Liquid Nitrogen Freezing in Microbiological Assay Systems

I. Preservation of Lactobacillus leichmannii for Direct Use in the Vitamin B₁₂ Assay

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ABSTRACT

Sokolski, W. T. (The Upjohn Co., Kalamazoo, Mich.), E. M. Stapert, and E. B. Ferrer. Liquid nitrogen freezing in microbiological assay systems. I: Preservation of Lactobacillus leichmannii for direct use in the vitamin B₁₂ assay. Appl. Microbiol. 12:327-329. 1964.—Suspensions of Lactobacillus leichmannii were stored in liquid nitrogen and were used as direct inocula in vitamin B₁₂ assays. Complete recovery of viable cells was obtained when the suspensions in basal B₁₂ medium were rapidly frozen by direct immersion into liquid nitrogen and rapidly thawed by agitating the suspensions in a water bath at 40 C. Greater than 90% destruction occurred when the suspensions were in saline. However, both suspensions were usable in the B₁₂ assay system. Assay results on a number of test materials indicated good correlation between freshly prepared suspensions and frozen suspensions in basal medium stored 3 months. Suspensions in saline stored for 1 year in liquid nitrogen showed no detectable difference from the first day after freezing. Suspensions frozen slowly at the rate of 1 degree per min from 4 to −40 C and subsequently immersed in liquid nitrogen had a longer lag period of growth and were not usable in the 18-hr assay incubation system. A major advantage of a stored inoculum for direct use in a microbiological assay is the reduced day-to-day variation in the inoculum.

Microorganisms may be preserved for long periods of time in many ways. The most widely used methods involve preservation in soil (Greene and Fred, 1934), under mineral oil (Morton and Pulaski, 1938), under paraffin oil (Ungermann, 1918; Hartsell, 1953), by freeze-drying (Keilin, 1959), and with direct freezing (Moline et al., 1963). Most of the methods are used for the maintenance of cultures in collection centers or of assay organisms for microbiological assays (Tanguay, 1959; Scholes, 1961). More recently, a combination of direct freezing and low-temperature storage has gained prominence for the preservation of semen and other biological materials. Since liquefied gases, particularly nitrogen, have become readily available, the use of low-temperature storage has gained impetus. In this paper, we describe the use of Lactobacillus leichmannii ATCC 7830 after freezing and storing in liquid nitrogen as a direct inoculum in vitamin B₁₂ assays.

Materials and Methods

L. leichmannii ATCC 7830 was cultured at 37 C for 18 hr in broth (U.S. Pharmacopeia, 16th ed., p. 888-892) containing: yeast extract, 0.75 g; peptone, 0.75 g; glucose, 1.0 g; monopotassium phosphate, 0.2 g; distilled water, 60 to 70 ml; clarified tomato juice, 10 ml; and polysorbate 80, 1 ml. The pH was adjusted to 6.8, and distilled water was added to 100 ml.

Tubes of fresh broth were inoculated with 1 drop of 18-hr culture per tube and were incubated for 7 hr at 37 C. The cultures were centrifuged, and the cells for each experiment were pooled and washed twice with 10-ml samples of 0.9 % saline or with vitamin B₁₂ basal medium (B₁₂-free) (U.S. Pharmacopeia, 16th ed., p. 888-892). The cells were resuspended to the original volume with the wash liquid. Each suspension was dispensed into 1.2-ml glass ampoules (Kimax Breeder, Owens Illinois Co., Toledo, Ohio), which were then immersed directly into liquid nitrogen.

In one experiment, the effect of rate of freezing on suspensions in saline was studied. One sample of a saline suspension was rapidly frozen by immersing ampoules of the suspension directly into liquid nitrogen, and another sample was subjected to a low freeze, the temperature being lowered at the rate of 1 degree per min from 4 to −40 C, and then the vials were immersed in liquid nitrogen. Both sets of ampoules were rapidly thawed by immersing them with agitation in a water bath at 40 C. These sets were compared with a fresh inoculum in the vitamin B₁₂ assay (Fig. 1).

To determine the effect of nitrogen freezing on the viability of L. leichmannii in saline and in basal medium, viable counts of suspensions were made before and after freezing. One sample of a 7-hr culture was washed twice and resuspended in saline, and another sample was washed twice and resuspended in basal medium. Samples for viable counting were taken from the 7-hr culture before washing, the saline and medium suspensions before freezing, and suspensions immersed in liquid nitrogen for 1 min and for 1 hr. Viable counts were determined by the usual pour-plate technique, with water as a diluent for diluting the suspensions and Tomato Juice Agar (Difco) as the plate medium. All plates were incubated at 37 C for 48 hr.

The frozen suspensions were compared with freshly prepared suspensions in the vitamin B₁₂ assay. All assays were run by the turbidimetric method described in the U.S. Pharmacopeia. Each fresh suspension was prepared on the day of assay. Each frozen suspension was removed from the liquid nitrogen and thawed rapidly by immedi-
ately immersing with agitation into a water bath at 40 C. The same batch of medium was used in assay for both suspensions on a given assay day, and each batch was used for 3 days.

