Death of *Lactobacillus bulgaricus* Resulting from Liquid Nitrogen Freezing

R. B. SMITTLE, S. E. GILLILAND, AND M. L. SPECK

*Department of Food Science, North Carolina State University, Raleigh, North Carolina 27607*

Received for publication 9 June 1972

Concentrated cell suspensions of *Lactobacillus bulgaricus* prepared from cells grown in semisynthetic media were frozen in liquid nitrogen. After storage for 24 hr, the cell suspensions were found to have decreased colony counts and acid-producing capacity in milk. The amount of loss varied among the different strains tested. The addition of known cryoprotective agents to cell suspensions of the most labile strain before freezing provided little or no protection to the cells. However, storage stability of all strains investigated was improved by supplementing the growth medium with Tween 80 (polyoxyethylene sorbitan monooleate). The concentration of Tween 80 necessary for maximal storage stability varied among strains.

Concentrated preparations of starter bacteria are being used successfully in the manufacture of various cultured milk products. Lactic streptococci (1, 7, 10, 12) and leuconostocs (6) can be successfully preserved and used after freezing and storage in liquid nitrogen. Optimal performance by the cultures is dependent on the stabilization of their viability and biological activity during freezing and storage. In projecting the use of concentrated starter cultures for additional foods, it is reasonable to expect that concentrated cultures of lactobacilli could find useful applications, particularly in the manufacture of products such as yogurt. Certain lactobacilli are not sufficiently resistant to freezing to permit their use as frozen concentrated starter cultures, especially for the direct inoculation of the final milk without intermediate starter preparation. In this study we report on factors involved in the susceptibility of *Lactobacillus bulgaricus* to freezing, and on means whereby the organism can be made more resistant to freezing and storage in liquid nitrogen.

**MATERIALS AND METHODS**

*Cultures*. The strains of *L. bulgaricus* selected for this study are used commercially in yogurt and Italian cheese manufacture. The cultures were routinely propagated in sterile litmus milk. A 1% inoculum was used with incubation at 37°C for 15 hr, and cultures were stored in a refrigerator between transfers. Three daily transfers in litmus milk were made before use in an experiment.

**Growth media**. The cultures were grown in lactobacilli MRS broth (Difco) or in a medium containing 2% tryptone (Difco), 1% yeast extract (BBL), and 2% lactose. The latter medium was designated as TYL and was used as the basal medium to study the effects of medium composition. In some experiments, this medium was supplemented with 0.5% (w/v) corn steep solids (TYLCS).

**Concentrate preparation**. Cultures of *L. bulgaricus* were grown statically in 100 ml of the test media (milk dilution bottles) at 37°C for 12 hr. The cultures were cooled in an ice-water bath for 10 min, and the cells were recovered by centrifugation for 20 min at 6,780 × g and 0°C. The resulting pellet was resuspended in sufficient cold sterile 10% (w/v) nonfat milk solids (NFMS) to produce a concentrated culture having approximately 10⁸ cells per ml. In experiments involving the use of cryoprotective agents, the cell pellets were resuspended in sterile NFMS (20%, w/v) and then diluted 1:1 with aqueous solutions of the protective agents.

The additives (final concentrations) investigated for their protective abilities were 4 and 2% monosodium glutamate; 10% glycerol; 5.0, 1.0, 0.4, 0.2, 0.1, and 0.05% Tween 80 (polyoxyethylene sorbitan monol-oleate); 2% polyvinylpyrrolidone; and an equal mixture of 2.5% dimethylsulfoxide and 2.5% glycerol.

The concentrated cell suspensions were aseptically weighed (1-g quantities) into 1.5-ml sterile plastic vials. The vials were sealed with a 1:1 mixture of acetone and chloroform, and were then submerged and held in liquid nitrogen (−196°C) for 24 hr.

