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Influence of Storage at Freezing and Subsequent Refrigeration Temperatures on β-Galactosidase Activity of *Lactobacillus acidophilus*†

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The ability of three strains of *Lactobacillus acidophilus* to survive and retain β-galactosidase activity during storage in liquid nitrogen at −196°C and during subsequent storage in milk at 5°C was tested. The level of β-galactosidase activity varied among the three strains (0.048 to 0.177 U/10^7 organisms). Freezing and storage at −196°C had much less adverse influence on viability and activity of the enzyme than did storage in milk at 5°C. The strains varied in the extent of the losses of viability and β-galactosidase activity during both types of storage. There was not a significant interaction between storage at −196°C and subsequent storage at 5°C. The strains that exhibited the greatest losses of β-galactosidase activity during storage in milk at 5°C also exhibited the greatest losses in viability at 5°C. However, the losses in viability were of much greater magnitude than were the losses of enzymatic activity. This indicates that some cells of *L. acidophilus* which failed to form colonies on the enumeration medium still possessed β-galactosidase activity. Cultures of *L. acidophilus* to be used as dietary adjuncts to improve lactose utilization in humans should be carefully selected to ensure that adequate β-galactosidase activity is provided.

Lactose malabsorption is a term used to describe a condition in certain humans who cannot adequately digest lactose. It results from amounts of lactase in the intestines insufficient to hydrolyze ingested lactose (1, 6). Because of the symptoms associated with the disorder, the lactose malabsorber usually deletes milk from the diet.

Nonfermented milk containing cells of *Lactobacillus acidophilus* has been shown to be beneficial for individuals classified as lactose malabsorbers (5). Such nonfermented milk is prepared by the addition of cells of *L. acidophilus* from a frozen concentrated culture to cold, freshly pasteurized milk. The product is then maintained at a refrigeration temperature until consumed. To be most effective in improving lactose utilization in lactose malabsorbers, it is desirable that the culture maintain the enzyme activity necessary to hydrolyze lactose during frozen storage of the concentrated culture and during subsequent refrigerated storage of the nonfermented refrigerated milk. Concentrated cultures of *L. acidophilus* prepared from cells grown at pH 6 are stable for extended periods of storage in liquid nitrogen (−196°C) (7). When thawed and added to milk at 5°C, the organisms retained viability for 10 to 14 days, and then the number of viable lactobacilli declined (7).

The lactobacilli contain either β-galactosidase or β-phosphogalactosidase or both as the enzyme responsible for enabling the organism to utilize lactose (3, 9). Related studies in our laboratory have shown that the cultures of *L. acidophilus* used in the present study possess only β-galactosidase (unpublished data).

The objective of this study was to determine the stability of β-galactosidase of *L. acidophilus* during frozen storage at −196°C and during subsequent storage in milk at 5°C.

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MATERIALS AND METHODS

Source and maintenance of cultures. *L. acidophilus* NCFM and *L. acidophilus* LA1 were from the laboratory stock culture collection. *L. acidophilus* RAM-1 was isolated from a commercially available low-fat nonfermented acidophilus milk product. All three strains were maintained by subculturing in reconstituted 10% nonfat milk solids with a 1% inoculum and 18 h of incubation at 37°C. The cultures were stored at 1 to 5°C between transfers.

Each culture was subcultured at least three times in Peptonized Milk Nutrient (PMN; Humko Sheffield) broth prior to experimental use. PMN broth contained 5% PMN, 2% lactose, 2% Primatone (Humko Sheffield), 0.1% yeast extract, and 0.1% polysorbitan monoleate (Tween 80; Sigma Chemical Co.). The medium was sterilized by heating for 15 min at 121°C.

