EFFECT OF FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE ON β-GALACTOSIDASE ACTIVITY, VIABILITY AND BILE RESISTANCE OF LACTOBACILLUS ACIDOPHILUS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

NFMS	=	Non Fat Milk Solids
PMN	=	Peptonized Milk Nutrient
PMNO	=	Peptonized Milk Nutrient plus Oxgall
ONPG	=	Ortho-Nitrophenyl-β-D-Galactopyranoside
ONP	-	Ortho-Nitrophenol
EA	=	Enzymatic Activity
CFU	=	Colony Forming Unit
ANOVA	=	Analysis of Variance

CHAPTER I

INTRODUCTION

Lactase is the common name for beta-galactosidase (β-D-galactoside galactohydrolase), which acts upon lactose hydrolyzing the β-1-4 glycosidic linkage and thus liberating the monomeric sugars, glucose and galactose. The enzyme also has transferase activity, catalyzing the formation of oligosaccharides by transferring of the glycone (galactose) moiety of the lactose molecule to various acceptors such as monosaccharides, polysaccharides, and alcohols (Richmond et al., 1981).

Since lactose is a major component and the only sugar present in mammalian milk, the hydrolytic activity of β-galactosidase is very important in the digestive system of newborn mammals. Digestion of lactose in humans takes place in the brush border of the small intestine, primarily in the jejunum (Semenza et al., 1965; Hsia et al., 1966). A review of published data on diverse populations supports the conclusion that human intestinal lactase activity reaches a maximum immediately after birth, exhibiting a decline during latter childhood and lower levels in the adult (Paige, 1981). Thus, older children and young adults are increasingly unable to digest even modest amounts of

lactose.

In the absence of appropriate lactase activity, or when the hydrolysis of lactose becomes the limiting factor (i.e. when the concentration of the sugar exceeds the hydrolytic capability of the enzyme), the undigested carbohydrate acts as a nonabsorbable substance creating a osmotic load or hyperosmosis (Kerns and Struthers, 1966; Wapnir, 1982). The retention of lactose in the intestinal lumen forces water and electrolytes to move into the lumen and increases the volume and the transit time of the small intestinal content (Launiala, 1968; Christopher and Bayless, 1971). The unhydrolyzed/unabsorbed disaccharide, upon reaching the colon, may be broken down by the action of bacterial enzymes with the production of organic acids and other fermentation products, including CO_2 and H_2 (Bond and Levitt, 1976; Bustos-Fernández et al., 1971; Christopher and Bailess, 1971). These fermentation products together with the large amount of water drawn into the intestine are responsible for varying degrees of flatulence, bloating, abdominal pain, and diarrhea (Newcomer and McGill, 1966; Bayless and Huang, 1969; Kretchmer, 1972). Persons experiencing such symptoms after the ingestion of lactose are referred in the literature as "lactose intolerants". Recognition of discomfort results in avoidance of milk or any other lactose-containing products.

Since the nutrient contribution of milk is too important to eliminate this food from the diet, researchers

have thought of suitable alternatives. Lactose-reduced dairy products, obtained by hydrolytic treatment of milk with microbial β-galactosidase, have been suggested as a means of dealing with the problem of lactose digestion. However, the availability of such products is limited by factors including flavor and economic feasibility (Hourigan, 1984). Cultured dairy foods have also been suggested as an alternative for lactose intolerant subjects to avoid the adverse symptoms experienced as a consequence of drinking regular milk (Gallagher et al., 1974).

A different approach to the previously described digestive deficiency is the consumption of non-fermented milk containing high numbers of Lactobacillus acidophilus. The preparation of this product involves the use of a culture which is grown under optimum conditions, concentrated, and stored at -196°C. The resulting frozen concentrated culture is thawed when needed and added to cold, pasteurized low fat milk; after which, the product is maintained under refrigeration until consumed. Milk containing high numbers of viable cells of L. acidophilus may be more digestible by lactose intolerant individuals because of the possible role of this microorganism in supplying a source of β-galactosidase for the intestinal tract (Kim and Gilliland, 1983).

For milk containing viable cells of L. acidophilus to be most beneficial for improving lactose digestion in humans, it is important to use strains possessing sufficient

β-galactosidase activity. Furthermore, it is important that the activity of the enzyme does not decline during stress conditions (freezing and refrigeration) imposed on the cultures during preparation and storage. To our knowledge, no information has been published concerning the effect of these conditions on the lactase system in L. acidophilus.

The primary objectives of this study were: to evaluate the β-galactosidase activity of three strains of L. acidophilus, and the stability of the enzymatic activity of concentrated cultures after being stored at -196°C and after subsequent refrigerated (5°C) storage in milk. In addition, the influence of these storage conditions on both survivability and bile resistance was evaluated for the three strains.

CHAPTER II

REVIEW OF LITERATURE

Relationship of Lactase Deficiency and Lactose Malabsorption to Lactose Intolerance

The subject of lactase deficiency has been a controversial issue for several years. Primarily confusion exists regarding terminology. Frequently, the terms "lactase deficiency", "lactose malabsorption", and "lactose intolerance" are used interchangeably. Further complication in the terminology is generated since milk is the primary source of lactose in the diet and many have concluded erroneously that lactose intolerance is synonymous with "milk intolerance". According to Lebenthal and Rossi (1982), this confusion has arisen in part because different procedures have been utilized to establish the diagnosis of lactase deficiency. Each method has its own limitations and comparing the results of indirect methods (e.g. lactose tolerance test; lactose breath tests; fecal measurements of stool pH, ^{14}C and/or reducing sugar) to the direct determination of intestinal lactase activity has been difficult (Solomons, 1981).

Although the debate continues about the overlapping

and proper use of the above terms, some definitions and diagnosis criteria reported in the literature will be presented in this section for the purpose of clarity.

Lactose malabsorption refers to lactose reaching the colon as a consequence of not being hydrolyzed and subsequently absorbed from the small intestine (Welsh, 1981). The oldest and most widely used indirect index of lactose absorption is the change in plasma glucose concentration following ingestion of an aqueous solution of lactose at a level of 2 g/Kg body weight, or 50 g total dose (McGill, 1983).. A 20 to 25 mg/dl increase in plasma glucose within the first two hours following lactose ingestion has been used as an indication of "normal lactose absorption" (Bayless and Rosensweig, 1966; Newcomer et al., 1975). Measurement of breath hydrogen excretion (Calloway et al., 1969) provides a more sensitive and objective index of inadequate lactose digestion than recording the symptoms (Solomons et al., 1977; McGill, 1983). Individuals exhibiting an increment of more than 20 ppm hydrogen excretion within two hours following ingestion of 12-50 g (0.25 - 2.0 g/Kg weight) are considered to be "lactose malabsorbers" (Caskey et al., 1977).

Lactose intolerance is the production of gastrointestinal symptoms, except nausea or constipation, associated with lactose ingestion (Welsh, 1981). The symptomatic response will depend to certain extent on the dose of lactose ingested, total intestinal β-galactosidase

activity, vehicle in which lactose is presented, variability in gastric emptying time, intestinal flora, general host health, and as yet unidentified factors (Launiala, 1968; Pirk and Skala, 1972; Bedine and Bayless, 1973; Leitcher, 1973; Gallagher et al., 1974; Bayless et al., 1975; Bond and Levitt, 1976; Welsh and Hall, 1977; Bond et al., 1980). For diagnostic testing, challenge doses of lactose are administered to the subject to correlate any laboratory index (such as rise in plasma glucose concentration, or breath hydrogen excretion) with the development of symptoms, such as bloating, abdominal pain, flatulence, eructations, and diarrhea (Solomons, 1981).

No precise definition is given for the term "milk intolerance". In general, a person who has experienced symptoms similar to those described for lactose intolerance following the consumption of one or two glasses of milk is considered to have an "awareness" of milk intolerance (Bayless, 1981). Researchers at the Massachusetts Institute of Technology (Haverbeg et al., 1980; Kwon et al., 1980) have stressed the necessity of including a placebo (i.e. lactose-free milk) to rule out any occurrence of milk intolerance due to a cause other than the presence of lactose.

Lactase deficiency or hypolactasia is defined as the state in which the total intestinal lactase activity is insufficient to hydrolyze usual amounts of dietary lactose or quantities of lactose used in acute testing (Lebenthal

and Rossi, 1982). Lactase deficiency may be primary or secondary. The secondary form is usually transient, occurring following disease entities, such as intestinal infectious diseases, kwashiorkor, etc. (Bayless and Huang, 1969). The primary form occurs as an isolated entity which may be congenital or developmental. The congenital form is an extremely rare enzyme defect present at birth and probably inherited. The developmental type of hypolactasia is a condition in which adequate lactase activity is present during infancy with a decline in the enzyme activity generally occurring in later childhood or adolescence, and activity levels greatly diminished in adulthood (Newcomer, 1981). Review of reported data supports the conclusion that high levels of lactase activity persist only in minority of human ethnic groups, including some Caucasians from Europe, Mongols of Central Asia, and some African tribes (Dahlqvist, 1983; Delmont, 1983).

An in vitro assay performed on a small intestinal mucosal biopsy specimen (Dahlqvist, 1964), provides explicit quantitative data for direct evaluation of lactase deficiency. A rate of hydrolysis of the lactose substrate of less than 2 μmoles (hydrolysis of 1 μmole of substrate per minute of 37^{o}C = 1 unit of lactase activity) per gram of wet tissue is considered to represent a deficiency (Solomons, 1981). Because of the difficulty in obtaining intestinal mucosal biopsy specimens, indirect methods have been more widely used for routine purposes. In practice,

the blood glucose test and/or the breath hydrogen test are used to detect lactose malabsorption from which, in the absence of biopsy, hypolactasia is inferred (Hourigan, 1984).

Significance of "Lactose Intolerance"

Milk is considered a nearly complete food which can provide 10% or more of the recommended dietary allowance for protein, calcium, riboflavin, niacin, vitamin D, vitamin B_{12}, phosphorus, and iodine (Phillips and Briggs, 1975). Being a relatively inexpensive source of nutrients, and easy to preserve and transport in dehydrated form, milk has been used in many programs of aid to malnourished populations around the world. However, the discovery that many individuals can not digest efficiently even modest amounts of dietary lactose has questioned the adequacy of such programs (Paige et al., 1972; Desai and Antia, 1973).

