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Frozen Concentrated Cultures from Cells of *Lactobacillus acidophilus* Grown in Pepsinized Whey Media

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Story in Brief

Three pepsinized whey based media were compared for growing cells of *Lactobacillus acidophilus* to be used in preparing frozen concentrated cultures. Concentrations of dried sweet whey tested in the media were 2.5, 5.0, and 7.5 percent. The pH of the media was maintained at 6.0 during growth of *L. acidophilus* at 37 C with a neutralizer consisting of 20 perecent Na_2CO_3 in 20 percent NH_4OH. As the level of whey solids was increased in the media, maximum populations attained during growth decreased. The culture reached maximum populations in the media containing 2.5 percent and 5.0 percent whey solids after 12 and 18 hr, respectively, then remained constant for the remainder of the 24-hr incubation period. However, in the medium containing 7.5 percent whey solids, the culture appeared to enter a death phase after only 12 hr of incubation. The maximum population in the 7.5 percent medium was also considerably lower than those in the other two media. Frozen concentrated cultures were prepared from cells of *L. acidophilus* grown in the media containing 2.5 percent and 5.0 percent whey solids and stored in liquid nitrogen at − 196 C. The viability and bile resistance of the cultures during storage in liquid nitrogen and subsequent storage in refrigerated milk was highest for cells which had been grown in the 2.5 percent pepsinized whey medium.

Introduction

Currently there is much interest in the use of *Lactobacillus acidophilus* as a dietary adjunct. Cells of *L. acidophilus*, in the form of a concentrated culture added to refrigerated low-fat milk, may help maintain intestinal health and improve intes-

tinal function. Consumption of milk containing this organism can help maintain a desirable balance among bacteria in the intestinal tract. It can also aid in preventing symptoms associated with lactose malabsorption in persons suffering from this malady.

Originally, acidophilus milk was available only as a fermented product which had an unpleasant flavor. Cells of *L. acidophilus* rapidly died during refrigerated storage in such a product. Because of low numbers of viable cells of *L. acidophilus* in the fermented milk and the milk's unpleasant flavor, an unfermented acidophilus milk was developed. A low-fat non-fermented milk product containing *L. acidophilus* was made commercially available in 1975. The milk is prepared by adding a concentrated culture of *L. acidophilus* to pasteurized low-fat milk. The flavor is the same as ordinary milk, but the milk contains several million viable and bile resistant cells of *L. acidophilus* per ml.

One of the most important considerations in preparing a frozen concentrated culture of *L. acidophilus* to be used as a dietary adjunct is the growth medium used to grow the cells. The growth medium should permit maximum growth of the bacterial cells and produce cells that will retain viability and their desirable characteristics during frozen storage of the resulting concentrated culture. These characteristics should also be maintained during subsequent refrigerated storage of the milk containing the cells of *L. acidophilus*.

The objective of this study was to develop a pepsinized whey based medium in which to grow cells of *L. acidophilus* to be used in the preparation of frozen concentrated cultures for use as dietary adjuncts.

Materials and Methods

Dried sweet whey (Associated Milk Producers Inc., Tulsa, OK) was reconstituted at the desired concentration (2.5 percent, 5.0 percent or 7.5 per percent) in 4 liters of distilled water containing 0.1 percent Tween 80 (Sigma Chemical Co.). The pH was adjusted to 3.0 with 5N phosphoric acid, and 1 g of pepsin (Sigma Chemical Co.) was added. This mixture was incubated 30 min in a 37 C water bath. The digested whey was adjusted to pH 7.0 with 5N ammonium hydroxide. One-tenth percent yeast extract and 1.0 percent thiotone were added to the mixture. After the added ingredients were dissolved, the medium was equally divided into two 4-liter flasks and autoclaved for 15 min at 121 C.

The 4 liters of sterile pepsinized whey medium were aseptically added to an empty sterile fermenter jar. The fermenter jar, equipped with a combination pH electrode, was connected to an automatic pH controller. The temperature of the whey was adjusted to 37 C, and the automatic pH controller was adjusted to maintain the growth medium at pH 6.0. The whey medium was then inoculated with 40 ml of an 18-hr milk culture (37 C) of *L. acidophilus* NCFM and incubated for 24 hr at 37 C. The medium was maintained at pH 6.0 during growth of the culture with a neutralizer consisting of 20 percent $Na_2 CO_3$ in 20 percent NH_4OH.