Preparation of frozen concentrated cultures. Procedures for producing cell crops of *L. acidophilus* and preparing frozen concentrated cultures were similar to those reported by Mitchell and Gilliland (7). One-liter volumes of PMN broth were inoculated (1%) with a freshly prepared PMN broth culture of *L. acidophilus*. The inoculated medium was incubated at 37°C and maintained at pH 6 for 12 h (end of exponential growth phase for each of the three strains). Neutralizer for pH control during growth of the cultures was composed of 10% sodium carbonate in 10% ammonium hydroxide. Cells of the lactobacilli were harvested by centrifugation (4,000 × *g* for 10 min at 0°C). The cell pellets were suspended in a volume of cold sterile reconstituted 10% nonfat milk solids equal to twice their weight as determined by weighing on a laboratory balance. The resulting concentrated culture was dispensed in 2-g quantities into screw-cap cryogenic vials, frozen, and stored in liquid nitrogen (−196°C). The concentrated cultures were evaluated before freezing and after 1, 14, and 28 days of frozen storage. For the evaluations, the frozen cultures were thawed by immersion of the vials in tap water at 21 to 22°C for 5 min.

Preparation of nonfermented milk containing *L. acidoph-*

TABLE 1. Viability of concentrated cultures of *L. acidophilus* during storage at −196°C

No. of days at −196°C	Log_{10} CFU/ml[a] of strain:		
	NCFM	LA1	RAM-1
0	10.32	10.50	10.60
1	10.33	10.50	10.63
14	10.33	10.46	10.58
28	10.31	10.44	10.55

[a] Each value is the average log_{10} CFU per milliliter from six trials, determined on PMN agar; no significant differences among counts for each culture ($P > 0.05$).

ilus. The reconstituted 10% nonfat milk solids used for these experiments were heated at 100°C for 30 min and cooled to 5°C before being used. The concentrated culture was diluted appropriately in the cold 10% nonfat milk solids to yield a population of 2×10^7 to 4×10^7 lactobacilli per ml. Four bottles, each containing 100 ml of the resulting nonfermented milk containing *L. acidophilus*, were stored at 5°C. Bottles were removed one at a time for assay on days 0, 7, 14, and 21. The assays included measurement of β-galactosidase activity and enumeration of viable lactobacilli.

Enumeration of lactobacilli. Appropriate dilutions prepared according to methods described in the *Compendium of Methods for the Microbiological Examination of Foods* (11) were plated by the pour plate method with PMN agar. PMN agar was prepared by the addition of 1.5% agar to PMN broth before sterilization. The plates were incubated for 48 h at 37°C, after which all colonies visible with the aid of a Quebec colony counter were counted.

Assay of β-galactosidase activity. A 1:10 dilution of the milk containing approximately 2×10^7 lactobacilli per ml was made with 0.05 M (pH 7.0) sodium phosphate buffer. Two milliliters of the dilution was added to a tube containing 0.2 ml of egg white lysozyme (grade III [Sigma]; 50-mg/ml buffer), mixed, and held in ice-water for 30 min. One milliliter of *o*-nitrophenyl-β-D-galactopyranoside (0.012 M in 0.05 M phosphate buffer [pH 7.0]) was added to the tube. The mixture was incubated at 37°C, and after the desired incubation time, 2 ml of 0.625 M sodium carbonate was added to stop the reaction. The contents of the tube were then centrifuged for 10 min at $2,300 \times g$ to remove the slight turbidity resulting from precipitated milk solids. The A_{420} was read against a reagent blank prepared in a like manner except that 0.2 ml of buffer was used in place of the lysozyme. The number of moles of *o*-nitrophenol released was based on the relationship of the A_{420} to a standard curve. Units of enzyme activity were expressed as micromoles of *o*-nitrophenol per minute (1 U = 1 μmol/min).

Statistical evaluation of data. Six trials for each of the three strains of *L. acidophilus* were included in the study. Data for viability and for β-galactosidase activity for each of the three strains were evaluated by analysis of variance for a split-plot design. The least-significant-difference test was used to compare means.

RESULTS

All three cultures of lactobacilli used in the present study were confirmed as *L. acidophilus* on the basis of the identifying characteristics of this species presented in *Bergey's Manual of Determinative Bacteriology* (2). All three cultures were composed of gram-positive rods which grew at 45°C but not at 15°C. All strains hydrolyzed esculin and fermented amygdalin, cellobiose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, salicin, sucrose, and trehalose. None produced ammonia from arginine or fermented arabinose, mannitol, melezitose, rhamnose, sorbitol, or xylose.