People with lactase deficiency may be intolerant of as little as 12 g of lactose (equivalent to the lactose content in 8 ounces of milk), and experience the symptoms associated with the incomplete digestion of lactose after consumption of one or two glasses of milk. As a countermeasure, these persons restrict their milk intake to levels which will not cause a laxative effect (Bayless and Huang, 1969), or in most cases develop some degree of milk rejection (Delmont, 1983). Evidence supporting the influence of lactase deficiency/lactose intolerance in milk drinking habits was

reported by Lebenthal et al. (1975). They stated that of 106 subjects interviewed with documented high intestinal lactase activity more than 90% admitted to ingest over one quart of milk per day while in 12 families with lactase deficiency, milk consumption was less than 250 ml per day. A similar trend has been observed in studies of black elementary school children (Paige et al., 1971) and American Indians (Newcomer et al., 1977).

Of considerable interest are the potential long-term complications associated with lactase deficiency/lactose intolerance. Although relatively little information regarding this subject has been reported, one of such long-term effects was described by Birge and coworkers in 1967. The authors indicated that nine of 19 osteoporotic patients had primary lactase deficiency while all the 13 control subjects had adequate lactase activity, suggesting the possible relationship between hypolactasia and osteoporosis (bone degeneration). Additional evidence supporting this idea was provided by Kocian et al. (1973), and Newcomer and colleagues (1978). According to Newcomer (1981), lactase deficiency could predispose a person to osteoporosis through either reduced milk intake or impaired calcium absorption. Since dairy products constitute the most concentrated and readily available form of dietary calcium, reduction of such products from the diet would expose the patient to a long term calcium-restricted diet which could lead to bone degeneration (Kocian et al., 1973).

Countermeasures to the Problem of Lactase Deficiency

The fact that lactase deficiency causing milk intolerance is prevalent among many people around the world is a matter of concern of scientists, nutritionists, health professionals, and policy planners. Recognition of the problem has led to continuous searching for means of providing affected individuals with an acceptable milk substitute. Suitable alternatives to regular milk that might benefit these patients include: low-lactose milk, cultured dairy products, and nonfermented acidophilus milk.

Production of low-lactose milk by ultrafiltration is one of the most recent technological means of supplying a substitute for those who can not digest regular amounts of dietary lactose. High cost, and loss of other nutrients during ultrafiltration are the main disadvantages of this product (Rao et al., 1984). Low-lactose milk can also be obtained by hydrolysis of the milk sugar with added, food grade microbial β-galactosidase. A number of studies have shown that consumption of lactose-hydrolyzed milk results in improved carbohydrate absorption and diminished symptoms of gastrointestinal discomfort (Paige et al., 1975; Cheng et al., 1979; Payne et al., 1981; Reasoner et al., 1981; Newcomer et al., 1983; Savaiano et al., 1985).

In the U.S.A., commercial microbial β-galactosidases are available for home use in liquid and powder form. The

disadvantage of modifying the milk at home is that the user must wait 24 hours for the lactase enzyme to act. Ready-to-drink low-lactose milk is sold in some cities of the U.S.A. at an affordable price [9 cents per 0.95 liter more than regular 2% milk (Richmond et al., 1981)]. However, consumer acceptability seems to be one of the factors limiting the success of the product. In a study by Reasoner et al. (1981) in which low-lactose milk was evaluated for test acceptance and clinical symptomatology, the beverage was described as being sweet and lacking body. The patients manifested preference for both regular skim milk and non-fermented acidophilus milk because of their flavor.

Although milk treated with microbial β-galactosidase has shown to be a satisfactory solution in many cases of milk intolerance due to lactase deficiency, this may not always be the best alternative. In many parts of the world where hypolactasia is common, the use of lactose-modified milk may be limited by factors such as high cost and/or consumer preference for traditional foods. Certainly, in many Asian and African countries, traditionally fermented dairy products are far more popular and better tolerated than milk, per se (Bayless and Huang, 1969; Hourigan, 1984).

The apparent increased digestibility of the lactose in fermented dairy products has been attributed in part to the reduced lactose content of these foods (Baer, 1970; Kilara and Shahani, 1976; Alm, 1982). It has also been proposed that the lactase-containing microflora of such products may

continue to be active in the intestinal tract and contribute to the hydrolysis of the ingested lactose (Gallagher, 1974; Kilara and Shahani, 1976).

Claims of the benefit of cultured dairy products in improving the digestion of dietary lactose has been challenged by several scientists. Gallagher et al. (1974) tested the lactase-deficient patients' tolerance to yogurt, cottage cheese, and buttermilk. They observed that the patients consumed such foods without experiencing symptoms of gastrointestinal discomfort, whereas ingestion of non-fermented dairy products caused moderate to severe symptoms. The authors suggested that the bacteria involved in the fermentation process continued to exert lactase activity in the intestine after ingestion. Evidence supporting this theory was obtained from a study in rats conducted by Goodenough and Kleyn (1976). They observed that rats fed with nonpasteurized yogurt were less symptomatic and showed greater intestinal lactase activity than those fed with pasteurized yogurt. It was stated that yogurt microflora may contribute to the successful metabolism of lactose.

Convincing results regarding this subject were provided recently by Kolars et al. (1984). These researchers used the breath hydrogen test to determine whether lactose mal-absorbers could digest lactose in yogurt better than lactose in milk. Ingestion of 18 g of lactose in the cultured food resulted in only about one third as much hydrogen excretion as an equivalent load of the disaccharide in milk or water.

Flatulence and diarrhea were reported by 80% of the test subjects drinking milk but by only 20% of those receiving yogurt. Assay of duodenal biopsy specimens for lactase activity showed an appreciable increase in the enzymatic activity for at least one hour after ingestion of yogurt. It was concluded that the lactase released by the yogurt bacteria could survive the passage through the stomach, resulting in an improved intraintestinal digestion of the lactose present in yogurt. These observations were confirmed in a study published almost simultaneously by Gilliland and Kim (1984). Consumption of pasteurized yogurt containing viable starter bacteria resulted in less breath hydrogen excretion when compared to that of pasteurized yogurt, indicating that lactose digestion was enhanced by the presence of viable bacteria in the unheated yogurt. On the other hand, utilization of part of the lactose by the starter culture bacteria during the manufacture of the cultured food was deemed to be sufficient to provide some beneficial effect. This was evidenced by both a 32% decrease in the lactose content of the yogurt mix during the fermentation process, and the fact that the test subjects produced less breath hydrogen when they received cultured yogurt (either heated or unheated) than when they were given direct acidified (uncultured) yogurt.

The beneficial effect of viable starter bacteria for those who can not tolerate usual amounts of dietary lactose seems not to be restricted to the consumption of fermented

dairy foods. A relatively recent approach is the suggestion that unfermented but culture-containing products, such as "sweet" acidophilus milk, would also be beneficial. In the manufacture of this beverage, a concentrated cell suspension of a bile resistant strain of L. acidophilus is added to pasteurized low-fat milk to achieve a population consisting of several millions of cells per milliliter. If the milk is maintained at a temperature below 15°C, the added lactobacilli do not multiply and the taste and appearance of the product remains undistinguishable from the original milk (Speck, 1980). Feeding trials have demonstrated that nonfermented acidophilus milk can be used as a source of L. acidophilus capable of surviving passage through the intestinal tract (Gilliland et al., 1978).

Evidence regarding the utility of L. acidophilus as a milk-dietary adjunct to aid lactose digestion was provided by Kim and Gilliland (1983). The effect of the bacterial supplement on the lactose digestion of individuals described as lactose malabsorbers was monitored by using the breath hydrogen test. Ingestion of whole milk (5 ml/Kg body weight) supplemented with either 2.5×10^6 or 2.5×10^8 cells/ml of L. acidophilus, resulted in a significantly reduced excretion of breath hydrogen when compared to that observed after the ingestion of uninoculated whole milk. The improved lactose digestion was found to be immediate, not requiring that the beverage be consumed daily. Contrary to this finding are the results of Nichols (1978) and Payne

et al. (1981). The former author tested commercial non-fermented acidophilus milk, containing 2.0 to 4.0 x 10^6 cells/ml of L. acidophilus, on lactose malabsorbers receiving 5 ml/Kg body weight. She observed no significant difference between hydrogen response whether the test subjects consumed regular low fat milk or commercial non-fermented acidophilus milk. In the study by Payne et al. (1981), lactose malabsorbers were challenged with 480 ml doses of regular low fat milk, lactase-treated (74-91% lactose hydrolysis) milk, and commercial nonfermented acidophilus milk. The authors indicated that only the lactase-treated milk significantly reduced breath hydrogen responses and symptomatic discomfort from the test subjects. Based strictly on symptomatic responses, Reasoner et al. (1981) and Newcomer et al. (1983) found that nonfermented acidophilus milk did not improve lactose-milk intolerance, nor in the latter case, did it relieve the symptoms of patients with irritable bowel syndrome.

In a recent publication, Savaiano et al. (1984) failed to demonstrate improved lactose digestion in lactose malabsorbers fed with 480 g of milk that had been inoculated with L. acidophilus. According to Kim and Gilliland (1983), the failure of some researchers to demonstrate the beneficial role of L. acidophilus in improving intestinal lactose digestion has involved issues including utilization of large (unphysiological) test doses, use of subjective measurements for evaluation, and lack of consideration of

both identity and physiological state of the culture in the product being evaluated. If the culture is to have a beneficial function in relation to lactose utilization, it is necessary that it be produced under conditions to ensure adequate β-galactosidase activity. Furthermore, it is necessary that the activity be maintained during frozen and subsequent refrigerated storage prior to consumption by the lactose intolerant person.

Quality of *L. acidophilus* Cultures for Use as Dietary Supplements

Recognition of desirable characteristics of microorganisms is important in the selection of cultures for use as dietary adjuncts. Some of these characteristics were described by Gilliland (1979) as follows. During storage in the food carrier, the culture should maintain its desirable biological properties. Once ingested, the microorganism should survive and produce the desired effect in the intestinal tract. Stability of the microbial supplement in the carrier can be greatly influenced by the conditions under which the cells were previously grown and handled. Survival in the intestinal tract would require, among other things, that the organism be able to resist both acidic conditions in the stomach and bile secretions in the intestine.

