Survival and Activity of Frozen Starter Cultures for Cheese Manufacture

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A study has been conducted on the effect of freezing and storage in liquid nitrogen on 13 strains of lactic streptococci. Cultures were frozen in droplet form and collected in mesh bags. After rapid thawing, the activity of the frozen cultures was compared with a culture of the same organism of the age usually used in cheese-making. The activities of the test and control cultures were traced simultaneously by continuous recording of the pH changes in inoculated milks. Viable counts were performed before and after freezing in liquid nitrogen and after storage in liquid nitrogen. There was no decrease in viable count or loss in activity of the cultures due to freezing and storage. Frozen cultures of some strains showed a shorter lag period after inoculation of milk than control cultures. Frozen concentrated cheese-starter cultures behaved normally in the manufacture of Cheddar cheese.

At the present time, there is a widespread interest in the preservation of cheese-starter cultures by freezing in liquid nitrogen and in the use of concentrated frozen starter cultures for direct inoculation into the vat in the manufacture of cheese (1, 4, 9, 12, 13). Already such cultures are in commercial use in the United States for the setting of bulk inoculum and for direct vat inoculation for long-set cheese manufacture.

The effects on the survival and activity of lactic streptococci, after freezing and thawing, of different methods, of different temperatures of freezing and storage, and of growth temperatures and pH prior to freezing have been the subjects of recent investigations (2, 5–8, 10, 11).

The present paper deals with a study of the activity of lactic starters as determined by the continuous recording of pH changes in milks inoculated with frozen starter and incubated for 18 hr at 30 C. Thirteen different strains of lactic streptococci were studied after periods of storage in liquid nitrogen, the behavior of the various strains being relevant to future commercial production of frozen concentrated starter cultures.

MATERIALS AND METHODS

Cultivation. The medium used for the cultivation of starter cultures contained 1,000 ml of low-calcium coprecipitate whey (0.04% calcium) and 0.005% bromocresol purple. The pH was adjusted to 6.8, and the medium was sterilized at 121 C for 10 min in 100-ml quantities.

Cultures were incubated for 6 hr at 30 C, neutralized with 0.1 N sodium hydroxide to the bromocresol purple end point, and immediately frozen. Culture HP was grown at 25 C.

Strains of cheese starters. The following strains of lactic streptococci were tested: Streptococcus lactis C2, C10, BA1; S. cremoris C1, C3, C11, C13, HP, E8, ML1; and S. diacetylactis DRC1, DRC2, DRC3. Culture C2 was used in cheese manufacturing experiments.

Method of freezing. The neutralized culture was frozen in droplet form by discharging drops from a 10-ml pipette into a terylene mesh bag suspended in a bath of liquid nitrogen. The bag was held in place in a specially constructed holder consisting of a stainless-steel tube with a mesh bottom. When 10 ml of culture had been frozen, the bag was closed by pulling a drawstring and was transferred to a liquid nitrogen refrigerator (type LR-10, Union Carbide).

To test the feasibility of freezing concentrated starter cultures in this way, cultures in the logarithmic phase of growth, produced by a continuous-culture technique, concentrated by centrifugation, and re-suspended in a whey culture medium (pH 6.3) with the aid of a high-speed blendor, were frozen as a thick slurry (approximately 10^11 cells/ml) in the same manner.

Method of testing the effects of freezing and thawing. Viable counts were performed on all cultures immediately after freezing, immediately after freezing, and at approximately monthly intervals during the storage period. The medium used for viable counts was tryptone-yeast extract-agar (TYA) of the following composition: tryptone, 3.0%; yeast extract, 1.0%; Lab-lemco, 0.2%; sodium chloride, 0.3%; dipotassium hydrogen phosphate, 0.5%; lactose, 0.5%; agar, 1.5%.

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Frozen cultures were checked for activity 24 hr after freezing and at approximately monthly intervals during storage, when viable counts were also made.