Frozen storage. Freezing and storage at −196°C in liquid nitrogen had no significant effect ($P > 0.05$) on the viability of *L. acidophilus* (Table 1). None of the three strains of *L. acidophilus* exhibited loss of viability during 28 days of storage. The data are presented as the average log_{10} CFU per milliliter from six trials for each culture.

The influence of various periods of storage in liquid nitrogen was evaluated by comparing on different days the levels of β-galactosidase in samples of nonfermented milk containing cells of *L. acidophilus*. The samples were tested immediately after being prepared from the frozen cultures (i.e., before storage at 5°C). The stability of enzymatic activity varied among the three cultures (Table 2). *L. acidophilus* NCFM had significantly lower ($P < 0.05$) β-galactosidase activity on day 28 than on days 0 and 1. No significant differences ($P > 0.05$) were observed in enzyme activity for strains LA1 and RAM-1 during the 28 days of storage. Although the units of activity were not expressed as units per cell number, the data suggest that *L. acidophilus* NCFM had higher enzymatic activity than did the other two strains. This was confirmed by a comparison of units of β-galactosidase activity per 10^7 lactobacilli for the three cultures. On this basis, strains NCFM, LA1, and RAM-1 possessed 0.117, 0.035, and 0.048 U/10^7 CFU, respectively. These results were based on measurements made on the cultures before freezing.

Refrigerated storage. Statistical evaluation of the data indicated no significant interaction ($P > 0.05$) between frozen and refrigerated storage for any of the three strains of *L. acidophilus*. However, the cultures behaved somewhat differently with regard to the stability of β-galactosidase activity during storage in milk at 5°C. Figures 1 through 3 show enzyme stability curves for the lactobacilli during refrigerated storage in milk subsequent to each period of frozen storage. *L. acidophilus* NCFM exhibited significantly reduced ($P < 0.05$) β-galactosidase activity with each 7-day storage interval at 5°C (Fig. 1). These reductions in activity were much greater than the reductions observed for either of the other two strains (Fig. 2 and 3).

A statistical comparison of the overall means of enzyme activity for *L. acidophilus* RAM-1 after each 7-day storage period indicated that there was significantly ($P < 0.05$) less activity on day 21 than on days 7 and 14 but no less activity than on day 1. However, from a practical standpoint this small difference probably would not be important. *L. acidophilus* LA1 exhibited significantly less ($P < 0.05$) activity

TABLE 2. Stability of β-galactosidase activity of *L. acidophilus* during storage at −196°C

No. of days at −196°C	Activity (U/ml)[a] of strain:		
	NCFM	LA1	RAM-1
0	0.245[b]	0.152[b]	0.140[b]
1	0.242[b]	0.148[b]	0.148[b]
14	0.208[b,c]	0.165[b]	0.148[b]
28	0.200[c]	0.153[b]	0.157[b]

[a] Cells were thawed and suspended at approximately 2×10^7/ml in milk at 5°C just before assay; 1 U is 1 μmol of *o*-nitrophenol released per min per ml of milk. Each value is an average from six trials.

[b,c] Values in the same column followed by different superscript letters differ significantly ($P < 0.05$).

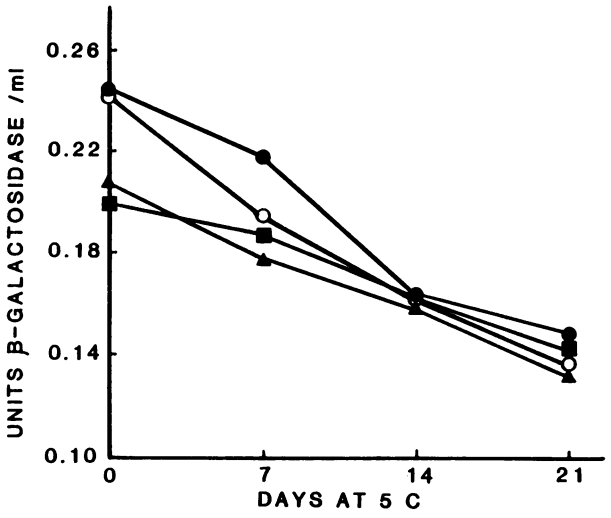

FIG. 1. Stability of β-galactosidase activity at 5°C in nonfermented acidophilus milk prepared from cells of *L. acidophilus* NCFM before storage (●) and after 1 (○), 14 (▲), and 28 (■) days of storage at −196°C. (Each point is an average from six trials.)