Media composition as well as growth conditions (such as pH, temperature, oxidation-reduction potential, etc.) are very important factors which can influence the

stability of the cultures during storage. Mitchell and Gilliland (1983) evaluated the influence of pepsinized-whey media containing three different whey concentrations (2.5, 5.0, and 7.5%) on the storage stability and bile resistance of L. acidophilus NCFM. Cells grown in the 2.5% whey medium exhibited the greatest viability and bile resistance after frozen (-196°C) and subsequent refrigerated storage. In a recent work, Rich and Gilliland (1986) reported on the influence of growth at different pH levels on the storage stability L. acidophilus. No significant changes in total or bile-resistant lactobacilli were observed over a 28-day period of storage in liquid nitrogen (-196°C). Growth at pH 5.0 resulted in higher cell yield and greater stability during refrigerated (5°C) storage in milk than cells grown at other pH levels (5.5, 6.0, 6.5, and 7.0).

Cultures of L. acidophilus for use as dietary supplement have been prepared as dry pharmaceutical products. Drying, however, may stress cells sufficiently to reduce their viability and bile resistance (Speck, 1980). Gilliland and Speck (1977a) evaluated 15 products sold as sources of bile resistant lactobacilli, seven of which were dry preparations. They observed that, among the dry products tested, only two contained considerably high numbers of viable, bile resistant lactobacilli (1.0×10^6 to 1.4×10^8/g). Identification procedures carried out for three of these dry products indicated that only one of them contained L. acidophilus.

Commercial production of nonfermented milk containing high numbers of viable bile resistant L. acidophilus has been possible by the development of frozen concentrated cultures. Although frozen storage causes stress to bacterial starter cells (Speck, 1980), thermophilic lactic acid bacteria such as L. acidophilus do not withstand drying and freeze-drying as well as they do freezing (Auclair and Accolas, 1983). To minimize stress during freezing, storage in liquid nitrogen has been recommended as the best method for maintaining a high level of viability and activity of the cultures (Baumann and Reinbold, 1966; Gilliland and Speck, 1974).

L. acidophilus used in the manufacture of nonfermented acidophilus milk is subjected to frozen storage during the preparation of fozen concentrated cultures, and subsequent refrigerated storage in the milk before consumption. However, it is uncertain whether or not the combined effect of these treatments can affect the metabolic capability of the cultures, in particular those related to enzymatic activities. Since one of the suggested benefits of nonfermented acidophilus milk relies on the lactose-hydrolyzing capability of L. acidophilus, it is important that the β-galactosidase system of this organism remains unaltered after storage in the food carrier.

Lactose-hydrolyzing Systems of the Lactobacilli

In the majority of microorganisms, including lactic acid bacteria, the mechanism by which lactose is transported into the cell determines the type of enzyme which hydrolyzes the sugar into its constituent monosaccharide moieties (Tinson et al., 1982). In most mesophilic dairy streptococci, for instance, lactose is translocated into the cell and phosphorylated by a phosphoenol pyruvate-dependent phosphotransferase system (PTS). The phosphorylated substrate is then hydrolyzed by phospho-β-galactosidase (phospho-β-gal) to release glucose and galactose-6-phosphate (McKay et al., 1969; Thompson, 1979; Kandler, 1983). In contrast, other lactic acid bacteria, such as Streptococcus thermophilus, utilize a system similar to that found in Escherichia coli. In these microorganisms, lactose is taken up by a permease-type system and then cleaved by β-galactosidase (β-gal) to release glucose and galactose (Kepes and Cohen, 1962; Tinson et al., 1982; Kandler, 1983; Herman and McKay, 1986). In addition, the presence of both β-gal and phospho-β-gal activities have been reported by Farrow (1980) and Crow and Thomas (1984) for slow lactose-fermenting strains of lactic streptococci.

The mode of lactose uptake has not been well defined for members of the genus Lactobacillus, except for some strains of L. casei. Most researchers have focused prima-

rily on characterization, and determinations of β-gal and phospho-β-gal activities. Based on the type of lactose-hydrolytic enzyme found, inferences about the corresponding uptake mechanism have been drawn. In general, it has been suggested that the transport and hydrolysis of lactose in lactobacilli may occur by two routes: 1) a PTS followed by phospho-β-gal activity and, 2) a permease-type system followed by β-gal activity (Kandler, 1983; Hickey et al., 1986).

Premi et al. (1972) reported on the lactose-hydrolyzing enzymes of lactobacilli. Out of 13 species examined, 11 had both β-gal and phospho-β-gal; the activity of the former being always greater than that of the latter enzyme. Similar results were obtained by Cesca et al. (1984) for 12 lactobacilli, including L. acidophilus 4356. Recently, Hickey et al. (1986) observed high β-gal activity and no significant phospho-β-gal activity for L. bulgaricus, L. lactis and L. acidophilus. The effect of various sugars on induction and repression of β-gal was reported only for L. helveticus 766. The levels of β-gal exhibited by this microorganism were respectively low, moderate, and high for cells grown on glucose, galactose, and lactose. There was a marked increase in the rate of β-gal synthesis after the addition of lactose to the culture growing in either glucose or galactose. However, the addition of glucose to cells growing on either lactose or galactose caused repression of β-gal synthesis.

Regulation of the uptake and hydrolysis of lactose in

L. casei was studied by Chassy and Thompson (1983). Initial screening tests indicated the presence of both lactose-PTS and phospho-β-gal activity in all the lactose-fermenting strains examined. For most cultures of L. casei, growth on several β-galactosides except galactose caused full induction of the lactose-PTS and phospho-β-gal. Glucose, on the other hand, appeared to exert a negative control on the lac genes (i.e., the genes coding for the lactose-PTS proteins and phospho-β-gal). Considerable variation in the ratio of PTS activity to phospho-β-gal activity was noticed when L. casei strains were grown under different conditions. The results from this experiment suggested the existence of at least two independent regulatory controls for modulation of the expression of lac genes in L. casei. Evidence supporting this idea was obtained in a previous work by Lee et al. (1982). These authors found that the PTS structural genes are not in an operon that includes the phospho-β-gal gene in L. casei 6 4H. The plasmid-encoded phospho-β-gal structural gene isolated from this strain was said to have its own transcription promoter and terminator.

CHAPTER III

MATERIALS AND METHODS

Source and Maintenance of Cultures

Three strains of Lactobacillus acidophilus were used in this study: L. acidophilus 1 and L. acidophilus NCFM were from the Dairy Foods Microbiology Laboratory at Oklahoma State University. L. acidophilus RAM-1 was isolated from a commercial sample of nonfermented acidophilus milk purchased from a local store. The strains were examined for purity and identity was confirmed by using Gram stain, catalase test, growth at 15 and 45°C, and Minitek system (Gilliland and Speck, 1977b).

Stock cultures were maintained by vacuum drying in 10% non-fat milk solids (NFMS) and stored dried at -20°C. When a subculture was desired, the dried stock culture was reactivated by at least three successive transfers in sterile 10% NFMS or in sterile 10% NFMS supplemented with 0.5% primatone (Kraft Inc., Norwich, NY). Incubation conditions for the reactivation of cultures were 37°C for 18 hours or longer, and formation of a firm curd in the milk was considered a positive test for growth. The sub-cultures were routinely propagated (at 37°C for 18 hrs.) by weekly transfers (1% v/v

inoculum) in the same media used for reactivation. Before using a particular strain in a scheduled experiment, the milk-propagated microorganism was transferred twice (1% v/v inoculum, incubation at 37°C for 18 hrs.) in Peptonized Milk Nutrient (PMN) broth, a medium described later in this chapter. All cultures were stored at 5°C between transfers.

Isolation and Identification of L. acidophilus From a Commercial Product

Lactobacillus Selection (LBS) agar was utilized as the selective medium for isolation of L. acidophilus from a commercial sample of nonfermented acidophilus milk purchased from a local supermarket. The medium was prepared by dissolving the following ingredients in one liter distilled water: 10 g Trypticase peptone (Baltimore Biological Laboratories, BBL: Cockeyville, MD), 5 g yeast extract (BBL), 6 g monopotassium phosphate (EM Science, Cherry Hill, NJ), 2 g ammonium citrate (MCB Manufacturing Chemist, Inc., Cincinnati, OH), 20 g dextrose (J.T. Baker Chemical Co., Phillipsburg, NJ), 1 g Tween 80 (Sigma Chemical Co., St. Louis, MO), 25 g sodium acetate trihydrate (J.T. Baker Chemical Co.). In addition to these ingredients, 5 ml of a sterile ten-fold concentrated mineral solution was added. [The mineral solution consisted of 11.77 g magnesium sulfate heptahydrate (J.T. Baker Chemical Co.), 1.34 g manganese sulfate monohydrate (J.T. Baker Chemical Co.) and 0.622 g

ferrous sulfate (J.T. Baker Chemical Co.) dissolved in 50 ml distilled water.] When all these ingredients were dissolved, the pH was adjusted to 5.5 using glacial acetic acid (J.T. Baker Chemical Co.) and 15 g of agar (Difco Lab.) was added. The resulting mixture was heated to boiling and held for two minutes with constant agitation. While still hot, the medium was aseptically dispensed into sterile dilution bottles, allowed to cool, and stored under refrigeration until used.

The protocol for the isolation of lactobacilli from a commercial sample of nonfermented acidophilus milk was similar to that reported by Martin and Gilliland (1980). The milk sample was diluted by preparing serial dilutions with 99 ml peptone dilution blanks (Gilliland et al., 1976), pour plated with LBS agar, and incubated at 37°C for 48 hours in a GasPak anaerobic system (BBL). At least three of different types of well isolated colonies were selected from the LBS agar plates and their characteristics recorded. Cells of the selected colonies were inoculated, with the aid of a sterile inoculating needle, into tubes containing 10 ml sterile PMN broth. The tubes were incubated at 37°C until growth was evidenced by turbidity. After incubation, Gram stain (Burke's modification, 1922) was performed for microscopic examination. Further purification was done by streaking the isolates on PMN agar plates with incubation at 37°C for 48 hours in anaerobic GasPak jars (BBL). The colonial morphology of each culture was observed as an

indication of whether or not the culture was pure.

