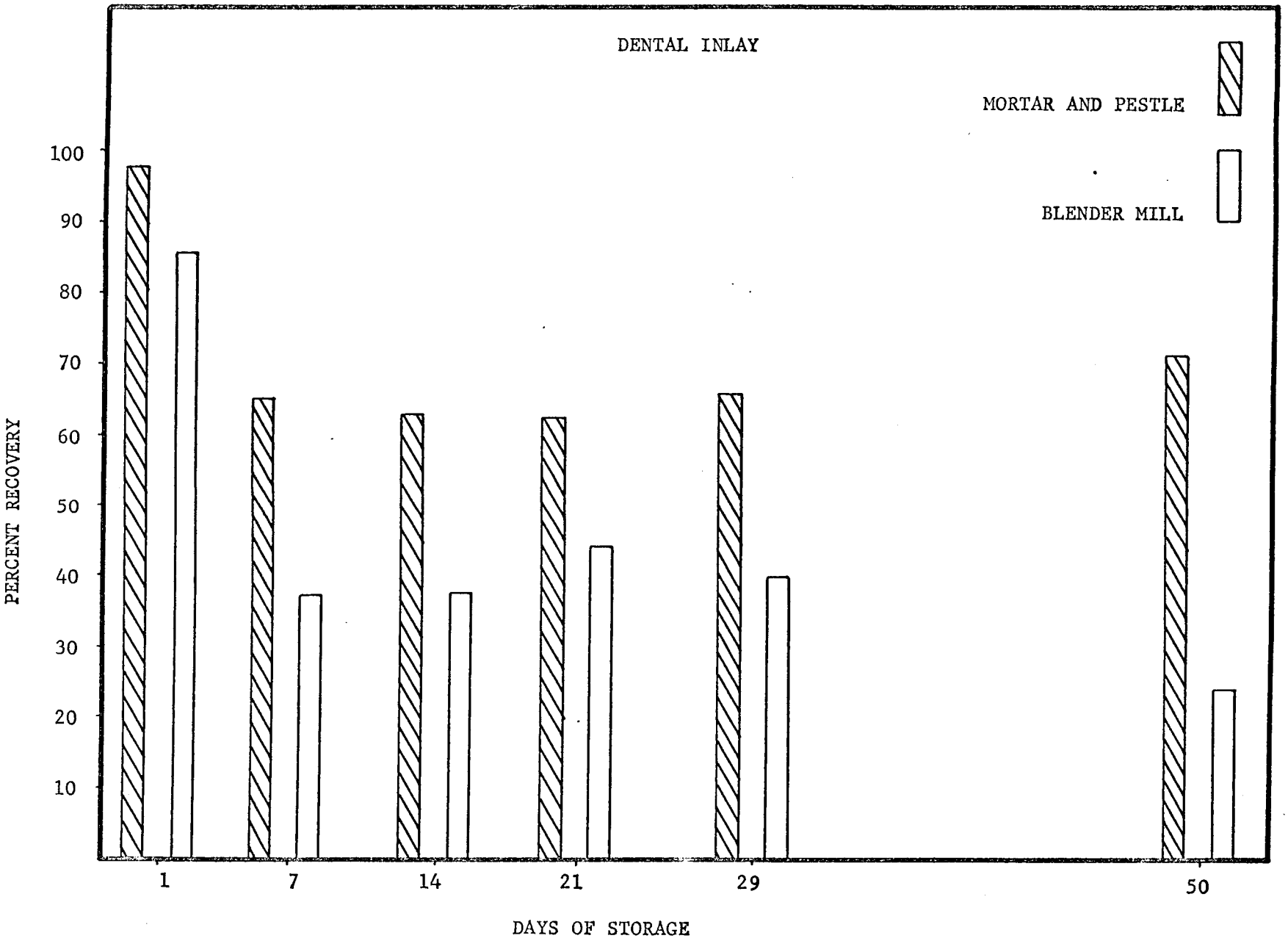

Fig. 5. Comparative recovery rates of microorganisms from dental inlay materials by mortar and pestle and blender-mill techniques.