Skim milk was used in checking the activity of the cultures, rather than reconstituted skim milk, because the behavior of the frozen starter in different milks was of interest in the experiment.

Two flasks, each containing 500-ml quantities of milk, were heated by being brought momentarily to 121°C and then cooled and inoculated with starter. One flask of milk was inoculated with the frozen 10 ml of starter, and the other with 10 ml of a coprecipitate whey suspension of a 16- to 18-hr milk culture. The amount of culture added to the coprecipitate whey medium was calculated so that the counts in the test and control flasks of milk would be comparable. Viable counts were made on the two flasks of milk immediately after inoculation and mixing.

The two flasks were placed in a water bath at 30°C and a combined calomel-glass pH electrode was placed in each flask. The two electrodes were connected to a Radiometer (model 22) pH meters which in turn were connected to a Hitachi (model QPD73) dual pen recorder. The pH change during the course of 16 to 18 hr of incubation was traced.

The activities of the normal starter and the frozen starter were compared by measuring the time taken for the first indication of pH change and the time taken for the pH to drop further to 5.6 in each flask.

Preliminary Cheddar cheesemaking trials. Frozen concentrated cheese starter (approximately 10^11 cells) was inoculated directly into 100 gal of milk in an experimental cheese vat. At the same time, another 100 gal of the same batch of milk was inoculated with 2% of the cheese-starter strain prepared in the normal way. Viable counts were made on the milk in each vat immediately after inoculation and mixing of the starter with the vat contents. Because chain disruption due to freezing and thawing has been reported (10), before counts were made, each sample of inoculated milk from the test and control vats was subjected to a blending at a standard speed with a high-speed blender to ensure maximum disruption of chains of the streptococci to make the viable counts in both samples comparable. Counts were also performed on untreated samples of the inoculated milks for comparison.

Cheddar cheese was manufactured in the two vats in the usual way, and the behavior of the normal and frozen starters during manufacture was compared. Only one strain of starter, S. lactis, C2, was tested.

RESULTS

Of the 13 strains of starter tested for viability before and after freezing, none showed a loss in numbers as measured by the plate-count method. None of the cultures stored for long periods, from 1 to 13 months, in liquid nitrogen showed a significant change in count during the period of storage. Microscopic examination of the culture before and after freezing showed no evidence of chain disruption due to freezing.

All cultures tested showed equal or better activity than their controls, except in the case of C13 where the difference in inoculum favored the control (Table 1). Figure 1 shows a typical chart tracing the activity of a frozen starter and its control.

Cheese manufacture with frozen starter proceeded normally, and the cheese was equal in quality to that made with the conventional starter, although, as expected, the higher pH in the frozen starter resulted in the renneted milk taking 10 min longer to coagulate in the test vat. Viable counts on the milk in the control and

| Culture code | Frozen culture | Control culture |
|--------------|----------------|----------------|
| | Age of frozen culture (months) | Period of lag (hr) | Time to drop pH to 5.6 (hr) | Counts/ml in milk at beginning of activity test | Period of lag (hr) | Time to drop pH to E.6 (hr) |
| C1 | 13 | 66 × 10^4 | 4.0 | 10.50 | 95 × 10^4 | 5.5 | 11.5 |
| C2 | 10 | 41 × 10^4 | 3.0 | 7.75 | 47 × 10^4 | 3.0 | 7.6 |
| C3 | 6 | 57 × 10^4 | 4.08 | 9.4 | 92 × 10^4 | 4.17 | 10.5 |
| C10 | 8 | 67 × 10^4 | 2.25 | 6.4 | 62 × 10^4 | 3.0 | 6.8 |
| HP | 4 | 27 × 10^4 | 3.5 | 8.6 | 28 × 10^4 | 5.3 | 11.7 |
| ML1 | 2 | 14 × 10^4 | 3.25 | 7.5 | 9 × 10^4 | 3.25 | 7.5 |
| E8 | 4 | 88 × 10^4 | 3.0 | 9.0 | 99 × 10^4 | 4.0 | 11.75 |
| C13 | 3 | 33 × 10^4 | 4.0 | 13.75 | 49 × 10^4 | 5.4 | 13.0 |
| C11 | 1 week | 71 × 10^4 | 3.5 | 12.8 | 66 × 10^4 | 4.5 | 12.8 |
| DRC1 | 9 | 67 × 10^4 | 1.8 | 9.4 | 45 × 10^4 | 2.75 | 10.2 |
| DRC2 | 2 | 32 × 10^4 | 2.25 | 8.2 | 44 × 10^4 | 3.25 | 8.4 |
| DRC3 | 2 | 40 × 10^4 | 3.5 | 7.4 | 45 × 10^4 | 3.75 | 8.3 |
| BA1 | 3 | 46 × 10^4 | 3.0 | 8.6 | 75 × 10^4 | 4.3 | 9.8 |

