Preservation of Microbiological Assay Organisms by Direct Freezing

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The effects of freezing on bacterial cultures have been investigated by numerous workers. The most commonly used method of preserving microorganisms is lyophilization (drying from the frozen state), but this is a drastic procedure for in most instances comparatively few organisms (2 to 3 per cent) survive the freeze drying process. Cultures preserved in this way have remained viable for as long as 35 years (Engley, 1956). However, the viability of the surviving organisms is low so that frequent serial transfers for 1 to 3 weeks are needed in order to reanimate the culture before use. It was felt that the adaptability, speed, and efficiency of microbiological assay procedures could be increased if a method of preserving test organisms in a ready state could be developed.

MATERIALS AND METHODS

In developing methods for the preservation of test inocula by direct freezing, the following principles, as enumerated by Squires and Hartsell (1955), were kept in mind. "The number of bacteria surviving subfreezing temperatures will be functions of:

1. Initial numbers of viable cells present when frozen.
2. The rate of the freezing and thawing process.
3. The temperature of storage, 0 to -20 C being more destructive than below -20 C.
4. The time or duration of the storage period.
5. The physical protection offered by the men brum in which the microorganisms are frozen."

Assay organisms selected for use in preparing frozen inocula were first tested for optimal sensitivity and growth response and, if necessary, were reactivated by serial transfers.

Cultures were washed in M/15 phosphate buffer, pH 7.0 (Squires and Hartsell, 1955), and the cells resuspended in phosphate buffer containing 15 per cent glycerol (Howard, 1956) prior to freezing.

The general procedure used for preparing frozen inocula is given below (refer to table 1 for any deviations from the general method).

(1) Inoculate 100 ml of the appropriate inoculum broth with one tube (10 ml) of a fresh 16 to 18 hr culture of the test organism. Incubate for 24 hr at temperature indicated (table 1).

(2) Centrifuge and wash culture in sterile phosphate buffer, volume for volume (table 2).

(3) Resuspend washed cells to 40 per cent of original volume in buffer containing 15 per cent glycerol (table 2). Use tryptose-saline for vitamin B12. Resuspend to 20 per cent of original volume for folic acid.

(4) Adjust cell concentration of suspension, if necessary, by adding phosphate buffer containing 15 per cent glycerol so that a 1- to 10-dilution of the suspension will give a light transmittance of 40 to 45 per cent against water on a Lumetron 402E colorimeter with a M640 filter. The inoculum is now ready for freezing.

(5) Add sufficient inoculum to sterile glass ampules (5- or 10-ml capacity) for 1 day's use only. Fill using a sterile syringe and do not fill more than one-half of ampule volume.

(6) Carefully heat-seal ampules, slant, and place in a deep-freeze at -40 C. The cell suspension should take approximately 15-30 min to freeze.

(7) The test inoculum, when stored at -40 C, should give a satisfactory growth response for 6 months to 1 year or more.

(8) For assay, thaw out inoculum by placing an ampule in lukewarm water (25 C). Shake well to mix contents, aseptically withdraw 1 ml, and dilute for assay as indicated in table 1.

(9) When a satisfactory assay response is no longer obtained, the test organism may be recultured on solid media for several transfers and new inoculum prepared.

The frozen inoculum was tested along with the parent inoculum, the same day as prepared, to detect any change due to the freezing process. In addition, several test standards were run to determine the best concentration of inoculum for the assay.

The frozen inoculum was tested against a control made from a regular monthly subculture, once a month for a period of 6 months and again at the end of 1 year. All assays were incubated for 17 hr (±15 min) except the tetracycline assay which has an incubation period of 3 hr.

RESULTS AND DISCUSSION

Preliminary experiments using a shell freezing technique (Dry-Ice plus acetone) were unsuccessful. Best survival was obtained by slow freezing (15 to 30 min) as recommended by Meryman (1956).

1 Photovolt Corporation, New York, New York.
Test organisms used for vitamin assay were prepared in two ways. The cell suspension was divided equally and one portion washed before freezing; the other, just before use. Cells washed after thawing gave little or no growth response, indicating nearly 100 per cent mortality.