A comparison of stored frozen suspensions in basal medium with freshly prepared suspensions in saline was made under routine quality-control assay conditions; 35 different preparations were assayed, 5 per each of 7 assay days, with both suspension. The standard solutions and media used were the same for both sets of assays on each assay day. Potencies of the products were estimated from standard dose-response curves for each suspension.

**RESULTS**

The viable count results (Table 1) indicated that the rapid-freeze and rapid-thaw process rendered more than 90% of organisms in saline nonviable and had little effect, if any, on the organisms in basal medium. Despite the marked reduction of viable cells, the saline suspension was still usable for assay. The usual 21-hr incubation period gave a lower dose-response curve than did the daily fresh suspension, but an additional 4-hr incubation resulted in a response close to the fresh suspension response (Fig. 1). The suspension which was slow-frozen gave a much lower response and slope (Fig. 1), and was considered not to be usable with this incubation period.

**TABLE 1. Viable counts on Lactobacillus leichmannii suspensions before and after storage in liquid nitrogen**

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Viable cells per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline suspension before freezing</td>
<td>$1.0 \times 10^8$</td>
</tr>
<tr>
<td>Saline suspension frozen 1 min</td>
<td>$1.6 \times 10^4$</td>
</tr>
<tr>
<td>Saline suspension frozen 1 hr</td>
<td>$3.7 \times 10^6$</td>
</tr>
<tr>
<td>Medium suspension before freezing</td>
<td>$1.1 \times 10^8$</td>
</tr>
<tr>
<td>Medium suspension frozen 1 min</td>
<td>$8.9 \times 10^7$</td>
</tr>
<tr>
<td>Medium suspension frozen 1 hr</td>
<td>$1.1 \times 10^8$</td>
</tr>
</tbody>
</table>

* Rapid-freeze and rapid-thaw process.

**FIG. 1. Standard dose-response curves with Lactobacillus leichmannii in saline.**

**FIG. 2. Standard dose-response curves with Lactobacillus leichmannii comparing a fresh suspension with a 3-month frozen suspension.**
The rapid-frozen bacterial suspension in saline was tested on 8 assay days at various intervals after storage in liquid nitrogen up to a period of 1 year. All assays, including the one after 1 year of storage, gave almost the same responses shown in Fig. 1.

The bacterial suspension in basal medium was compared with the daily fresh suspension in saline periodically after storage in liquid nitrogen for up to a period of 3 months. The dose-response curves for both suspensions were nearly alike. Figure 2 shows the comparison between the fresh suspension in saline and the suspension in medium after 3 months of storage in liquid nitrogen. Note that the curves are almost parallel, even to the smallest dose.

The results of the 35 multiple vitamin products assayed with the frozen suspension compared favorably with the routine product assay system with fresh suspensions. There was no trend of bias. The comparative results on 16 of the products were within 1% of each other, and all except 2 products were within 3%. The largest variation was with one lot of a multiple vitamin tablet which assayed 2.40 μg of B₁₂ per tablet with the fresh suspensions and 2.65 μg with the frozen inoculum.

**Discussion**

The rapid-freezing process appeared to sustain suspensions of _L. leichmannii_ in saline for the vitamin B₁₂ assay better than a slow-freezing process, as evidenced by the dose response curves in Fig. 1. The cells in the slow-freeze process grew poorly, probably owing to a reduction in numbers. This is contrary to the general opinion that a slow cool (1 to 5°C per min) and a rapid thaw give the highest survival rate of cells. However, we have only studied two cooling rates, and these were done only in saline. We do not contend that the rapid freeze would give the better recovery of _L. leichmannii_ in all media. Optimal cooling rates and media were reported to vary with different organisms.

Evidence for the complete recovery of viable cells of _L. leichmannii_ in basal medium with the rapid-freeze and rapid-thaw process is given in Table 1. This treatment permits the storage of vitamin B₁₂ assay inoculum for use directly into the assay system. The comparative dose-response curves between the frozen suspensions in basal medium and the freshly prepared suspensions in saline were nearly alike in every assay. Figure 2 shows parallel curves with some distance between them. That they were not superimposable may be attributed to the efficiency in washing the suspensions and residual B₁₂ in the suspensions. Note that the same difference occurs at the smallest B₁₂ dose, which indicates that the fresh saline suspension contained more B₁₂ than did the frozen suspension. The washing step is probably critical because of the relatively small amounts of the vitamin needed for growth.

Stored frozen suspensions would be usable in the vitamin B₁₂ assay even if the kill-rate was as much as 90%. Our first efforts at rapid freezing with suspensions in saline gave usable lower dose-response curves with the same assay conditions (Fig. 1).

There are a number of advantages for a stable microbial suspension ready for use directly into the assay system. The day-to-day variation in inoculum is reduced. Time is saved in the daily preparation of inoculum and in the maintenance of the test organism. Once the stored inoculum is prepared, the assay procedures may be followed without elaborate detail concerning care and preparation of the test organism. The test organism may be regarded as another reagent, and any analytical technician may run the assay. Time is saved in training technicians to run the assay.

**Acknowledgments**

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**Literature Cited**