* Period of lag is time taken in hours to show first indication of a pH change.
test vats after inoculation were very similar (4 × 10^2 ml in both vats). There was no evidence of chain disruption by concentration and freezing of the streptococcal culture.

**DISCUSSION**

The freezing of the starter culture in liquid nitrogen in droplet form presents no problems with regard to viability and activity of the culture. The better activity of the frozen culture, in terms of time taken to show the first indication of pH change in the milk, could be attributed to the fact that the starters were frozen in an active phase of growth (6 hr of incubation), whereas the control cultures were in the stationary phase of growth when transferred to the milk, as is normal with the starter inoculum prepared by conventional methods for cheese manufacture. If, in the future, cultures are grown by continuous-culture techniques, the freezing of cells in their active phase of growth could be safely achieved. A selection of more active variants may also result from continuous culture. It is possible, therefore, that a reduction in the number of cells required for vat inoculation could be made for some strains of lactic streptococci.

The medium used in the present study was selected because of its possible use in the production of starter by continuous-culture technique. Its protein content was very low. In spite of this, the cells suffered no apparent damage during the processes of freezing and thawing. The fact that cultures can be stored successfully for 13 months in liquid nitrogen would suggest that they might in fact be stored indefinitely.

The addition of a protective substance to the culture before freezing in liquid nitrogen is unnecessary. This has also been demonstrated by other workers (3). If the cultures are frozen or stored at higher temperatures, protective substances are necessary (7). The absence of protective substances not derived from milk constituents in a frozen starter is advantageous because such additives might not be permissible under some food regulations.

Preliminary cheesemaking trials showed that, at this stage of the investigation, the concentration of cheese starters by centrifugation and their subsequent rapid freezing in liquid nitrogen followed by rapid thawing appear to present no problems in cheesemaking. This can be assumed, provided that in the continuous culture there is no selection of cells which might be lacking in enzymes essential for fast acid production or for the maturing of cheese.

It is foreseen that in commercial production the concentrated suspension of a starter could be dropped at a constant rate into bags of a similar type but larger than the ones used in the present experiment. The bath of liquid nitrogen would be slowly moving to ensure that the drops do not fall in the same place consecutively to avoid clumping.

Another possibility would be to discharge the concentrate on a moving Teflon belt through a bath of liquid nitrogen. The culture would be frozen as a thin film on the belt. The belt would impinge upon a scraping device which would remove the frozen concentrate as a powder into a plastic bag.

Although it is possible that the frozen cultures could be stored at temperatures above that of liquid nitrogen and below −130°C without the presence of protective substances, there are advantages in the use of liquid nitrogen refrigeration. Provided the supply of liquid nitrogen is assured, there is little likelihood of breakdown of refrigeration equipment.

Liquid nitrogen is not a sterile product, and its sterilization would probably be too costly for commercial use. The large numbers of quickly
revived cells in the concentrated frozen starter would be expected to suppress small numbers of contaminants. In the trials carried out in the present study, although no specific tests were made, there has been no evidence of bacterial or phage contamination in the milk inoculated with starters which have been in direct contact with the liquid nitrogen.

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