Relationship of Cellular Components to the Stability of Concentrated Lactic Streptococcus Cultures at \(-17\) C\(^1\)

S. E. GILLIAND and M. L. SPECK

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

Received for publication 15 January 1974

Concentrated cultures of lactic streptococci varied with respect to survival at \(-17\) C. Cells of each strain grown at pH 6.0 were more stable to freezing than were those grown statically. The lipid fraction of the cells from static cultures was important in preventing death during freezing. As the percentage of octadecenoic acid in the cellular lipids from different cultures increased, the percentage of survivors decreased. Capsular material associated with cells from cultures grown both statically and at pH 6.0 was also important in protecting the cells at \(-17\) C. The amount of capsular material, measured as percentage of cellular glucose, varied among the cultures tested. Cultures containing larger amounts of the capsular material were more resistant to the stress of freezing than those containing low levels.

The production of many fermented foods now routinely involves the use of concentrated starter cultures produced by commercial culture suppliers. Such cultures must be stable to storage if they are to be used successfully. At present, concentrated cultures are shipped and stored in a frozen state to preserve their activity and to insure their satisfactory performance in preparing cultured products. Storage in liquid nitrogen has been accepted as the best method of preserving culture activity. Such storage requires a certain amount of special equipment and a supply of liquid nitrogen.

The feasibility of storing frozen lactic streptococci at temperatures higher than that of liquid nitrogen has been studied by several groups (1, 3, 4, 7, 11). Individual strains of lactic streptococci vary with respect to their ability to survive freezing at or near \(-20\) C (3, 7, 11). Information explaining such strain variations is lacking in the literature. Smittle et al. (9) have shown variations in fatty-acid composition among strains of *Lactobacillus bulgaricus* which correlate to their resistance to freezing.

This investigation was undertaken to study possible relationships between cellular composition of the lactic streptococci and resistance to freezing at \(-17\) C.

**MATERIALS AND METHODS**

**Cultures.** The single strain cultures of lactic streptococci (AC, AC, \(E_n\), and ML) used in this study had been maintained in the North Carolina State University Food Microbiology stock culture collection. Cultures were routinely propagated in sterile litmus milk using a 1% inoculum and incubation at 21 C for 18 to 20 h. The cultures were held in a refrigerator (5 C) between transfers and subcultured at least three times before being used experimentally.

**Preparation of concentrated cultures.** The growth medium and procedures for preparing the concentrated cultures were the same as those presented by Peebles et al. (8), except that smaller volumes of broth (650 ml) were employed in fermentors having 1-liter capacities. Incubation was for 16 h at 25 C so that all cultures were in the stationary phase of growth. Ten percent NH\(_4\)OH was used as the neutralizer for pH control. Concentrated cultures of the desired cell density (\(5 \times 10^6\) to \(1 \times 10^{10}/g\)) were prepared from cells grown statically and from cells grown at pH 6.0. The cells were harvested by centrifugation and resuspended in the desired amount of cold, sterile 10% non-fat-milk solids (8). These concentrated cultures were sealed in plastic vials (1 g) and placed in a freezer at \(-17\) C for storage.

**Determination of survivors.** Colony counts were made before freezing and after 7 days of storage using methods described previously (8).

**Cellular fatty acid composition.** Free lipids were extracted from cells of the lactic streptococci and methyl esters were prepared using the methods described by Smittle et al. (9). The methyl esters of the fatty acids were identified and quantitated using gas chromatographic equipment and methods described by Moereck and Ball (6).

**Cellular sugar content.** The contents of three vials of frozen concentrated culture were thawed and added to 6 ml of cold distilled water. One milliliter of 20% sodium citrate was added to solubilize the milk protein after which the cells were removed by centrif...
ugation (12,000 × g for 15 min at 0 C). Each cell pellet was washed once with 20 ml of cold distilled water. The cells were resuspended in 8 ml of cold distilled water and held in an ice-water bath until assayed. Dry weight determinations (milligrams per milliliter cell suspension) were made by drying 2-ml portions of the cell suspensions in dry, tared aluminum weighing pans overnight at 110 C in a forced air oven and weighing on an analytical balance. The cell suspensions were also analyzed for sugar content using the anthrone method with glucose as the standard. Four milliliters of 0.2% anthrone in concentrated sulfuric acid was mixed with 2 ml of cell suspension or glucose solution and heated for 5 min at 100 C. After cooling, the optical density was measured at 540 nm. The milligrams of glucose per milligram dry cell weight was calculated, and the results were expressed as percentage of glucose.

Statistical analyses of data. The regressions of percent survivors on percent fatty acid or percent glucose were determined, and the variances about the regression were analyzed by methods described in Statistical Methods (10).

RESULTS

Effect of freezing at −17 C on lactic streptococci. Variations were observed among the four strains of lactic streptococci with respect to the percentages surviving 7 days of storage at −17 C (Table 1). For the concentrates prepared from static cultures, strains AC1 and AC11 were most resistant, ML1 was least resistant, and culture E5 was intermediate. Concentrates prepared from cells grown at pH 6 were more resistant than those from cells grown without pH control for each strain.