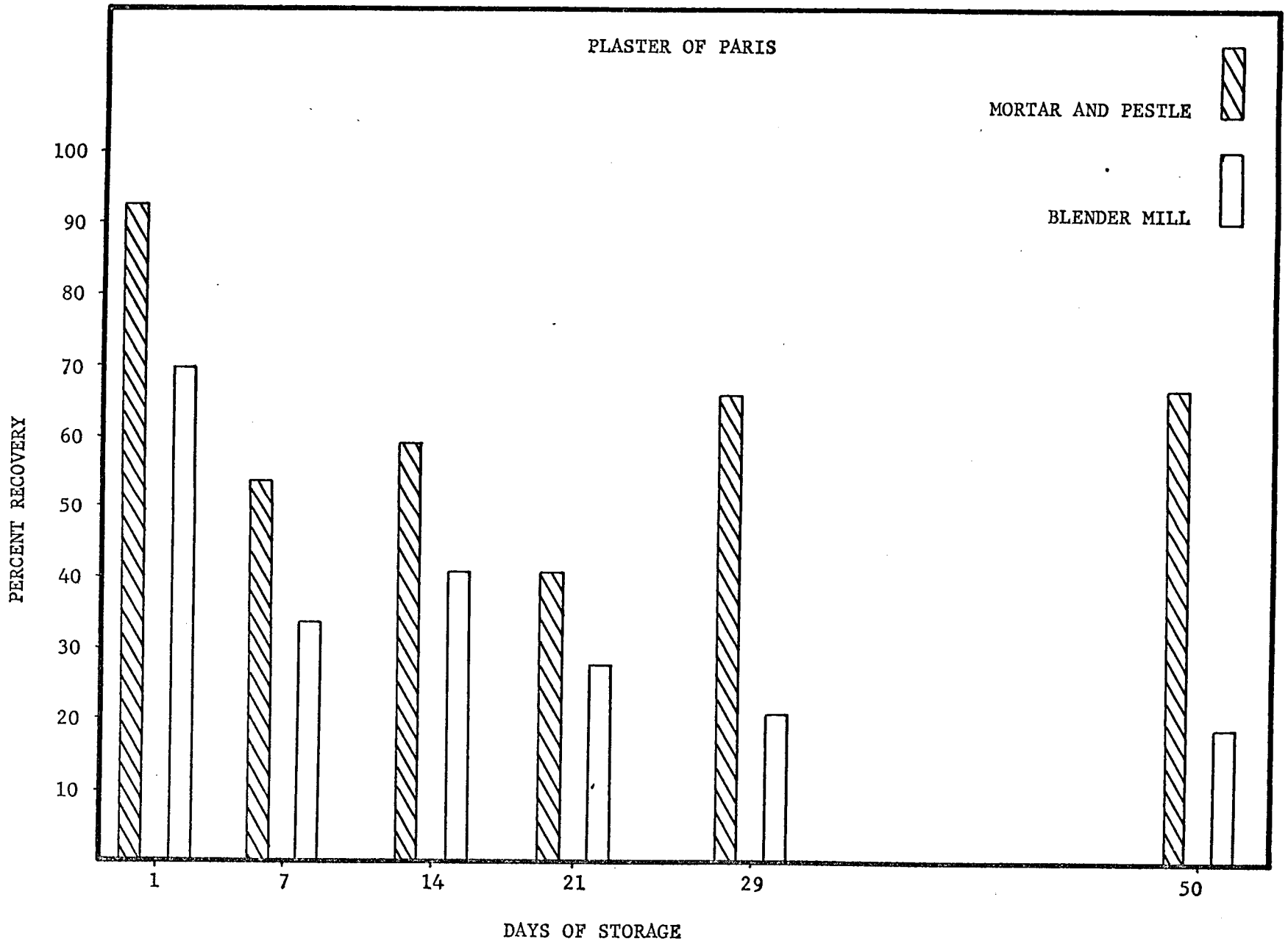

Fig. 6. Comparative recovery rates of microorganisms from plaster of Paris material by mortar and pestle and blender-mill techniques.