**Colony counts**. Colony counts were determined before and after freezing by the pour-plate method. The vials of frozen cells were thawed by immersion
in 1 liter of tap water at 17 C for 5 min. The initial dilution (1:100) was made by adding 1 g of cell suspension to 99 ml of cold 10% NFMS (steamed for 30 min on the day of use). Additional dilutions were prepared with sterile 99-ml dilution blanks containing 0.1% NFMS and 0.01% silicone antifoamer (Sigma Chemical Co.). Duplicate plates were prepared from the required dilutions. The plating media and incubation times were selected to maximize the count for each strain. The YEPT plating medium contained 5 g of yeast extract (BBL), 5 g of Phytone (BBL), 5 g of Trypticase (BBL), 5 g of dextrose, 4 g of K₂HPO₄, 15 g of agar, and 1,000 ml of distilled water. The mixture was adjusted to pH 7.0 before autoclaving at 121 C for 15 min. This medium was used for L. bulgaricus strains NCS1 and NCS2. Lactic agar (3) was employed for strain NCS3. The medium of Rogosa et al. (11), modified by omitting the acetic acid and autoclavng at 15 min at 121 C, was used for strain NCS4. The plates for strain NCS1 were incubated for 48 hr at 37 C; those for the remaining strains were incubated for 72 hr at 37 C. All colonies were counted with the aid of a Quebec colony counter.

**Acid production.** Portions (10 ml) of the initial dilution of the cell suspensions were incubated at 45 C for 4 hr. The acid produced in each sample was measured by titration with 0.1 N NaOH to pH 8.6 by use of a Radiometer automatic titrator. Compensation was made for titratable acid present in uninoculated 10% NFMS. The percent acid production lost was then calculated by the following formula:

\[
\text{percent acid production lost} = \frac{\text{acid produced by unfrozen cells} - \text{acid produced by frozen cells}}{\text{acid produced by unfrozen cells}} \times 100
\]

**RESULTS**

**Effect of liquid nitrogen freezing on L. bulgaricus.** Cultures varied in their stability to freezing and storage at ~196 C (Table 1). Strain NCS1 was very liable to freezing, whereas NCS4 was essentially unaffected. Since strain NCS1 was the most sensitive to freezing, it was chosen for further study.

**Freezing menstruum additives.** The addition of 2.0% monosodium glutamate, 10% glycerol, or 2.0% polyvinylpyrrolidone to the menstrum provided little or no protection of the cells against freezing (Table 2). The number of lactobacilli killed was slightly less in the samples supplemented with 4% monosodium glutamate and a mixture of 2.5% glycerol and dimethylsulfoxide. The mixture of glycerol and dimethylsulfoxide afforded slight protection of the acid-producing capacity. However, the apparent protection resulting from these additives was not great enough to be significant in preserving the cultures.

**Effects of growth medium composition on storage stability.** The effects of growth medium composition on stability of cells to subsequent freezing and storing in liquid nitrogen was examined in further attempts to improve the stability of the lactobacilli. Cells of L. bulgaricus NCS1 grown in lactobacilli MRS broth were resistant to freezing, whereas those grown in TYLCS were not (Table 3). The major differences in these media with respect to the types of ingredients appeared to be the presence of Tween 80, supplementary mineral salts, and buffer in the MRS medium. Based on this information, MRS broth was prepared with these components omitted, and TYLCS was supplemented with the components singly and combined. The results (Table 3) revealed that 0.1% Tween 80 was the ingredient primarily responsible for the resistance to freezing. supplementation with ammonium citrate or MgSO₄, improved resistance to some extent.

**Relationship of growth time to storage stability.** Since the incubation time for

---

**Table 1. Stability of Lactobacillus bulgaricus cultures to freezing in liquid nitrogen**

| Culture             | Storage time | 1 day | 2 days |
|---------------------|--------------|-------|--------|
|                      | Death (%)    | Acid production lost (%) | Death (%)    | Acid production lost (%) |
| L. bulgaricus NCS1  | 95           | 73    | 99     | 69     |
| L. bulgaricus NCS3  | 54           | 31    | 72     | 32     |
| L. bulgaricus NCS4  | 0            | 8     | 0      | 8      |

* Cultures had been grown in TYLCS before freezing.