Beginning at 10-hr and at 2-hr intervals thereafter, 10 ml samples of the culture were aseptically removed from the fermenter using sterile pipettes and placed into sterile screw-capped test tubes in an ice-water bath. The total numbers of lactobacilli and bile resistant lactobacilli were determined by plating appropriate dilutions of the samples on MRS and MRSO agar media. The MRS agar provided total numbers of lactobacilli, and the MRSO agar (MRS plus 0.1 percent oxgall) provided numbers of bile resistant lactobacilli.

Cells were harvested after 16 hr of growth from 800 ml of the cultures of *L. acidophilus* grown in the 2.5 and 5 percent whey media. The cells were harvested

by centrifugation and resuspended in twice their weight of cold sterile 10 percent NFMS. The resulting concentrated cultures were dispensed into sterile freezing vials (2 g each), then frozen and stored in liquid nitrogen (−196 C).

Numbers of *L. acidophilus* in the concentrated cultures were determined prior to freezing (Day 0) and after 1, 14 and 28 days of storage in liquid nitrogen by plating appropriate dilutions on MRS and MRSO agar. The vials of frozen concentrated cultures were thawed by submerging them in 1 liter of tap water at 30 C for 5 min.

Milk for preparing nonfermented acidophilus milk was prepared by dividing 400 ml of 10 percent reconstituted nonfat milk solids (NFMS) into four bottles and heating at 100 C for 30 min. The heated milk was immediately cooled to 5 C. Thawed concentrated culture was diluted, as required, and added to each of four bottles containing the cold 10 percent NFMS to achieve a population of about 2 x 10^6/ml. The milk was then stored at 5 C. Bottles were removed for examination on Days 1, 7, 14 and 21. The total numbers of lactobacilli and bile resistant lactobacilli were determined.

Results

Considerable variation was observed in the growth of *L. acidophilus* NCFM in the media containing different concentrations of whey solids (Figure 1). In this figure the $\log_{10}$ of counts on MRS agar (solid lines) and on MRSO agar (broken lines) are plotted against incubation time. The highest population attained, based on counts on MRS agar, was 1 x 10^9/ml in the 2.5 percent whey medium, which was significantly higher than that obtained in the 5 percent or the 7.5 percent media ($P<.05$). Growth of *L. acidophilus* in the 2.5 percent and 5.0 percent whey media reached maximum numbers at 16-18 hr and then remained constant with little or no reduction in numbers for the remainder of the incubation time. The growth of *L. acidophilus* in the medium containing 7.5 percent whey solids, however, reached its maximum at approximately 12 hr and then decreased throughout the remainder of the incubation period. It is apparent from this data that as the level of whey solids was increased in the media, the maximum populations attained during growth at pH 6.0 decreased. The differences between MRS and MRSO counts for the culture growing in each level of whey solids increased as the level of whey solids was increased. Due to the poor performance of the culture in the 7.5 percent medium, further experiments with this medium were not done.

The survival of the concentrated cultures of *L. acidophilus* NCFM during storage in liquid nitrogen is shown in Table 1. The data in this table represent the average $\log_{10}$ counts from six trials. For the concentrated cultures prepared from cells grown in the medium containing 2.5 percent whey solids, counts on both the

Table 1. Survival of *Lactobacillus acidophilus* NCFM in concentrated cultures during storage in liquid nitrogen[a]

Days in liquid nitrogen	2.5% Whey solids		5.0% Whey solids	
	MRS	MRSO	MRS	MRSO
0	10.31	10.26	9.81	9.65
1	10.27	10.13	9.67	9.53
14	10.28	10.24	9.61	9.51
28	10.27	10.21	9.63	9.36

[a]Each value is the average $\log_{10}$ count/g from 6 trials.