Each isolate showing signs of purity—as indicated by the formation of colonies of similar characteristics, and same cellular arrangement and morphology (i.e. Gram positive, rod-shaped cells arranged in a particular pattern)—was restreaked onto duplicate plates of PMN agar and incubated under the same conditions described above. After incubation, one plate was saved for both Gram stain and catalase reaction while the other one was utilized as inocula for monitoring growth at 15 and 45^{o}C, and fermentation patterns. These tests were carried out to verify the identity of the cultures.

The Gram stain procedure used was that modified by Burke (1922). In checking for catalase reaction, the colonies on one of the PMN plates were flooded with 3% hydrogen peroxide, and production of effervescence from the colonies was considered a positive test for the presence of catalase.

Cells from growth on the remaining plate were collected with the aid of a sterile polyester-fiber swab (American Scientific Products Inc., MacGraw Park, IL) and transferred into 2.5 ml of sterile basal broth, a medium similar to lactobacilli MRS broth (Difco Lab.) without glucose and beef extract adjusted to pH 7.2 (Gilliland and Speck, 1977b). This cell suspension was utilized to inoculate Minitek plates containing a series of substrate disks, used in determining the fermentation patterns of the isolates according with the method described by Gilliland and Speck (1977b).

The following reactions were tested for each culture: hydrolysis of esculin, deamination of arginine and fermentation of amygdalin, arabinose, cellobiose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. The inoculated basal broth was also used to inoculate (1% v/v inoculum) two 10-ml PMN broth tubes per culture. One tube was maintained at 15°C for one week while the other one was held at 45°C for 48 hours. In both cases, visual turbidity was a positive test for growth.

Preparation and Storage of Concentrated Cultures

The procedures used in the production of the cell crops used in preparation of concentrated cultures were similar to those reported by Mitchell and Gilliland (1983). The cells were grown for 12 hrs. at pH 6.0 and 37°C in a 1-liter fermentor vessel which contained ports for neutralization, inoculation, and removal of samples. The pH was maintained by a Horizon pH control unit (Horizon Ecology Co., Chicago, IL) with a 465 Ingold pH electrode (Ingold Electrodes Inc., Andover, MA) and an automatic pump for adding neutralizer as needed. The growth medium was kept under constant moderate agitation by a stirrer adapted to the head of the fermentor to disperse the neutralizer. For sterilization, one liter of distilled water was placed in the vessel and the entire assembly autoclaved at 121°C for 45 min. The

water was removed from the fermentor jar prior to adding the sterile growth medium.

Peptonized-milk Nutrient (PMN) broth was utilized for growing the cells in the fermentor. The medium was prepared by dissolving the following ingredients in one liter of distilled water: 50 g Peptonized-milk Nutrient (Kraft Inc.), Norwich, NY), 20 g lactose (BBL), 20 g primatone (Kraft Inc.), one g yeast extract (Difco Labs.), and one g Tween 80 (Sigma Chemical Co.). The broth was sterilized by autoclaving at 121^{o}C for 15 min., then allowed to cool at room temperature.

In setting the fermentor for inoculation, the sterile PMN broth was added to the sterile jar, the temperature allowed to equilibrate at 37^{o}C, and the pH control unit adjusted to maintain the growth medium at pH 6.0. The medium was then inoculated (1% v/v) with a 18-hr old culture of L. acidophilus grown at 37^{o}C in PMN broth.

A basic solution consisting of 10% Na_2CO_3 in 10% NH_4OH was utilized to neutralize the acid produced by the cells during growth. In the preparation of the base, 58.5 g $Na_2CO_3.H_2O$ were dissolved in 417.5 ml distilled water, autoclaved at 121^{o}C for 15 min., and allowed to cool at room temperature. The sodium carbonate solution was then mixed with 82.5 ml of concentrated NH_4OH to get the final neutralizer mixture. The neutralizer was placed in a sterile reservoir, connected to the pH control unit, and added as needed to the culture medium by peristaltic pumps controlled by the pH control module.

Cells were harvested at the end of the logarithmic phase of growth, which corresponded with 12 hours of incubation. For L. acidophilus 1 and L. acidophilus RAM-1, this time was established from growth curves (Figs. 5 and 6, Appendix A) obtained in preliminary trials of the present experiment. For L. acidophilus NCFM, the time of harvesting was estimated in the course of another research project (in progress) conducted by this laboratory.

Cultures grown in the fermentor for 12 hours were immediately chilled by placing the fermentor jar for 30 minutes in ice water. The cells were subsequently harvested by centrifugation at 4,080 x g for 10 minutes at 0°C in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge (Fran Sorvall, Inc., Norwalk, CO). Thereafter, the supernatant fluid was discarded and the cell pellets were resuspended, with the aid of sterile glass beads (0.3 cm diameter), in cold sterile 10% NFMS in a proportion of 2:1 by weight (Mitchell and Gilliland, 1983).

The concentrated cultures were prepared for freezing by dispensing 2-gram quantities into sterile 2-ml polyethylene screw cap cryogenic vials (Dinatech Laboratories, Inc., Alexandria, VA). Two vials containing unfrozen concentrates (F_0), were placed in ice water until use the same day for inoculation of the corresponding set of refrigerated milk samples. The other vials were frozen and stored for 1 day (F_1), 14 days (F_{14}) and 28 days (F_{28}) at -196°C in the liquid phase of liquid nitrogen (Fig. 1).

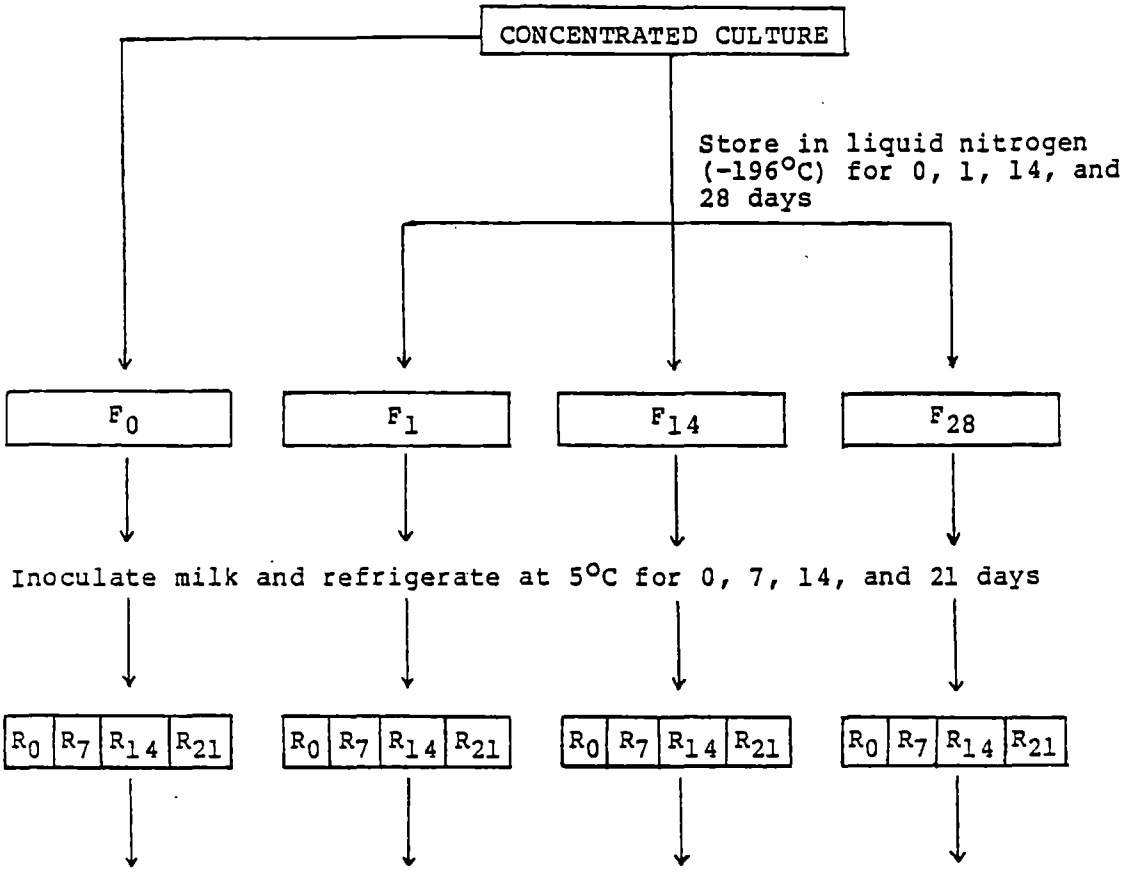

Figure 1. Flow Chart of Enumeration of Cultures of L. acidophilus and Evaluation of β-galactosidase Activity After Frozen and Subsequent Refrigerated Storage.

Preparation and Storage of Nonfermented Acidophilus Milk

Before refrigeration and after each period of frozen storage, the concentrated cultures were thawed by immersing the vials for five minutes in 500 ml tap water (21-22^{o}C), and placed in ice water until used for inoculation. Prior to opening, the exterior of the vials was sanitized by dipping them in ethanol and the excess of ethanol removed by air-drying.

Four sets of four milk sub-samples each were prepared for each trial when assessing the effect of refrigeration on β-galactosidase activity and viability of L. acidophilus. Within a set, the sub-samples were obtained by dividing 500 ml reconstituted 10% NFMS into one-99 ml and four-90 ml portions dispensed into five dilution bottles. After which, the bottles were heated for 30 minutes in a boiling water bath (GCA Precision Scientific Co, Chicago, IL) and cooled to 5^{o}C. One gram of the corresponding thawed concentrated culture was aseptically placed into the bottle containing 99 ml cold milk, and the contents mixed by inverting six times. A 10 ml portion of the resulting 1:100 dilution was then aseptically added to each of the four bottles containing 90 ml cold milk to achieve a population of about 2.0×10^{7} cells/ml. One of these inoculated milk sub-samples, designated as R_0, was kept in ice water until used the same day for measuring β-galactosidase

activity and viability of lactobacilli on day 0 of refrigerated storage. The rest of the inoculated milk sub-samples were stored at 5°C in a walk-in cooler (Bally Case and Cooler, Inc., Bally, PA). One bottle of each was removed for enumeration of bacterial numbers and assessing β-galactosidase activity on days 5, 14, and 21 (Fig. 1).