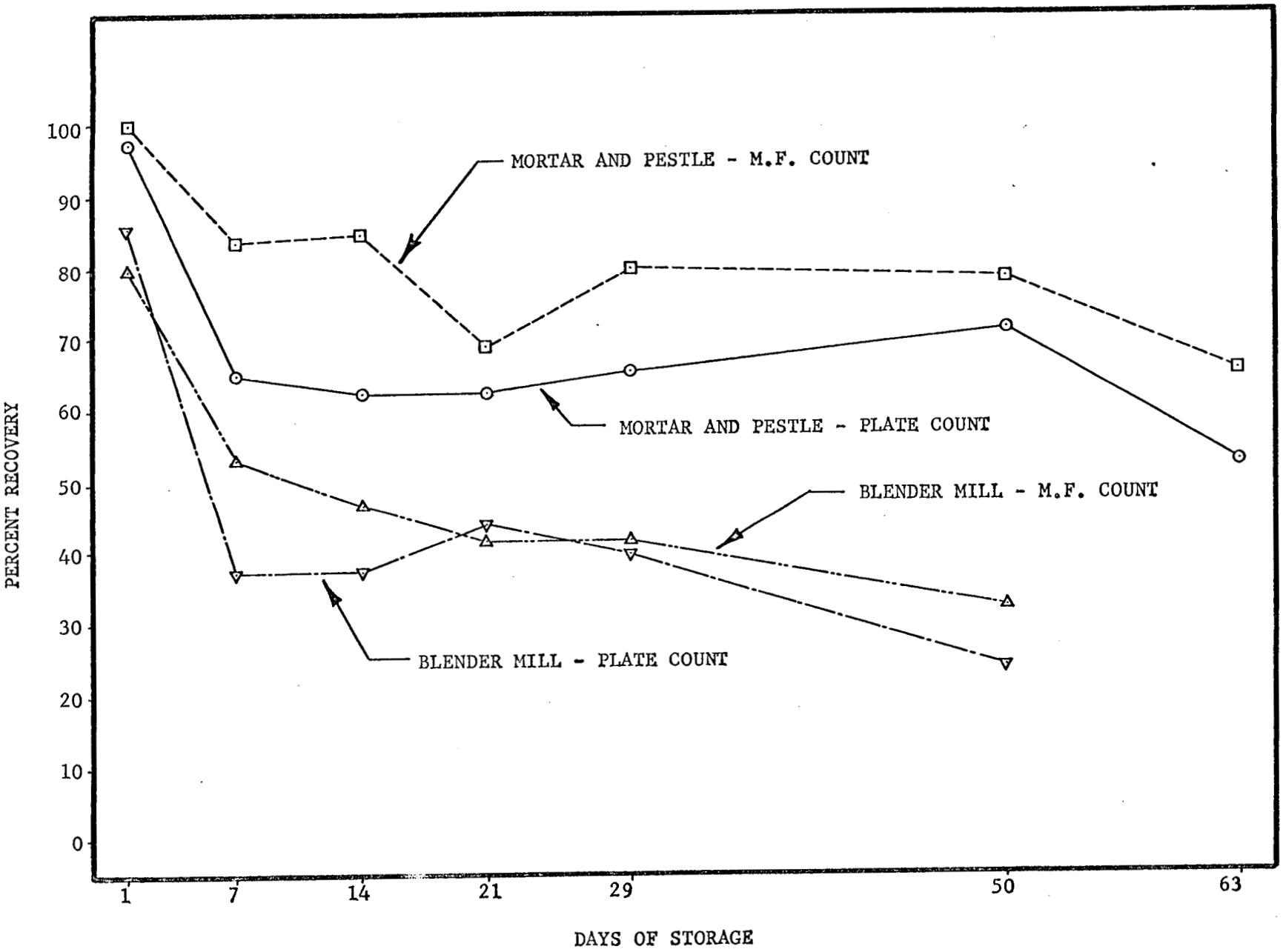

Fig. 7. Comparative rates of recovery of spores of B. subtilis var. niger from dental inlay material when assayed by the standard plate count or membrane filter techniques during various storage periods.