**Table 2. Effects of cryoprotective agents on the stability of Lactobacillus bulgaricus NCS1 during frozen storage**

| Freezing menstruum additive | Death (%) | Acid production (%) |
|-----------------------------|-----------|---------------------|
| None                        | 87        | 47                  |
| Monosodium glutamate, 2.0%  | 92        | 60                  |
| Monosodium glutamate, 4.0%  | 73        | 51                  |
| Glycerol, 10%               | 89        | 43                  |
| Polyvinylpyrrolidone, 2.0%  | 96        | 64                  |
| Glycerol, 2.5% + dimethylsulfoxide, 2.5% | 86 | 36 |

* Cells grown in TYLCS; suspended in 10% nonfat milk solids for freezing.
The instability of concentrated cultures of *L. bulgaricus* to freezing in liquid nitrogen is in contrast to characteristics reported for concentrated cultures of lactic streptococci and *Leuconostoc citrovorum* (1, 6, 7, 10, 12). The unsatisfactory storage stability of the lactobacilli imposes severe restrictions on the commercial use of concentrated cultures prepared from them.

The use of additives to protect bacterial cells from damage due to freezing has been investigated. For these experiments, the cells were grown in TYL broth and resuspended in sterile 10% NFMS containing various levels of Tween 80. The incorporation of 0.05% Tween 80 into the freezing menstruum did not protect *L. bulgaricus* NCS1 from damage caused by freezing. Use of the TYLCS medium was discontinued at this point because the variable composition of corn steep did not permit a predictable yield of cells.

### Table 3. Effects of growth medium composition on stability of Lactobacillus bulgaricus NCS1 to freezing and storage in liquid nitrogen

| Growth medium | Supplement                        | Death (%) |
|---------------|-----------------------------------|-----------|
| TYLCS         | None                              | 68        |
| MRS           | None                              | 5         |
| MRS minus minerals and Tween 80 | Minerals and Tween 80  | 2         |
| TYLCS         | Tween 80                          | 5         |
| TYLCS         | Sodium acetate                    | 69        |
| TYLCS         | Ammonium citrate                  | 44        |
| TYLCS         | *MgSO₄*                           | 59        |
| TYLCS         | *MnSO₄*                           | 79        |
| TYLCS         | *K₂HPO₄*                          | 83        |

Growing the cultures in media with and without Tween 80 was 12 hr (late exponential phase), the possibility existed that the age of the culture might affect their stability to freezing. Based on this, experiments were conducted in which cells grown in TYLCS and TYLCS plus Tween 80 were harvested at various intervals and frozen to evaluate the effect of age on their stability. The results indicated no difference in the freezing stability of cells harvested during a range of 8 to 14 hr of growth.

### Table 4. Tween 80 supplementation of growth medium and its effects on stability of Lactobacillus bulgaricus to freezing in liquid nitrogen

| Culture            | Growth medium | Death (%) | Acid production lost (%) |
|--------------------|---------------|-----------|--------------------------|
| *L. bulgaricus* NCS1 | TYL           | 67        | 55                       |
| *L. bulgaricus* NCS2 | TYL           | 83        | 32                       |
| *L. bulgaricus* NCS3 | TYL           | 38        | 9                       |
| *L. bulgaricus* NCS4 | TLY           | 65        | 70                       |
|                    | TLYT80        | 49        | 40                       |

*Cells suspended in 10% nonfat milk solids for liquid nitrogen freezing.*

*TYL medium plus 0.1% Tween 80.