Relationship of fatty acid composition to cellular stability. Smittle et al. (9) reported a close relationship between resistance to freezing and cellular fatty acid composition for L. bulgaricus. Based on this, efforts were made to determine if such a relationship existed for the lactic streptococci. Table 2 shows the fatty acid composition of the free lipids extracted from cells of the lactic streptococci grown statically and at pH 6.0. The fatty acids identified in the lipid material were essentially the same as those reported by Macleod et al. (5) for Streptococcus lactis. There were considerable variations in the fatty-acid composition of the individual strains. The major fatty acids appeared to be hexadecanoic acid (16:0), octadecenoic acid (18:1), and a 19-carbon cyclopropane acid (Δ19:0). Of the major fatty acids, the percentages of 18:1 and Δ19:0 acids varied more among strains than did the 16:0 acid. To see if relationships existed between resistance to freezing and the cellular fatty-acid compositions, regression coefficients were determined for each combination of fatty acid and percent survivor. With the exception of 18:1 fatty acid, none of the fatty acids were significantly related to resistance of the cells to freezing. Figure 1 shows the regression of percentages of survivors on percent 18:1 fatty acid for cultures of lactic streptococci grown statically. As the cellular content of this fatty acid increased, the percentages of survivors decreased. The regression coefficient was significant (P < 0.05), indicating a close relationship between the 18:1 fatty acid content and resistance to freezing. The regression of percentages of survivors on percentage 18:1 fatty acid for cells grown at pH 6 did not indicate a close relationship (Fig. 2). The regression coefficient for these data was not significant (P > 0.1).

Relationship of cellular glucose to survival at −17 C. During the preparation of the concentrated cultures, it was noted that centrifugation of the broth cultures did not always result in the formation of a firm pellet. We suspected that the formation of capsular material by the streptococci during growth was responsible for this. The presence of capsular material on the cells in

| Culture | Conditions of growth | Fatty acid composition (%) |
|---------|----------------------|-----------------------------|
|         |                      | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | Δ19:0 | NI* |
| AC1     | Static              | 2.8  | 15.9| 3.6  | 2.3  | 17.8| 43.3 | 14.3 |
| AC11    | Static              | 4.2  | 19.2| 5.8  | 6.9  | 58.5| 4.8  |
| E5      | Static              | 4.4  | 17.7| 4.3  | 1.2  | 16.8| 49.3 | 6.6  |
| ML1     | Static              | 1.4  | 16.3| 4.3  | 1.5  | 29.8| 44.2 | 2.6  |
| Avg     |                     | 3.2  | 17.3| 4.5  | 1.3  | 17.8| 48.8 | 7.1  |
| AC1     | pH 6.0              | 1.7  | 16.6| 3.5  | 3.0  | 34.3| 34.3 | 6.5  |
| AC11    | pH 6.0              | 3.8  | 17.1| 4.8  | 14.5| 58.4| 1.2  |
| E5      | pH 6.0              | 4.8  | 13.3| 4.1  | 3.4  | 15.0| 43.8| 55.8 |
| ML1     | pH 6.0              | 1.5  | 17.6| 3.1  | 0.7  | 42.9| 32.4| 1.9  |
| Avg     |                     | 3.0  | 16.2| 3.9  | 1.8  | 26.7| 42.2| 6.4  |

*NI, Percentage represented by several small unidentified peaks on chromatograms.

Table 1. Survival of concentrated cultures of lactic streptococci prepared from cells grown statically and at pH 6.0

| Culture | Survivors (%) |
|---------|---------------|
|         | Static | pH 6.0 |
| AC1     | 73*    | 92    |
| AC11    | 74     | 82    |
| E5      | 67     | 100   |
| ML1     | 27     | 67    |

* Each value represents the average from two experiments.
STABILITY OF LACTIC STREPTOCOCCUS CULTURES

The cellular content of glucose was selected as a basis for comparing the cultures, since most bacterial capsules are primarily composed of polysaccharide material. The percentages of

- Fig. 1. Regression of percent survivors on percent 18:1 fatty acid in cells of lactic streptococci grown without pH control and frozen at -17 C (regression coefficient, -1.9465; significant at P < 0.05). Each point represents data from an individual cell crop.

- Fig. 2. Regression of percent survivors on percent 18:1 fatty acid in cells of lactic streptococci grown at pH 6 and frozen at -17 C (regression coefficient, -0.3414; not significant at P < 0.1). Each point represents data from an individual cell crop.

- Fig. 3. Regression of percent survivors on percent cellular glucose of lactic streptococci grown without pH control and frozen at -17 C (regression coefficient, +15.23; significant at P < 0.001). Each point represents data from an individual cell crop.