All cultures, except *Lactobacillus leichmannii*, remained stable when frozen in M/15 phosphate buffer 

<table>
<thead>
<tr>
<th>Assay</th>
<th>Test Organism</th>
<th>Culture Medium</th>
<th>Inoculum</th>
<th>Suspending Agent</th>
<th>Lumetron 402E Adjustment of Cell Conc</th>
<th>For Assay Dilute Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>B&lt;sub&gt;12&lt;/sub&gt;</td>
<td><em>Lactobacillus leichmannii</em> strain ATCC 7830</td>
<td>B&lt;sub&gt;12&lt;/sub&gt; inoculum broth USP (Difco)</td>
<td>24 hr</td>
<td>37 C</td>
<td>Tryptose-saline + 15% glycerol</td>
<td>1 to 10 dilution of suspension gives 40 to 45% LT*</td>
</tr>
<tr>
<td>Folic acid</td>
<td><em>Streptococcus faecalis</em> strain ATCC 8043</td>
<td>Tomato juice 20.00 ml Tryptone 0.50 g Peptone 1.00 g Liver extract conc 0.05 g Folic acid 0.05 µg Dist H&lt;sub&gt;2&lt;/sub&gt;O 100.00 ml</td>
<td>24</td>
<td>37 C</td>
<td>Phosphate buffer + 15% glycerol Resuspend cells to 20% of original vol</td>
<td>1 to 10 dilution of suspension gives 35 to 40% LT (M640 filter)</td>
</tr>
<tr>
<td>Inositol</td>
<td><em>Saccharomyces carlsbergensis</em> strain ATCC 9080</td>
<td>Yeast extract 0.4 g Malt extract broth 0.1 g Glucose 0.4 g Dist H&lt;sub&gt;2&lt;/sub&gt;O 100.0 ml</td>
<td>24</td>
<td>30 C (shaker)</td>
<td>Phosphate buffer + 15% glycerol Resuspend cells to 40% of original vol</td>
<td>1 to 10 dilution of suspension gives 40 to 45% LT (M640 filter)</td>
</tr>
<tr>
<td>Lysine</td>
<td><em>Leuconostoc mesenteroides</em> strain ATCC 8042</td>
<td>Micro inoculum broth (Difco)</td>
<td>24</td>
<td>37 C</td>
<td>None Same as above</td>
<td>1 to 10 dilution of suspension gives 40 to 45% LT (M640 filter)</td>
</tr>
<tr>
<td>Panthenol</td>
<td><em>Acetobacter suboxydans</em> strain ATCC 621H</td>
<td>Mannitol 5.0 g Yeast extract 0.5 g Peptone 0.3 g Dist H&lt;sub&gt;2&lt;/sub&gt;O 100.0 ml</td>
<td>24</td>
<td>30 C (shaker)</td>
<td>Same as above</td>
<td>1 to 10 dilution of suspension gives 10 to 15% LT (M580 filter)</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td><em>Lactobacillus arabinosus</em> strain ATCC 8014</td>
<td>Micro inoculum broth (Difco)</td>
<td>24</td>
<td>37 C</td>
<td>Same as above</td>
<td>1 to 10 dilution of suspension gives 40 to 45% LT (M640 filter)</td>
</tr>
<tr>
<td>Riboflavin</td>
<td><em>Lactobacillus casei</em> strain ATCC 7469</td>
<td>Micro inoculum broth (Difco)</td>
<td>24</td>
<td>37 C</td>
<td>Same as above</td>
<td>1 to 10 dilution of suspension gives 40 to 45% LT (M640 filter)</td>
</tr>
<tr>
<td>Tetracycline and chlorotetracycline</td>
<td><em>Micrococcus pyogenes</em> var. <em>aureus</em> strain ATCC 6538 P</td>
<td>Penassay seed agar (Difco)</td>
<td>24</td>
<td>37 C</td>
<td>Same as above</td>
<td>0.1 to 10 dilution of suspension gives 40 to 45% LT (M640 filter)</td>
</tr>
</tbody>
</table>

* LT = Light transmittance.
† Difco Laboratories, Inc., Detroit, Michigan.
‡ RT = Room temperature.
TABLE 2
Solutions needed for preparation of frozen cultures

<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer pH 7.0 (m/15)</td>
<td></td>
</tr>
<tr>
<td>Monopotassium phosphate KH₂PO₄</td>
<td>79.0 mg</td>
</tr>
<tr>
<td>Dipotassium phosphate K₂HPO₄</td>
<td>100.0 mg</td>
</tr>
<tr>
<td>Distilled water q.s</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Phosphate buffer pH 7.0 with 15 per cent glycerol.
To make buffer containing 15 per cent glycerol add 15 ml of glycerol to above ingredients and dilute to 100 ml with distilled water.
Adjust pH, if necessary, to pH 7.0 ± 0.1.