Measurement of β-galactosidase Activity

Six trials were conducted for evaluating the β-galactosidase activity of L. acidophilus. The evaluation was done prior to freezing (F_0) and after 1 (F_1), 14 (F_{14}), and 28 (F_{28}) days of storage of the concentrated cultures in liquid nitrogen (-196°C). In addition, stability of the enzyme was monitored during subsequent refrigerated (5°C) storage of the cultures for 7 (R_7), 14 (R_{14}), and 21 (R_{21}) days in milk (Fig. 1).

The chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) was used to measure the activity of the enzyme (Citti et al., 1964). Solutions of 0.012 M ONPG (Sigma Chemical Co.) were prepared by dissolving the substrate in 0.05 M, pH 7.0, sodium phosphate buffer. Release of β-galactosidase from the cells was accomplished by the use of lysozyme. Stock solutions (50 mg/ml) of this enzyme were made from egg white lysozyme (Grade III; Sigma Chemical Co.) dissolved in the above indicated buffer.

A 10-ml portion of the appropriate refrigerated, inoculated milk sample was added to a dilution bottle containing

90 ml cold, sterile, 0.05 M, pH 7.0, sodium phosphate buffer. The resulting 1:10 dilution, containing about 2 x 10^6 cells/ml, was used for assessing the enzymatic activity. The assay was carried out in test tubes to which the reagents were incorporated as follows: 1.0 ml ONPG, 2.0 ml diluted cell suspension, and 0.2 ml of lysozyme. All the tubes were kept in ice water until the addition of lysozyme, then they were vortexed for two seconds and incubated at 37°C in a New Brunswick Model 6072 water bath shaker (New Brunswick Scientific Co., Edison, NJ) adjusted to produce one stroke/second. Two controls, one without lysozyme and the other one without ONPG, were prepared in the same manner as the rest of the assay samples. The hydrolytic reaction was terminated at 20 minutes for L. acidophilus 1 and L. acidophilus NCFM, and at 30 minutes for L. acidophilus RAM-1, by the addition of 2 ml 0.625 M Na_2CO_3. Thereafter, the assay mixtures were centrifuged at 23,300 x g for 10 minutes at room temperature (20-22°C), and the absorbance of the supernatant fluid was measured at 420 nm in a Bausch and Lomb Spectronic 20 spectrophotometer (Bausch and Lomb Optical Company, Rochester, NY) to determine the amount of 0-nitrophenol (ONP) released by the action of β-galactosidase on ONPG and converted to 0-nitrophenolate ion by the addition of 0.625 M Na_2CO_3 (Fig. 7; Appendix E).

The moles of ONP were calculated by means of the linear regression equation:

$$Y = 0.010 + 2.165X$$

where Y = absorbance at 420 nm and X = μmoles ONP released/ml. This equation was determined from data (Table VII, Appendix B) obtained from 16 trials in which the absorbance produced by varying standard concentrations of ONP (Sigma Chemical Co.) was measured at 420 nm. The hydrolytic activity of β-galactosidase, expressed in terms of units of enzymatic activity, was estimated as follows:

$$\text{Units enzymatic activity (in } \mu\text{moles/min/ml milk)} = \frac{A}{B} \times C$$

$A = \mu$moles ONP released/min

$$= \frac{\mu\text{moles ONP/ml, from regression equation} \times \text{ml assay mixture}}{\text{incubation time in minutes}}$$

B = ml cell suspension used in assay

C = dilution factor

Viability of L. acidophilus After Freezing and Subsequent Refrigerated Storage in Milk

Six trials were conducted to evaluate the viability during frozen and subsequent refrigerated storage of the three strains of L. acidophilus. The evaluation was carried out prior to freezing (F_0) and after 1 (F_1), 14 (F_{14}) and 28 (F_{28}) days of storage of concentrated cultures in liquid nitrogen (-196°C). In addition, numbers of viable cells were determined after subsequent refrigerated (5°C)

storage of the cultures for 7 (R_7), 14 (R_{14}), and 21 (R_{21}) days in milk (Fig. 1).

Enumeration of lactobacilli was accomplished by the use of a pour plate technique. Appropriate dilutions of the milk samples containing cells of L. acidophilus were made utilizing 99-ml peptone dilution blanks according to the procedures described in the Compendium of Methods for the Microbiological Examination of Foods (Gilliland et al., 1976). The diluted samples were then plated in duplicate with melted PMN agar tempered at 45°C. The PMN agar was prepared by dissolving 1.5% Bacto-Agar (Difco Labs.) in PMN broth prior to autoclaving (15 min. at 121°C). Numbers of bile resistant lactobacilli were determined by a pour plate procedure utilizing PMNO agar, a medium composed of the same ingredients of PMN plus 0.1% Bacto-Oxgall (Difco Labs.). All plates were incubated at 37°C for 48 hours, after which colonies were counted with the aid of a Quebec colony counter and in accordance with the aerobic plate count procedure described by Gilliland et al. (1976).

The percentage loss in viability during storage was calculated from the average of non-transformed data, using the following equation:

$$\% \text{ loss in viability} = 100 - \left(\frac{B}{A} \times 100\right)$$

A = the mean CFU/ml from PMN counts obtained on day 0.

B = the mean CFU/ml from PMN counts obtained after a

particular period of storage.

The percentage loss in bile resistance at each period of storage was derived from the average of non-transformed data, using the following formula:

$$\% \text{ loss in bile resistance} = 100 - \left(\frac{D}{C} \times 100\right)$$

C = the mean CFU/ml from PMN counts

D = the mean CFU/ml from PMNO counts.

Statistical Analyses

The influence of frozen and subsequent refrigerated storage on both survivability and β-galactosidase-hydrolytic activity of cells of L. acidophilus was evaluated by means of an analysis of variance for a split plot design. Bile resistance of cells submitted to frozen and subsequent refrigerated treatments were studied by comparing PMN vs PMNO counts by means of an analysis of variance for a split plot design. Additionally, Least Significant Difference (LSD) tests were conducted for analyzing differences between means. The procedures for these analyses are outlined in Statistical Methods (Snedecor and Cochran, 1967).

CHAPTER IV

RESULTS

Isolation and Confirmation of Identity of Cultures

Lactobacillus acidophilus RAM-1 was isolated from a commercial sample of nonfermented acidophilus milk purchased from a local store. The microorganism formed lens-shaped, sub-surface colonies when pour plated with LBS agar and incubated at 37°C for 48 hr in a GasPak anaerobic system. Like L. acidophilus 1, but contrary to L. acidophilus NCFM, the L. acidophilus RAM-1 isolate did not grow well in autoclaved, reconstituted non-fat milk (10% NFMS) unless supplemented with 0.5% Primatone (a protein hydrolysate from animal origin; Kraft Inc.).

The results of the tests used in the identification of L. acidophilus RAM-1 are presented in Table I. The table also shows the results of the tests for the confirmation of the identity of stock cultures of L. acidophilus 1 and L. acidophilus NCFM used in this research. As is seen from the Table, the characteristics of the three strains match those described for L. acidophilus in Bergey's Manual of Determinative Bacteriology, 8th Edition (Buchanan, 1974).

TABLE I

IDENTIFYING CHARACTERISTICS OF CULTURES OF *L. acidophilus*

Test	Strain			
	1[a]	NCFM[a]	RAM-1[b]	Bergey's[c]
Gram Stain	+	+	+	+
Cellular Morphology	rods	rods	rods	rods
Catalase	-	-	-	-
Growth at 15°C	-	-	-	-
Growth at 45°C	+	+	+	+
NH_3 from Arginine	-	-	-	-
Acid from				
Amygdalin	+	+	+	+
Arabinose	-	-	-	-
Cellobiose	+	+	+	+
Galactose	+	+	+	+
Glucose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	-	-	-	-
Mannose	+	+	+	+
Melezitose	-	-	-	-
Melibiose	+	+	+	±
Raffinose	+	+	+	±
Rhamnose	-	-	-	-
Salicin	+	+	+	+
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	-	-	-	-
Esculin Hydrolysis	+	+	+	+

[a] Obtained from the Dairy Food Microbiology Laboratory at Oklahoma State University.

[b] Isolated from a commercial sample of non-fermented acidophilus milk.

[c] Characteristics of *L. acidophilus* as indicated in *Bergey's Manual of Determinative Bacteriology*, 8th Edition (Buchanan, 1974).

Influence of Frozen and Refrigerated Storage on the β-Galactosidase Activity of L. acidophilus

The hydrolytic activity of β-galactosidase was studied for three strains of L. acidophilus. The activity of the enzyme, expressed as units of enzymatic activity (EA) in μmoles o-nitrophenol released/min/ml milk, was monitored after frozen (-196°C) and subsequent refrigerated (5°C) storage of the cultures in milk. Results of the analysis of variance on data for each strain are given in Tables XIV, XV, and XVI in Appendix D. Since no significant freezing x refrigeration interaction was found for any of the cultures, the influence of each of these treatment factors on the EA are presented separately. Thus, when considering the effect of frozen storage on the EA, the results are summarized and compared (Table II) in terms of means obtained from 24 observations, averaging over the six replicates of the experiment and across the four periods of refrigerated storage (Tables VIII, IX, and X; Appendix C). The same criterion is used in reporting the influence of refrigerated storage on the β-galactosidase hydrolytic activity (Table III) of the strains, except that the means were calculated by averaging across the four periods of frozen storage (Tables VIII, IX, and X; Appendix C).

As seen in Table II, cells of L. acidophilus 1 and L. acidophilus RAM-1 exhibited a fairly stable enzymatic

TABLE II

EFFECT OF FROZEN STORAGE IN LIQUID NITROGEN (-196°C) ON β-GALACTOSIDASE ACTIVITY[a] OF *L. acidophilus*

	FREEZING (days)[b]			
STRAIN	0	1	14	28
1	0.140	0.137	0.140	0.136
NCFM	0.194a	0.183ab	0.170b	0.172b
RAM-1	0.150	0.157	0.155	0.148

[a] Expressed in units of enzymatic activity (EA). One unit of enzymatic activity is equivalent to one μmole of o-nitrophenol released/min/ml milk.