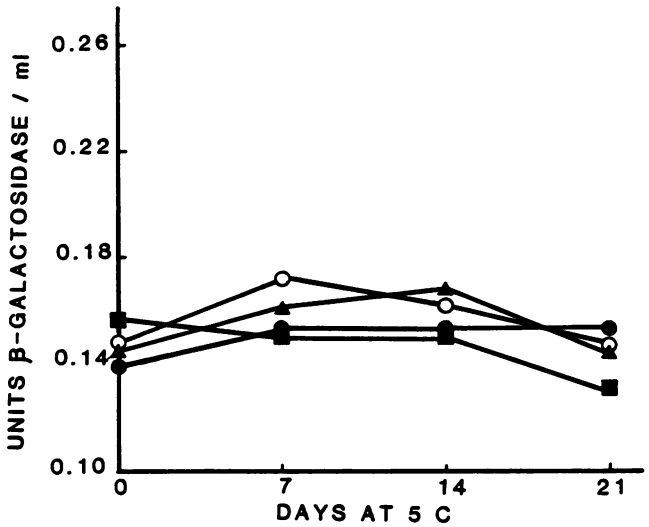

FIG. 3. Stability of β-galactosidase activity at 5°C in nonfermented acidophilus milk prepared from cells of *L. acidophilus* RAM-1 before storage (●) and after 1 (○), 14 (▲), and 28 (■) days of storage at −196°C. (Each point is an average from six trials.)

with each successive day of the storage period. However, the losses were not as great as those observed for *L. acidophilus* NCFM.

The magnitudes of losses of viability of each strain in milk stored at 5°C subsequent to each period of storage in liquid nitrogen varied (Fig. 4–6). *L. acidophilus* NCFM exhibited the greatest losses in viability (Fig. 4), followed by *L. acidophilus* LA1 (Fig. 5). Statistical comparisons of the overall mean $\log_{10}$ CFU per milliliter for each of these two strains at each storage period revealed significant reductions ($P < 0.05$) in viability with each successive 7-day storage period. *L. acidophilus* RAM-1 appeared to be the most stable of the three strains (Fig. 6). A significant loss in viability for this strain did not occur until the period from day 14 to 21 ($P < 0.05$).

DISCUSSION

The relatively small influence of freezing and storage in liquid nitrogen on the viability of the three strains of *L. acidophilus* indicates that in general this organism is stable in storage under these conditions. On the other hand, the significant reduction in β-galactosidase activity by one (strain NCFM) of the three strains shows that variation exists among strains with regard to stability of enzyme activity during storage at −196°C. Additionally, considerable differences were observed among the three strains with regard to stability during subsequent storage in milk at 5°C. The lack of significant interaction between the influence of storage at −196°C and subsequent storage at 5°C suggests that the detrimental effect of storage at the higher tempera-

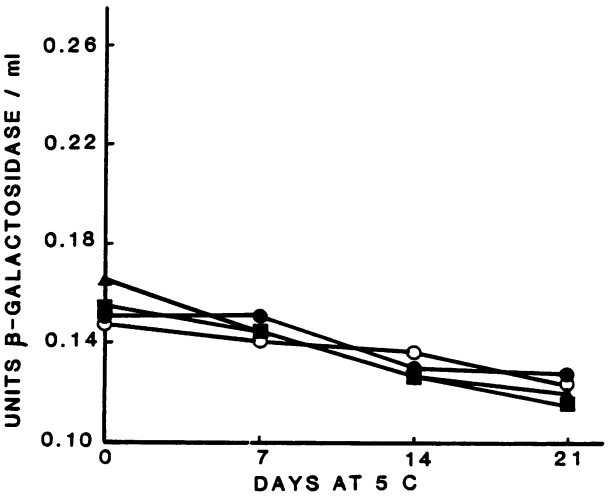

FIG. 2. Stability of β-galactosidase activity at 5°C in nonfermented acidophilus milk prepared from cells of *L. acidophilus* LA1 before storage (●) and after 1 (○), 14 (▲), and 28 (■) days of storage at −196°C. (Each point is an average from six trials.)