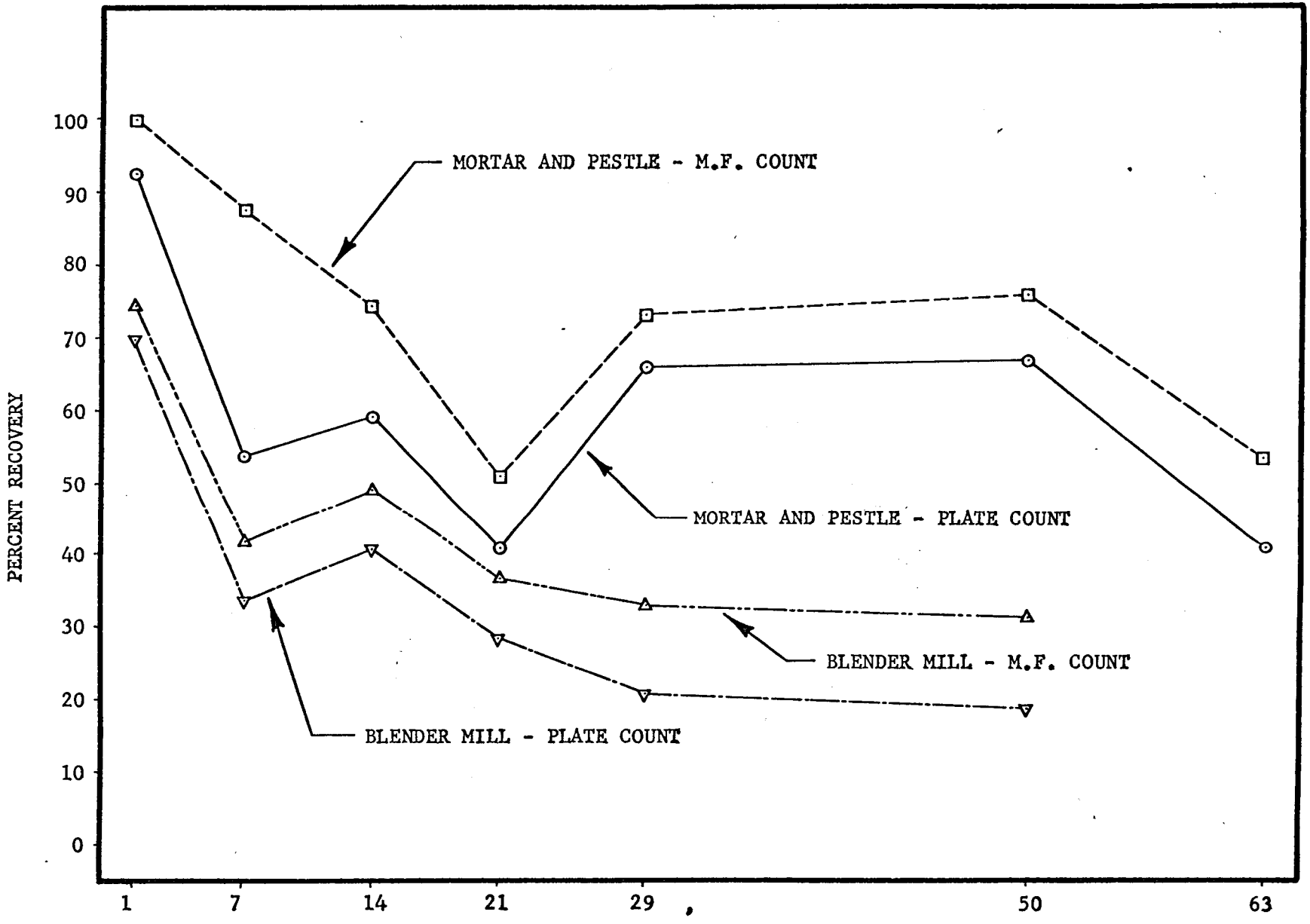

Fig. 8. Comparative rates of recovery of spores of B. subtilis var. niger from plaster of Paris when assayed by the standard plate count or the membrane filter techniques during various storage periods.