[b] Each value is the average EA from 24 observations (Tables VIII, IX, and X; Appendix C) across all periods of refrigerated storage. Means in a row followed by different letters are significantly different ($P<0.05$).

activity after they had been held in liquid nitrogen in that no significant reduction in activity was observed during 28 days of storage ($P>0.05$). The values of the EA means varied from 0.140 (day 0) to 0.136 (day 28) for strain L. acidophilus 1, and from 0.150 (day 0) to 0.148 (day 28) for strain L. acidophilus RAM-1. A different behavior was observed for L. acidophilus NCFM, which displayed a higher although less stable β-galactosidase activity. This was evidenced by a significant 12% decrease ($P<0.05$) in the mean EA obtained from cells frozen for 14 days; after which, the activity of the enzyme remained almost stationary.

Table III and Figure 2 illustrate the effect of storage at 5°C on the β-galactosidase activity of cells of L. acidophilus. The results indicate that, although the activity of the enzyme of L. acidophilus NCFM was higher than that of L. acidophilus 1, both cultures exhibited the same trend of decreasing ($P<0.05$) enzymatic activity with increasing time of refrigerated storage. The average values corresponding to the day 0, 7, 14, and 21 were respectively 0.224, 0.195, 0.161, and 0.140 for the former strain; and 0.155, 0.146, 0.130, and 0.122 for the latter strain. The progressive decrease in the mean values after 7, 14, and 21 days of refrigerated storage represent reductions in enzymatic activity of about 6, 16, and 21%, respectively for L. acidophilus 1; and 13, 28, and 38%, respectively for L. acidophilus NCFM. Different results were found for the strain L. acidophilus RAM-1. The

TABLE III

EFFECT OF REFRIGERATED STORAGE (5°C) IN MILK ON β-GALACTOSIDASE ACTIVITY[a] OF *L. acidophilus*

	REFRIGERATION (days)[b]			
STRAIN	0	7	14	21
1	0.155a	0.146b	0.130c	0.122d
NCFM	0.224a	0.195b	0.161c	0.140d
RAM-1	0.148ab	0.160a	0.158a	0.144b

[a] Expressed in units of enzymatic activity (EA). One unit of enzymatic activity is equivalent to one μmole of o-nitrophenol released/min/ml milk.

[b] (Tables VIII, IX, and X; Appendix C) across all periods of refrigerated storage. Means in a row followed by different letters are significantly different ($P<0.05$).

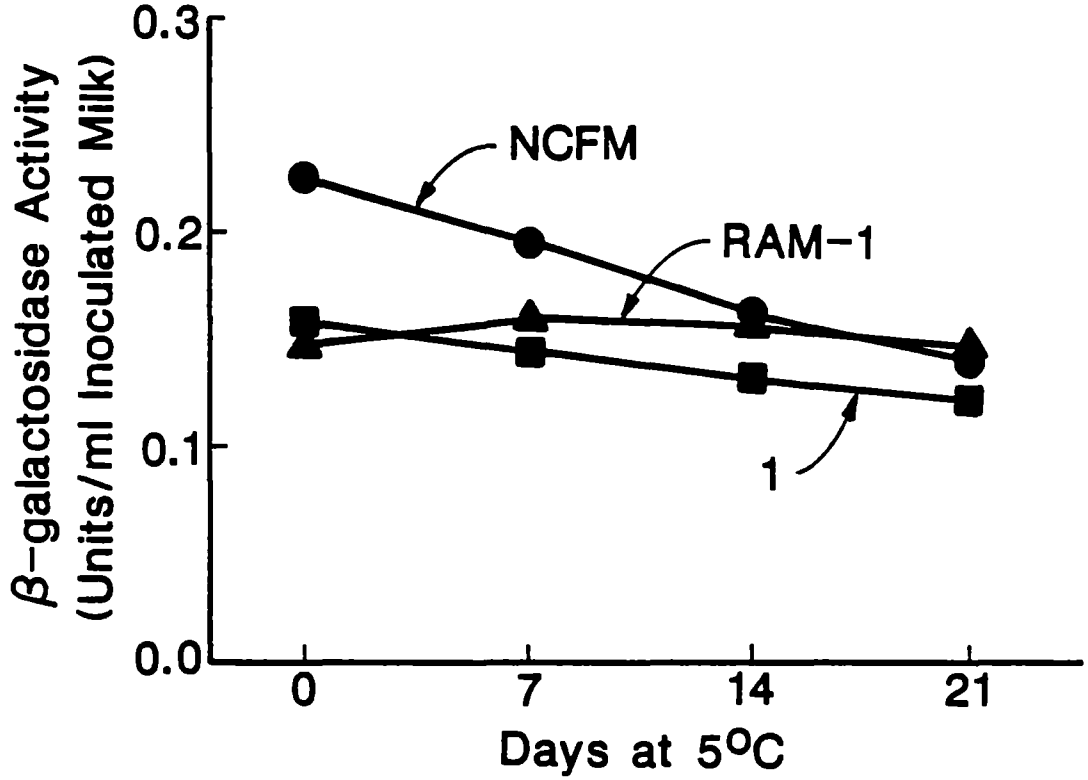

Figure 2. β-galactosidase Activity of Three Strains of L. acidophilus Stored in Milk at 5°C. Each Point is the Average Enzymatic Activity (EA), Expressed in Units/ml Inoculated Milk, Obtained From 24 Observations. One Unit of EA Equals to 1 μmole ONP Released/min/ml Milk.

analysis of variance (Table VI; Appendix D) indicated a significant effect of refrigeration on the activity of the enzyme. However, comparison among the means showed the average on days 7 and 14 (0.160 and 0.158, respectively), were not significantly different ($P>0.05$) but, a significant difference between these two means and that corresponding to 21 days of storage at $5^{o}C$ was indicated ($P<0.05$, Table III). Nevertheless, since the average EA value for the control (0.148) could not be separated from any of the other means, the significant difference in the enzymatic activity detected between the averages for days 7 and 14 compared to that of day 21 could be considered not important from a practical standpoint. Further evidence supporting such an assumption could be obtained by examining the results presented in Table X (Appendix C). Means in the first row of this Table were estimated from data of unfrozen samples which were refrigerated for either 0 (control), 7, 14, or 21 days; therefore, they constitute a more direct indication of the effect of refrigerated storage on the β-galactosidase activity of cells of L. acidophilus RAM-1. By looking at these means one can notice that, after a slight increase on day 7, the mean values remained unchanged over the 21-day storage period.

Viability of L. acidophilus During Storage

Colony counts of L. acidophilus on PMN agar were made to evaluate the stability of cells during frozen and subsequent refrigerated storage. The bacterial counts expressed as colony forming units (CFU)/ml were converted to logarithm base 10 and subjected to analysis of variance. No significant interaction between frozen and refrigerated storage was found for any of the cultures included in this study (Tables XVII, XVIII, and XIX; Appendix D). This absence of interaction makes it possible to report separately the influence of each of those treatment factors on the viability of the cultures.

The survivability of frozen cultures of L. acidophilus based on plate counts done on PMN agar is shown in Table IV. The results are presented as means of $\log_{10}$ CFU/ml estimated from 24 observations, averaging over the six trials of the experiment and across all periods of refrigerated storage. Whereas variations from 7.13 (day 0) to 7.04 (day 28) for L. acidophilus 1, and from 6.56 (day 0) to 6.36 (day 28) for L. acidophilus NCFM were not significant ($P>0.05$), a decrease in the mean $\log_{10}$ CFU/ml from 7.50 on day 0 to 7.42 on day 28 was deemed to be statistically significant for L. acidophilus RAM-1 ($P<0.05$). Despite the statistically significant loss in viability (about 17%) detected for this strain, if one

TABLE IV

EFFECT OF STORAGE IN LIQUID NITROGEN (-196°C) ON THE VIABILITY OF L. acidophilus BASED ON COLONY COUNTS ON PMN AGAR

	FREEZING (days)[a]			
STRAIN	0	1	14	28
1	7.13	7.06	7.04	7.04
NCFM	6.56	6.31	6.40	6.36
RAM-1	7.50a	7.53a	7.47ab	7.42b

[a] Each value is the average $\log_{10}$(CFU)/ml obtained from 24 observations as indicated on Tables XI, XII, and XIII (Appendix C). Means in a row followed by different letters are significantly different ($P<0.05$).

compares the individual arithmetic counts (i.e. the antilog of the $\log_{10}$ CFU/ml) obtained from the control samples with those corresponding to each period of frozen storage (data not shown), the difference between these counts are small.

A more complete picture of the stability of cells of L. acidophilus during storage is provided in Tables XI, XII, and XIII (Appendix C). The average PMN counts included in the first column of these tables were from samples which were not refrigerated but maintained in liquid nitrogen for specific periods of time; therefore, they reflect more directly the isolated effect of frozen storage on the viability of the cultures. A close examination of such mean values indicates very small variations in cell viability over the 28-days frozen storage period for all the strains.

Table V shows the influence of refrigerated storage on the viability of L. acidophilus. The results are tabulated as means of $\log_{10}$ CFU/ml and each value is the average from 24 observations. The number of viable cells of L. acidophilus 1 and L. acidophilus NCFM decreased significantly ($P<0.05$) with each 7 days of successive storage. This is evidenced by viability losses of 38, 72, and 86%; and 48, 93, and 99% for the former and the latter strain, repectively, after 7, 14, and 21 days of storage at $5^{o}C$. Cells of L. acidophilus RAM-1, on the other hand, were more resistant to refrigeration as indicated by the highest recovery of viable bacteria and the lowest, although signif-

TABLE V

EFFECT OF REFRIGERATED STORAGE (5°C) IN MILK ON THE VIABILITY OF *L. acidophilus* BASED ON COLONY COUNTS ON PMN AGAR

	REFRIGERATION (days)[a]			
STRAIN	0	7	14	21
1	7.47a	7.26b	6.92c	6.61d
NCFM	7.32a	7.04b	6.15c	5.12d
RAM-1	7.59a	7.55ab	7.49b	7.30c

[a] Each value is the average $\log_{10}$(CFU)/ml obtained from 24 observations across all periods of frozen storage (Tables XI, XII, and XIII; Appendix C). Means in a row followed by different letters are significantly different ($P<0.05$).