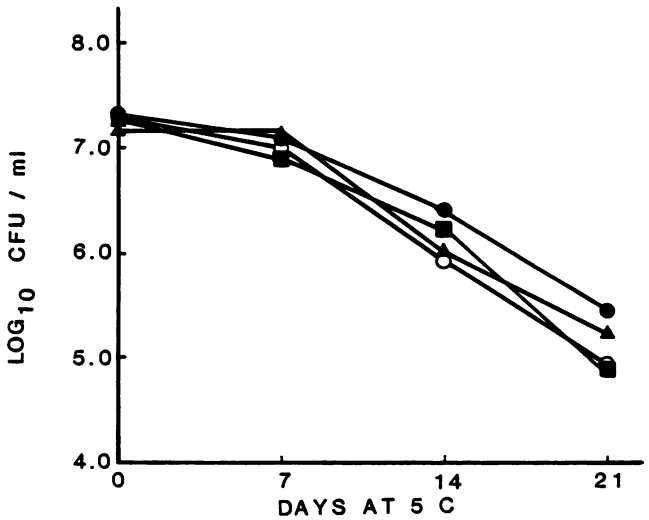

FIG. 4. Viability of *L. acidophilus* NCFM at 5°C in nonfermented acidophilus milk prepared from cells before storage (●) and after 1 (○), 14 (▲), and 28 (■) days of storage at −196°C. (Each point is an average from six trials.)

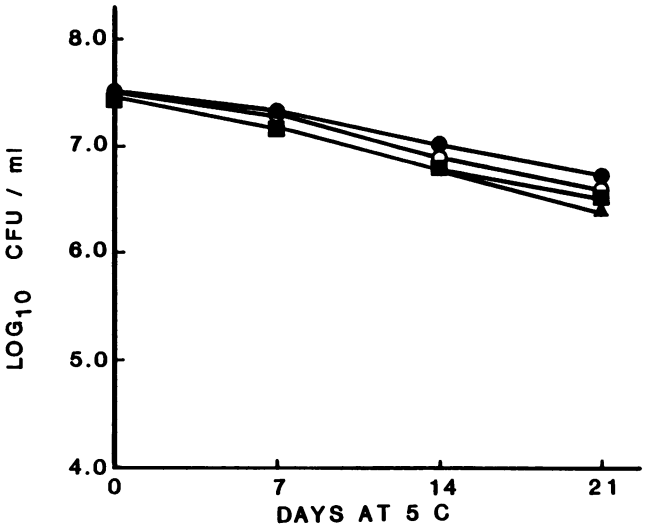

FIG. 5. Viability of *L. acidophilus* LA1 at 5°C in nonfermented acidophilus milk prepared from cells before storage (●) and after 1 (○), 14 (▲), and 28 (■) days of storage at −196°C. (Each point is an average from six trials.)

ture is not influenced by prior storage at the lower temperature. The more detrimental effect of storage at refrigeration temperature compared with that of storage at −196°C is in general agreement with previous reports concerning the stability of starter culture bacteria at freezing or refrigeration temperatures (4). Although there was some reduction in enzymatic activity for *L. acidophilus* NCFM during storage at −196°C, the most drastic influence on this strain occurred during storage at 5°C. Furthermore, the amount of enzymatic activity remaining in milk containing this strain after 14 and 21 days of storage at 5°C was not much different regardless of how long the culture had been previously stored in liquid nitrogen. Even though *L. acidophilus* NCFM exhibited the greatest decrease in enzymatic activity during storage at 5°C, it still exhibited as much activity as or more