### Table 5. Relationship of Tween 80 concentration in the growth medium to the stability of Lactobacillus bulgaricus NCS3 frozen in liquid nitrogen

| Concen of Tween 80 (%) | Death (%) |
|------------------------|-----------|
| 0                      | 67        |
| 0.025                  | 15        |
| 0.050                  | 21        |
| 0.100                  | 46        |
| 0.200                  | 50        |

*Cultures had been grown in TYL with and without Tween 80 before freezing.*
extensively studied (2, 4, 5, 8, 9; D. A. Gabis, Ph.D. thesis, North Carolina State Univ., Raleigh, 1970). Glycerol and dimethylsulfoxide are believed to protect cells by reducing electrolyte concentrations around the cells during freezing (8). The lack of response to 10% glycerol and the poor response to 2.5% glycerol and dimethylsulfoxide indicated that storage instability of lactobacilli is probably not due to an excessive electrolyte during freezing. Morichi et al. (9) found that L. bulgaricus was not adequately protected by glutamic acid during the process of freeze-drying; our results with monosodium glutamate were similar. Although monosodium glutamate and glycerol provided some protection, the concentrated cell suspensions of L. bulgaricus were not sufficiently protected for commercial application.

The mechanism whereby Tween 80 imparts freezing stability to the lactobacilli has not been elucidated. Calcott and Postgate (2) reported that Tween 80 incorporated into the freezing menstrum was effective in protecting Aerobacter aerogenes from freezing damage. The results from our study, however, indicate that Tween 80 used in this manner did not protect the lactobacilli during freezing. Storage stability of L. bulgaricus cells was markedly improved only when the cells were grown in broth supplemented with Tween 80, which has long been known to stimulate the growth of the lactobacilli (13). However, little information is available concerning its physiological role. Such material may be directly involved in lipid metabolism or may have a physical effect on the cells. Most of the lipid material in gram-positive microorganisms is associated with the cell membrane; thus, if Tween 80 is metabolized, it could play a role in developing cell membranes whose integrity is maintained during freezing and storing of L. bulgaricus in liquid nitrogen.

LITERATURE CITED
1. Accolas, J. P., and J. Auclair. 1967. Storage of highly concentrated suspensions of lactic acid bacteria in the frozen state. I. Mesophilic lactic acid bacteria. Lait 47:253-60.
2. Calcott, P. H., and J. R. Postgate. 1971. Protection of Aerobacter aerogenes by nonionic detergents from freezing and thawing damage. Cryobiology 7:238-242.
3. Elliker, P. R., A. W. Anderson, and G. Hannesson. 1966. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci. 50:1611-1612.
4. Foster, E. M. 1962. Symposium on lactic starter cultures. VI. Culture preservation. J. Dairy Sci. 45:1290-1294.
5. Gibson, C. A., G. B. Landerkin, and P. M. Morse. 1966. Effects of additives on the survival of lactic streptococci in frozen storage. Appl. Microbiol. 14:965-969.
6. Gilliland, S. E., E. D. Anna, and M. L. Speck. 1970. Concentrated cultures of Leuconostoc citrovorum. Appl. Microbiol. 19:890-893.
7. Lamprech, E. D., and E. M. Foster. 1963. The survival of starter organisms in concentrated suspensions. J. Appl. Bacteriol. 26:359-369.
8. Mazur, P. 1970. Cryobiology: The freezing of biological systems. Science 168:939-949.
9. Morichi, T. B. Irse, N. Yano, and H. Kembo. 1963. Protective effect of glutamic acid and related compounds on bacterial cells subjected to freeze-drying. J. Gen. Appl. Microbiol. 9:149-161.
10. Peebles, M. M., S. E. Gilliland, and M. L. Speck. 1969. Preparation of concentrated lactic streptococcus starters. Appl. Microbiol. 17:805-810.
11. Rogosa, M., J. A. Mitchell, and R. F. Wiseman. 1961. A selective medium for the isolation and enumeration of oral and fecal lactobacilli. J. Bacteriol. 82:132-133.
12. Stadhouders, L. A., and J. G. Hup. 1971. A study of the optimum conditions of freezing and storing concentrated mesophilic starters. Neth. Milk Dairy J. 26:229-239.
13. Williams, V. R., and E. A. Fieger. 1947. Further studies on lipide stimulation of Lactobacillus casei. J. Biol. Chem. 170:399-411.