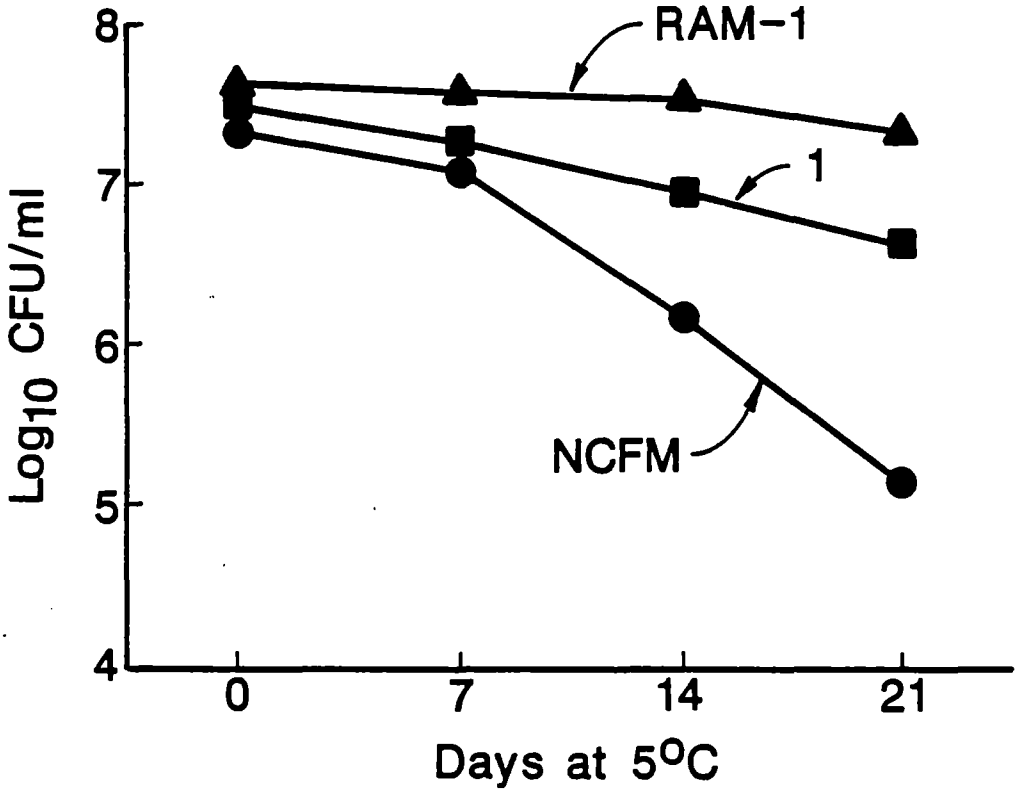

Figure 3. Storage Stability of Three Strains of L. acidophilus in Milk at 5°C. Each Point is the Average Log_{10} CFU/ml From 24 Observations.

icant ($P<0.05$), reduction (21 and 49%, respectively) in viability on days 14 and 21.

The results in Table V are also graphically illustrated in Figure 3, where the means $\log_{10}$ CFU/ml relative to the different refrigeration periods are plotted for each strain. The consistency of high numbers of viable cells can be visualized for L. acidophilus RAM-1. The magnitude of the viability loss with increased time of storage is easily seen for L. acidophilus 1 and L. acidophilus NCFM. In fact, it can be noticed that after a decrease on day 7 which was similar for these two cultures, on days 14 and 21 the counts of L. acidophilus NCFM suffered a considerably higher reduction than those of L. acidophilus 1.

Bile Resistance of L. acidophilus During Cold Storage

Comparison of PMN vs PMNO counts were done in an attempt to evaluate the ability of different cultures of L. acidophilus to grow in the presence of bile salts and retain this characteristic after being submitted to the stresses of frozen (-196°C) and subsequent refrigerated (5°C) storage. The three-way tables showing the counts, expressed as means of $\log_{10}$ CFU/ml, as influenced by freezing, refrigeration, and plating media are presented in Tables XI, XII, and XIII in Appendix C; and the corresponding ANOVA are in Tables XX, XXI, and XXII in Appendix D. For L. acidophilus 1 and L. acidophilus NCFM, the main

effects of refrigeration and media, and the refrigeration x media interaction were significant ($P<0.001$); while the other interactions (freezing x refrigeration, and freezing x refrigeration x media) were negligible (Tables XX and XXI, respectively; Appendix D). For strain L. acidophilus RAM-1, on the other hand, only the main effects of refrigeration and media are significant at the 1% level (Table XXII; Appendix D). Based on the preceding statistical results, the following assumptions are made. First of all, the survivability and bile resistance of cells was unaffected by storage at $-196^{o}C$ in this experiment regardless of the time of frozen storage. Second, all the information in the experiment showing any significant influence of refrigeration and/or media is contained in the corresponding two-way table of means (Table VI). Third, the significant Refrigeration x Media interaction found for L. acidophilus 1 and L. acidophilus NCFM indicates that their survival based on counts on PMNO relative to PMN agar is not the same at every period of refrigerated storage; therefore, further comparisons (carried out separately at each level of refrigerated storage) between the mean $\log_{10}$ CFU/ml for the two media are also shown in the two-way table. Fourth, in the case of L. acidophilus RAM-1, the absence of any significant interaction involving media allows the comparison between overall PMN vs PMNO means (averaged over all levels of the other factors).

The ability of L. acidophilus to grow in the presence

of bile salts and maintain such characteristic after refrigerated storage in milk varied among cultures (Table VI, Figure 4). Strain L. acidophilus 1 grew well in the selective medium containing bile salts after being held for seven days at 5°C, as evidenced by similar ($P>0.05$) number of CFU on PMN and PMNO agars on days 0 and 7. Afterwards, the population of bacteria in the selective medium gradually decreased ($P<0.05$) with respect to that of the non-selective one. On day 14, the means $\log_{10}$ CFU/ml were respectively 6.92 and 6.70 for PMN and PMNO, while on day 21 the corresponding average counts were 6.61 and 6.29. These values represent losses of bile resistant organisms of 40 and 52%, respectively after 14 and 21 days of storage at 5°C (Table VI, Figure 4).

Non-refrigerated cells of L. acidophilus NCFM were resistant to the action of bile salts as indicated by similar ($P>0.05$) numbers of CFU on PMN and PMNO agars at day 0 (Table VI, Figure 4). The culture, however, exhibited increasing differences between PMN and PMNO counts with each increase in the period of refrigerated storage. The means of $\log_{10}$ CFU/ml corresponding to PMN and PMNO counts were, respectively: 7.04 and 6.81 (day 7), 6.15 and 5.66 (day 14), and 5.12 and 4.51 (day 21). The differences between the counts represent losses of bile resistant organisms of about 41, 68, and 76%, respectively over the 21-day storage period.

Numbers of cells of L. acidophilus RAM-1 surviving on

TABLE VI

COMPARISON OF PMN vs PMNO COUNTS OF *L. acidophilus* AFTER STORAGE OF CULTURES IN MILK AT 5°C

STRAIN[c]	MEDIA	REFRIGERATION (days)[a] 0	7	14	21	OVERALL[b] MEANS
1	PMN	7.47a	7.26a	6.92a	6.61a	7.07
	PMNO	7.43a	7.21a	6.70b	6.29b	6.91
NCFM	PMN	7.32a	7.04a	6.15a	5.12a	6.41
	PMNO	7.30a	6.81B	5.66b	4.51b	6.07
RAM-1	PMN	7.59	7.55	7.49	7.30	7.48a
	PMNO	7.48	7.40	7.40	7.17	7.39b

[a] Each value is the average $\log_{10}$(CFU)/ml obtained from 24 observations (Tables XI, XII, and XIII; Appendix C).

[b] Each value is a mean from 96 observations.

[c] For a particular strain, means in a column followed by different letters are statistically different ($P<0.05$).