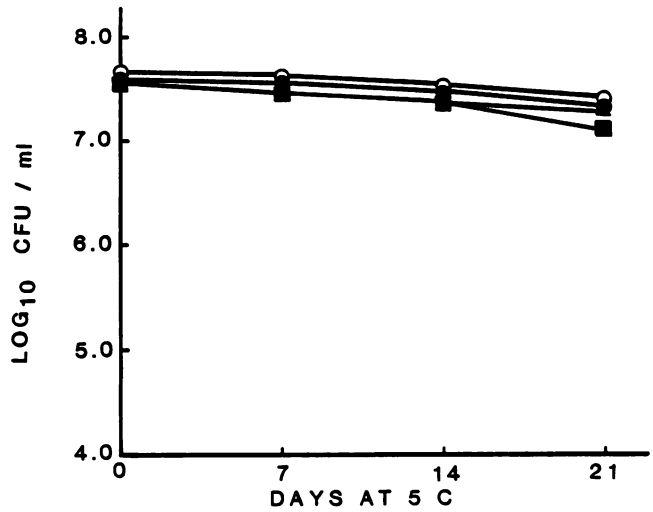

FIG. 6. Viability of *L. acidophilus* RAM-1 at 5°C in nonfermented acidophilus milk prepared from cells before storage (●) and after 1 (○), 14 (▲), and 28 (■) days of storage at −196°C. (Each point is an average from six trials.)

than the other two strains did following 14 to 21 days of storage.

There was an indication of a relationship between the viability of the strains during refrigerated storage and the β-galactosidase activity. *L. acidophilus* NCFM, which exhibited the greatest decrease in enzymatic activity during refrigerated storage, also exhibited the greatest decline in numbers of viable lactobacilli. However, the loss of viability for NCFM was of greater magnitude than was the loss of β-galactosidase activity. Thus, some cells that failed to form colonies on the PMN agar after storage apparently still possessed β-galactosidase activity. For strain LA1, intermediate reductions in enzyme activity and intermediate losses in viability were observed. Again, the loss in viability was of greater magnitude than the loss in enzyme activity. *L. acidophilus* RAM-1 exhibited the best stability with regard to both viability and β-galactosidase activity during the entire refrigerated storage period in that only minor changes were observed.

Storage conditions, particularly refrigerated storage, of milk containing cells of *L. acidophilus* can stress the cells sufficiently to have an adverse effect on certain desirable characteristics of the organism. If nonfermented milk containing *L. acidophilus* is to be useful in improving lactose utilization in lactose malabsorbers, it is important that the organism maintain both viability and enzymatic activity during storage of the product. It is important to consider the level of β-galactosidase activity when strains of the organism are selected for use as a dietary adjunct.

The results of the study further point out the importance of the need for additional research to discover means for extending the shelf life of nonfermented milk containing *L. acidophilus*. It would be highly desirable to have such a product which would retain both viability and enzymatic activity for periods longer than those observed in the present study. This would be especially true for a strain such as *L. acidophilus* NCFM, which exhibited the highest level of β-galactosidase activity of the three strains before storage. The behavior of the other strains of *L. acidophilus* in the study suggests that it would be possible to select strains of the organism which would exhibit much greater stability in storage. Of course, it would be even more desirable to have an organism which exhibited the higher degree of β-galactosidase activity, such as *L. acidophilus* NCFM, and which at the same time maintained that level of activity throughout storage of the product.

The loss of β-galactosidase activity or viability of cells of *L. acidophilus* in nonfermented milk during refrigerated storage may have contributed to the lack of beneficial effect of the milk on lactose malabsorption in some previous studies (8, 10). In these studies, neither the age of the culture nor the exact time of refrigerated storage was revealed. Previous studies in our laboratory (5) which indicated a benefit for lactose malabsorbers from consuming milk containing cells of *L. acidophilus* involved the use of NCFM cultures. In that study, the milk containing the lactobacilli was prepared freshly at 3-day intervals for the test subjects. Thus, there would have been minimal loss in viability and enzymatic activity.

Although the three strains involved in the present study exhibited apparent differences in levels of enzymatic activity and stability during storage of the product, we cannot say with certainty at this point which strain would be best to use as a dietary adjunct. In addition to having adequate enzymatic activity when consumed, cells of *L. acidophilus* must be able to survive and initiate rapid growth in the small

intestine in order to provide the fullest benefit in improving lactose utilization.

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