Tryptose-saline (suspending medium for B₁₂ inoculum)
Bacto-tryptose                         2.0 g
Sodium chloride                        0.5 g
Glycerol                               15.0 ml
Distilled water q.s                   100.0 ml
Adjust pH, if necessary, to pH 6.8 ± 0.1.
Sterilize all solutions at 121 C for 15 min.

Figure 1. Response of Lactobacillus leichmannii strain ATCC 7830 to vitamin B₁₂ after storage at −40 C for several months. The control represents the growth response of the test organism before freezing.

Figure 2. Response of Streptococcus faecalis R strain ATCC 8043 to folic acid after storage at −40 C for several months. The control represents the growth response of the test organism before freezing.

Figure 3. Response of Saccharomyces carlsbergensis strain ATCC 9080 to inositol after storage at −40 C for several months. The control represents the growth response of the test organism before freezing. Regular inoculum is the inoculum prepared from a fresh 16- to 24-hr test culture.
fluctuation in growth response. However, since the control (regular inoculum) responded in the same way, these differences in growth response are probably the result of variations in the nutrient qualities of the laboratory prepared media. These fluctuations were not evident in those assays where commercially prepared media were used (figures 1, 2, 4, and 6-8).

It is interesting to note that Streptococcus faecalis (figure 2) gave a greater growth response at the end of 1 year's storage at -40 C than the original culture.

![Figure 4](https://example.com/figure4.png)  
*Figure 4. Response of Leuconostoc mesenteroides strain ATCC 8042 to L-lysine after storage at -40 C for several months. The control represents the growth response of the test organism before freezing.*

![Figure 5](https://example.com/figure5.png)  
*Figure 5. Response of Acetobacter suboxydans strain ATCC 621H to pantothenol after storage at -40 C for several months. The control represents the growth response of the test organism before freezing. Regular inoculum is the inoculum prepared from a fresh 16- to 24-hr test culture.*

![Figure 6](https://example.com/figure6.png)  
*Figure 6. Response of Lactobacillus arabinosus strain ATCC 8014 to calcium pantothenate after storage at -40 C for several months. The control represents the growth response of the test organism before freezing.*

![Figure 7](https://example.com/figure7.png)  
*Figure 7. Response of Lactobacillus casei strain ATCC 7469 to riboflavin after storage at -40 C for several months. The control represents the growth response of the test organism before freezing.*
Sarcina lutea strain ATCC 9341 also exhibited an accelerated growth rate after 3 months’ storage at $-40$ C. This increased growth rate was so pronounced that the amount of inoculum for assay was reduced by one-third. This effect may be the same as that reported by Squires and Hartsell (1955), who found that thawed cells of Escherichia coli grew at a much faster rate than the parent culture. This was believed to be due to the accumulation of stimulating materials within the cell during frozen storage at $-9$ C. This delayed response for S. faecalis and S. lutea could well be the result of the lower temperature of storage ($-40$ C) resulting in a slower rate of assimilation of stimulating materials. No significant differences in assay results were noted on comparing the frozen inoculum with the regular inoculum. With the exception of Lactobacillus casei (figure 7), all the organisms tested maintained a satisfactory assay response for 1 year or more (figures 1–4, 6, and 8). Further studies now in progress indicate that the test organisms may retain a satisfactory level of viability for as long as 2 years.

**SUMMARY**

The direct freezing of test organisms in an appropriate suspending agent has made it possible to keep test cultures on hand, ready for instant use. Test inoculum, frozen and stored at $-40$ C, maintained a satisfactory growth response for periods ranging from 6 months to 1 year or more.

Main advantages of this technique are: (a) Ability to run assays at any time without the necessity of preparing daily inoculum or maintaining the sensitivity of test organisms. This would be a great advantage to those laboratories that do microbiological assays only occasionally. (b) Standardization of assay responses. Day to day fluctuations in growth responses would be largely eliminated as well as invalid assays due to inoculum failure.

**REFERENCES**