- Fig. 4. Regression of percent survivors on percent cellular glucose of lactic streptococci grown at pH 6.0 and frozen at -17 C (regression coefficient, +14.7; significant at P < 0.01). Each point represents data from an individual cell crop.

The concentrated cultures was confirmed by performing capsule stains (2). Microscope examination revealed that the amount of capsular material varied for the different cultures. To determine if any relationship existed between the resistance to freezing and the capsular material, efforts were made to quantitate the amount associated with the cells of each cul-

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survivors after 7 days at −17 °C are plotted in Fig. 3, against the amount of cellular glucose for lactic streptococci grown without pH control. Generally, as the amount of glucose associated with the cells increased, so did the percentage of survivors. The regression coefficient for the data was significant (P < 0.001), indicating that the capsular material was important in protecting the cells during frozen storage. The data comparing the survivors to cellular glucose for the cultures grown at pH 6 (Fig. 3) also had a positive regression coefficient which was significant at P < 0.001.

Even though survival during storage at −17 °C was generally higher for the cultures grown at pH 6 (Fig. 3) than for cultures grown statically (Fig. 4), the amounts of cellular glucose for the two sets of cultures were within the same range. The average cellular content of glucose was 6.8% for the cultures grown statically and 6.6% for the cultures grown at pH 6.

**DISCUSSION**

The cellular content of Δ19:0 fatty acid has been shown to influence the survival of *Lactic acid bacteria* during freezing (6). Cells of the lactobacilli which contained low levels of Δ19:0 fatty acid were most sensitive to freezing. Such a relationship was not found for the lactic streptococci. This may be because all the lactic streptococci included in our study contained much higher levels of this fatty acid than was reported for the lactobacilli (6).

Lipids of gram-positive bacteria are located primarily in the membrane. Therefore, a close relationship between the cellular content of 18:1 fatty acid and survival, as observed for cells grown statically, indicates that the fatty acid composition of the membrane is important in preventing damage to the lactic streptococci during freezing. Stability of the membrane probably involves more than just the 18:1 fatty acid. Stability or resistance to damage from freezing most likely involves an overall favorable balance of fatty acids in the lipid portion of the membrane.

Cellular components other than lipids are obviously involved in protecting the streptococci from damage during freezing, since a close relationship was observed between the amount of capsular material and survival. The mechanism whereby the capsular material provides protection at −17 °C is not known. However, it has been generally accepted that capsules provide some protection to the bacterial cells. The capsular material may provide structural stability to the cells so that they can better withstand the stresses of freezing.

An explanation as to why concentrated cultures of lactic streptococci prepared from cells grown at pH 6 are more stable to freezing than are concentrated preparations of cells grown statically is not clear. The greater stability is, however, an added advantage of growing cells for preparing concentrated cultures at pH 6. Peebles et al. (8) reported that better cell yields are obtained by growing the lactic streptococci at pH 6 than without pH control.

Both the fatty acid composition and the amount of capsular material associated with the lactic streptococci appear to be important in minimizing cellular damage resulting from freezing at −17 °C. Since these cellular components are important, more stable concentrated cultures could be produced by selecting and using cultures having the desired cellular composition for maximum survival. On the other hand, it may be possible to alter the growth conditions for cells used in preparing concentrated cultures to enhance the production of these beneficial components.

**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 47:253–260.
2. Anthony, C. E. 1931. A note on capsule staining. Science 73:319.
3. Cowman, R. A., and M. L. Speck. 1965. Ultra-low temperature storage of lactic streptococci. J. Dairy Sci. 48:1531–1532.
4. Lamprecht, E. D., and E. M. Foster. 1963. The survival of starter organisms in concentrated suspensions. J. Appl. Bacteriol. 26:359–369.
5. Macleod, Patricia, R. G. Jensen, G. W. Gander, and J. Sampugna. 1962. Quantity and fatty acid composition of lipid extracted from cells of *Streptococcus lactis*. J. Bacteriol. 83:806–810.
6. Moerck, K. E., and H. R. Ball, Jr. 1973. Lipids and fatty acids of chicken bone marrow. J. Food Sci. 38:978–980.
7. Moss, C. W., and M. L. Speck. 1963. Injury and death of *Streptococcus lactis* due to freezing and frozen storage. Appl. Microbiol. 11:326–329.
8. Peebles, M. M., S. E. Gilliland, and M. L. Speck. 1969. Preparation of concentrated lactic streptococci starters. Appl. Microbiol. 17:395–3810.
9. Smittle, R. B., S. E. Gilliland, M. L. Speck, and W. M. Walter, Jr. 1974. Relationship of cellular fatty acid composition to survival of *Lactobacillus bulgaricus* in liquid nitrogen. Appl. Microbiol. 27: April.
10. Snedecor, G. W., and W. G. Cochran. 1971. Statistical methods, 6th ed. The Iowa State University Press, Ames, Iowa.
11. Stadhouders, J., L. A. Jansen, and G. Hup. 1969. Preservation of starters and mass production of starter bacteria. Neth. Milk Dairy J. 23:192–199.