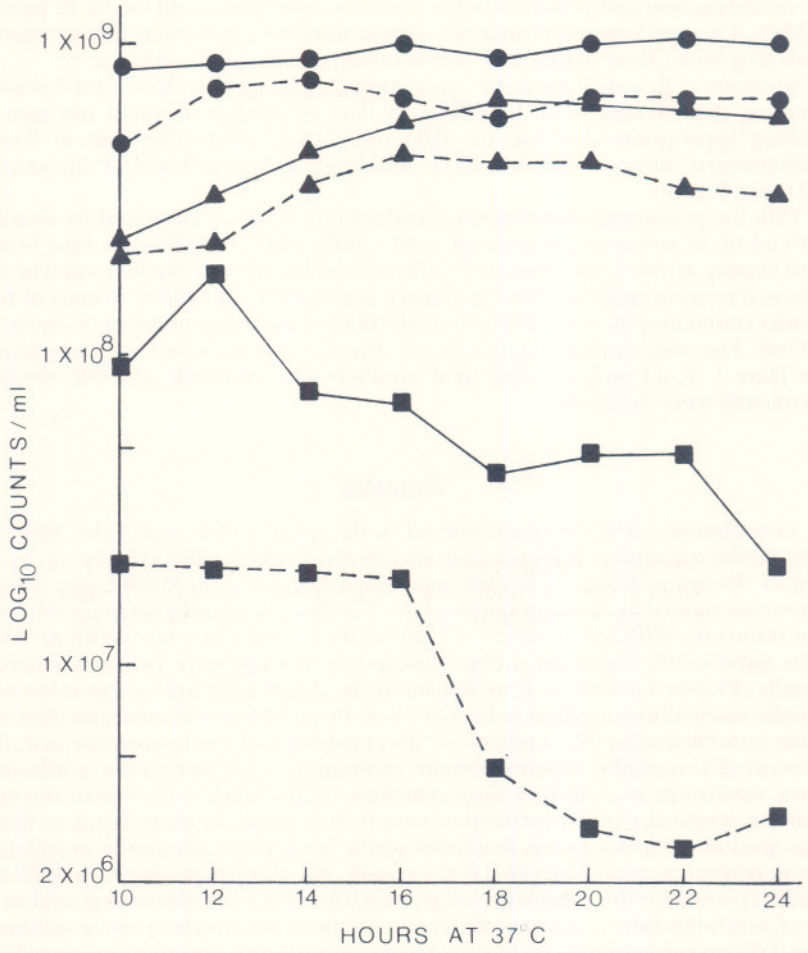

Figure 1. Growth of *Lactobacillus acidophilus* NCFM at pH 6.0 in pepsinized whey based media. ● 2.5% whey solids (averages from 6 trials); ▲ 5.0% whey solids (averages from 6 trials); ■ 7.5% whey (averages from 4 trials); plotted on semi-logarithmic paper; solid lines = counts on MRS agar; broken lines = counts on MRSO agar

MRS and MRSO agar remained fairly constant, exhibiting only slight reductions during 28 days of storage in liquid nitrogen. However, the MRS and MRSO counts for the concentrated cultures prepared from cells grown in the 5.0 percent medium showed greater reductions after 1 day of storage in liquid nitorgen. The MRS counts then remained about the same from Day 1 to Day 28, but the MRSO count was further reduced after 28 days of storage in liquid nitrogen.

Figure 2 shows the effect of storage in refrigerated milk on the viability and bile resistance of cells of *L. acidophilus* NCFM which were grown in the 2.5 percent

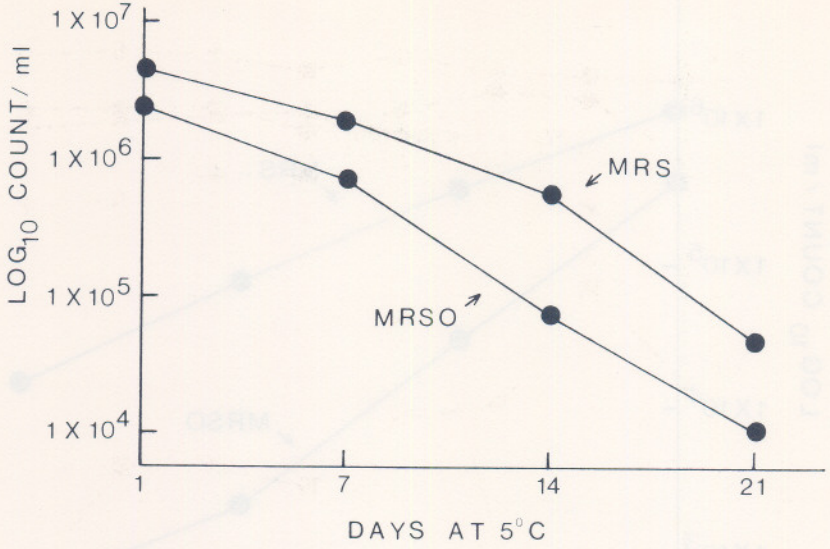

Figure 2. Stability in refrigerated milk of cells of *Lactobacillus acidophilus* NCFM grown in medium containing 2.5% pepsinized whey solids. (Averages from 6 trials, plotted on semi-logarithmic paper)

medium. The concentrated culture used to prepare the milk had been stored in liquid nitrogen for 28 days. Counts obtained on MRS and MRSO agar decreased during storage in the milk at 5 C. The MRS counts showed the sharpest decrease from Day 14 to Day 21. The counts obtained on MRS agar were significantly higher than the MRSO counts ($P<0.005$), and the greatest difference in counts was observed after 14 and 21 days of refrigerated storage.

The storage stability in refrigerated milk of cells of *L. acidophilus* grown in the medium containing 5.0 percent whey solids is presented in Figure 3. Once again the milk was prepared using concentrated cultures which had been stored 28 days in liquid nitrogen. Both the MRS and MRSO agar counts dropped steadily during storage in milk at 5 C, but the MRSO counts dropped much more rapidly than the MRS agar counts. The counts obtained on MRS agar were significantly higher than the MRSO agar counts ($P<0.005$). As in the 2.5 percent medium, the greatest difference between MRS and MRSO counts was seen after 14 and 21 days of refrigerated storage.

Discussion

The reductions in maximum populations observed as the amount of whey solids in the growth medium was increased could be due to inhibitory metabolic products produced from the higher concentration of whey components. Increasing concentrations of whey components might also have created an "imbalance" among nutrients in the medium. For instance, the media with higher levels of whey solids might require different levels of yeast extract, thiotone, or Tween 80 to attain maximum growth of lactobacilli.

The type of neutralizer used to maintain the pH at 6.0 during growth of the culture could influence the maximum populations attained. The type of neutralizer has been shown to influence the growth of other lactic acid bacteria. It is

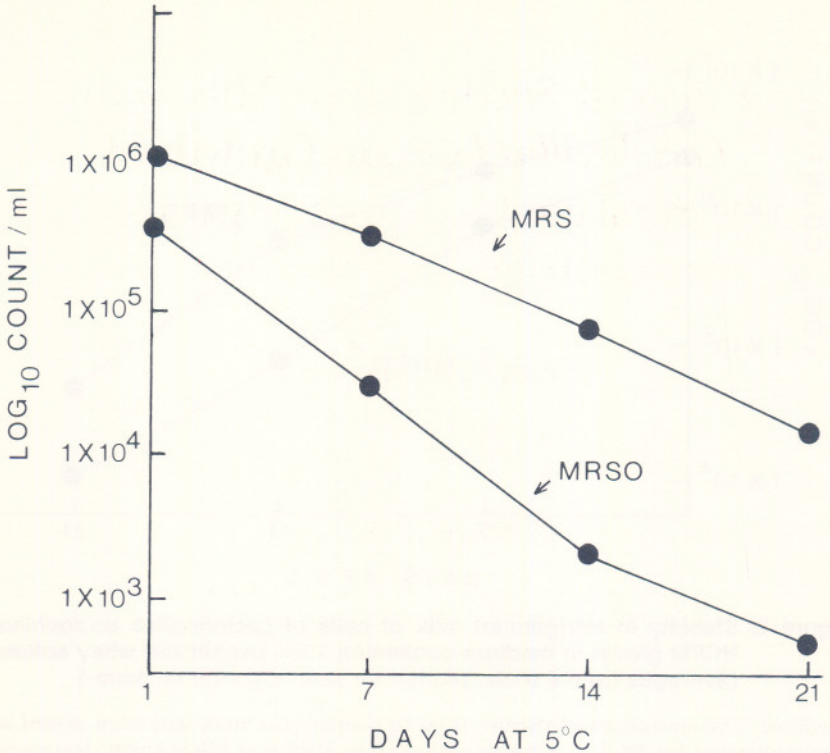

Figure 3. Stability in refrigerated milk of cells of *Lactobacillus acidophilus* NCFM grown in medium containing 5.0% pepsinized whey solids (averages from 6 trials, plotted on semi-logarithmic paper)

possible that a different neutralizer may have been more effective in the media containing higher concentrations of whey. While there is no information available as to the optimum level of various minerals and/or salts on the growth of *L. acidophilus*, an alteration in the amounts of the various salts caused by increasing the whey solids could have influenced the culture.

The lower stability of the concentrated cultures in liquid nitrogen prepared from cells grown in the 5.0 percent medium could be due to some damage to the cells during growth at 37 C. Damage or injury to the cells is also indicated by larger differences between MRS and MRSO agar counts for the culture grown in the 5.0 percent medium than for the cells grown in the 2.5 percent medium. The lower counts on MRSO agar indicated reduced resistance of the culture to bile salts.

In summary, the medium containing 2.5 percent pepsinized whey solids would be the most desirable for producing cells of *L. acidophilus* for preparing frozen, concentrated cultures to be used as dietary adjuncts. However, the survival of the culture during storage in refrigerated milk needs to be improved. Factors other than the level of whey--such as pH during growth, neutralizer used for maintaining pH, and growth temperature need to be researched.