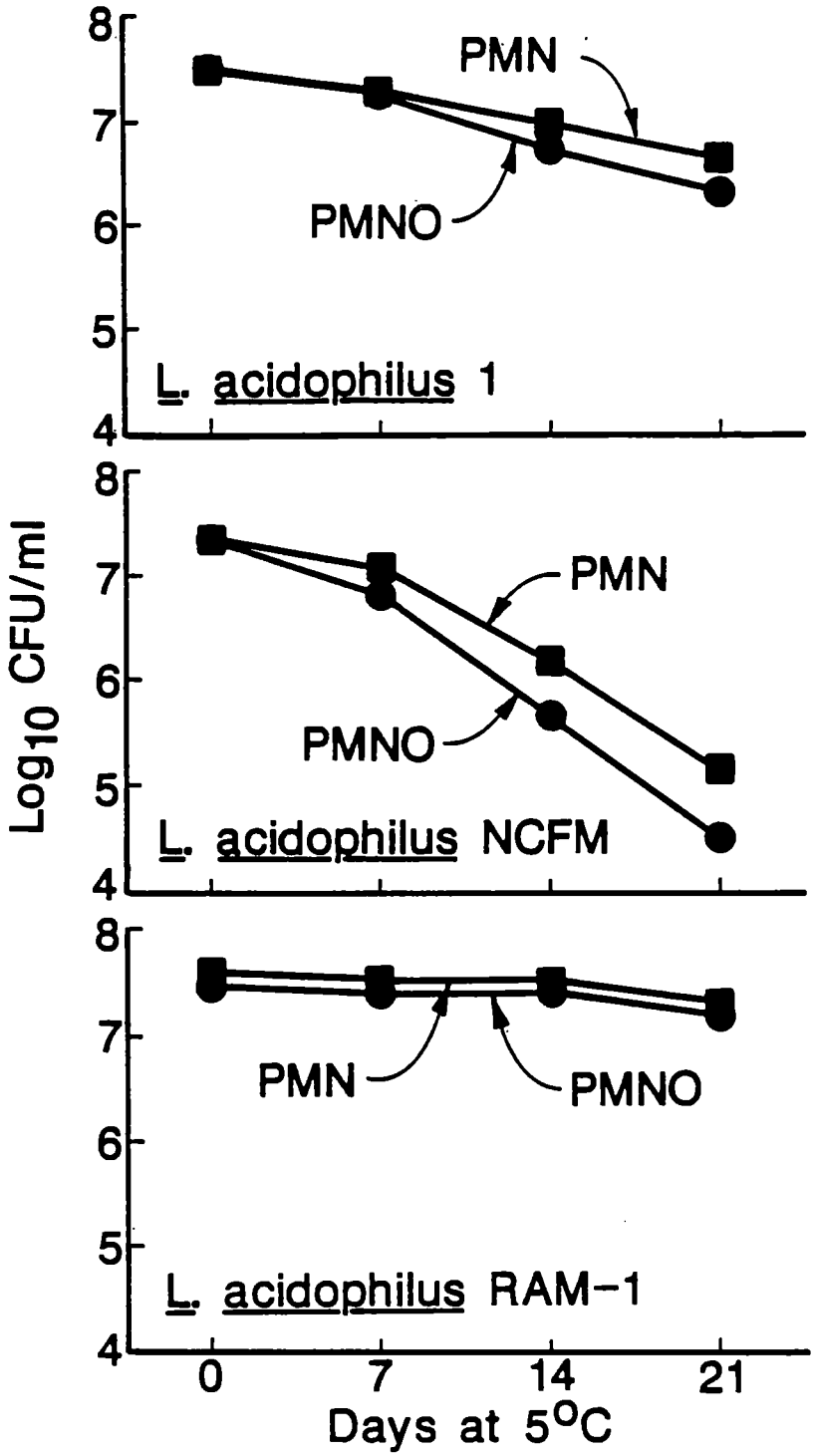

Figure 4. Viability of L. acidophilus on PMN and PMNO Agar After Storage of Milk at 5°C. Each Point is the Average Log_{10} CFU/ml From 24 Observations.

PMNO agar were significantly ($P<0.05$) lower than those on PMN agar regardless of the time the strain was maintained at 5^{o}C (Table VI, Figure 4). However, it is important to point out that, among the three cultures, L. acidophilus RAM-1 exhibited the highest survivability (in the two media) and the greatest stability during refrigerated storage in milk (Figure 4).

CHAPTER V

DISCUSSION

Utilization of lactose in milk by lactic acid bacteria, especially by microorganisms of the genera Streptococcus and Lactobacillus, is important for the dairy industry. Fermentation of this sugar makes an important contribution to the flavor, aroma, texture and preservation of cultured dairy foods (Smith and Palumbo, 1982; Cesca et al., 1984). Beyond the fermentative ability of such bacteria, an additional therapeutic role has been attributed to species of Lactobacillus which are natural components of the intestinal microflora (Metchnikoff, 1908; Kopeloff, 1926; Myers, 1931; Speck, 1980). The potential benefits derived from the activity of lactobacilli has suggested their use as a dietary supplement for maintenance of intestinal health, treatment of intestinal diseases, and as a source of β-galactosidase for lactose-intolerant persons (Sandine, 1979; Kilara, 1983). The species which have been most often suggested as beneficial dietary adjuncts include L. acidophilus, L. casei and L. bifidus (Gilliland, 1979).

Ability to resist gastric and bile acids is an important criterion in choosing the cultures to be used as

dietary adjunct (Sandine, 1979; Klaenhammer, 1982; Kilara, 1983). On the other hand, if the microbial supplement is to be used for improving lactose digestion, additional desirable characteristics would be required. For instance, the microorganism(s) must possess an adequate lactose-hydrolyzing system. Furthermore, the quality of the microbial supplement should be maintained during its preparation and storage in the food carrier prior to consumption. In fact, for large scale production, attention must be given to the physiological state of the cultures, which can be affected by culturing, preparation and storage conditions (Speck, 1980).

Among the lactobacilli, some species hydrolyze lactose via β-galactosidase while others have exhibited both β-galactosidase and phospho-β-galactosidase activity (Premi et al., 1972). For L. casei and L. plantarum, on the other hand, only phospho-βgalactosidase has been detected (Romano et al., 1979). Recently, Hickey, Hillier and Jago (1986) reported on transport and metabolism of lactose in L. helveticus, L. bulgaricus, L. lactis and L. acidophilus. All four species tested by these authors contained β-galactosidase activity, and no significant phospho-β-galactosidase activity. In a research work by Nielsen and Gilliland (in progress) on the characteristics of the lactose-hydrolyzing system of several strains of L. acidophilus, phospho-β-galactosidase activity has not been detected for any of the strains. The present study

reports on the β-galactosidase activity of frozen concentrated cultures of L. acidophilus, thawed and added to cold milk stored under refrigeration, simulating the conditions used in the preparation of commercial non-fermented acidophilus milk.

Although this work was not statistically designed to make comparisons among the strains, the results indicate differences regarding the β-galactosidase activity of the cultures. For instance, prior to frozen and/or refrigerated storage, the enzyme activity of L. acidophilus NCFM was almost twice that obtained from cells of L. acidophilus RAM-1; while intermediate enzyme activity levels were observed for L. acidophilus 1. These results suggest the need of screening cultures to select one having, among other attributes, an efficient lactose-hydrolyzing system for use as a dietary supplement.

Environmental stresses to which bacterial populations are subjected may induce adverse changes in the biological functions of bacterial cells (Cowman and Speck, 1969). Cellular damage may be manifested as injury or death. This differentiation is usually based on the ability to multiply and form colonies on different types of solid media (Speck and Ray, 1977). Injury may be manifested, for instance, as loss of the ability to form colonies on a selective medium (such as one containing bile salts); and/or damage of cellular enzyme systems (Cowman and Speck, 1965, 1969). Bacteria which are no longer able to multiply on non selective media

are regarded as dead (Speck and Ray, 1977).

Low temperature has been recognized as an environmental stress in relation to long-term preservation of biological systems (Mazur, 1965; Graham and Pace, 1967). Typical viability losses of 50 to 80% have been reported for the lactobacilli during freezing at conventional temperature of -20°C to -40°C (Sharp, 1979). Injury or death of mesophilic bacteria by chilling without freezing has also been described (Macleod and Calcott, 1976). In the present study, we were interested in the influence of low temperature as an environmental stress on some biological functions of L. acidophilus, in particular the effect of storage in liquid nitrogen (-196°C) and subsequent storage at 5°C on the β-galactosidase hydrolytic activity, viability and bile resistance of three strains of this species. Relatively small variations of these characteristics were detected after the bacterial cells had been held for a 28-day period in liquid nitrogen. On the other hand, maintenance of the strains L. acidophilus NCFM and L. acidophilus 1 for more than seven days in milk at 5°C caused an increasingly significant reductions in their β-galactosidase activity, viability and bile resistance. These observations are in agreement with the general idea of considering storage in liquid nitrogen as a means of maintaining high levels of culture activity and survivability (Mazur, 1965; Cowman and Speck, 1969; Peebles et al., 1969; Gilliland et al., 1974); and with the stress or injury

described for other microorganisms upon chilling without freezing (Strange and Dark, 1962; Cowman and Speck, 1965; 1968; Ring, 1965; Smeaton and Elliot, 1967; Jackson, 1974).

It is interesting to notice that, in addition to the difference in the initial β-galactosidase activity, the stability of the enzyme activity upon refrigerated storage also varied among the strains. In fact, L. acidophilus NCFM, which showed the greatest values of enzymatic activity up to 14 days of storage at 5°C, was also the culture suffering the highest reduction in β-galactosidase activity when maintained at this temperature. The opposite was found for L. acidophilus RAM-1 with fairly stable, although somewhat lower, enzyme activity levels over the entire 21-day period in milk at 5°C. Cells of L. acidophilus 1, on the other hand, suffered a significant, but less pronounced decrease in the enzyme activity with prolonged refrigerated storage.

An apparent relationship between the stability of both numbers of viable cells and β-galactosidase activity was noticed for the lactobacilli after being submitted to 21 days of refrigeration at 5°C. This interpretation is based on the following observations. L. acidophilus NCFM whose enzymatic activity suffered the highest decrease after each refrigerated storage interval also showed the highest decline in numbers of viable cells. For L. acidophilus 1, intermediate reductions in enzyme activity values corresponded to intermediate losses; while L.

acidophilus RAM-1 exhibited the greatest recovery in viable cell numbers, and the lowest decrease in β-galactosidase activity during the entire period of refrigerated storage.

Before explaining some of the possible mechanisms involved in the behavior of L. acidophilus cultures after being submitted to low temperature stress conditions, it is worthy to point out that substrate uptake is considered one of the factors governing the ability of microorganisms to grow at low temperature. Experimental evidence supporting this theory was provided by Baxter and Gibbons (1962) when comparing the effect of temperature on the respiratory activity of two yeast: one psychrotrophic Candida specie, and one mesophilic strain of C. lypolytica. The psychrotroph oxidized exogenous glucose at an appreciable rate at near 0°C. Endogenous substrate was, however, metabolized by C. lypolytica below this temperature. The authors concluded that the ability of the psychrotroph to transport the sugar near 0°C enabled this organism to grow at temperatures at which the mesophile could not. Similar findings were published by Rose and Evison (1965) using mesophilic strains of C. utilis and psychrotrophic isolates of Arthrobacter.

Based on the theory which correlates substrate uptake with ability to grow at low temperature, an important aspect for discussion is the fact that one of the characteristics of L. acidophilus is the inability to grow at or below 15°C. Therefore, one may suspect that storage of the

cultures for several days in milk at 5°C may cause inactivation of the mechanism for translocation of lactose into the cells; and, consequently, put them under a starvation-like state. Viability under such conditions would probably be maintained at expenses of endogenous substrates. However, with prolonged refrigerated storage many complex biochemical changes may start as injury and then end in the death of the microorganisms. One of these biochemical changes may involve the activation of autolytic enzymes, including proteases which may destroy β-galactosidase. Autolytic enzymes are believed to be present in most bacteria, and appear to be activated whenever normal growth of the organism is disrupted (Thomas et al., 1969). It is also conceivable that at temperatures approaching the minimum for growth one or more key enzymes may become cold inactivated. For instance, it may be that the β-galactosidase of some strains of L. acidophilus undergoes structural changes (e.g. changes in the tertiary and/or the quaternary structure) from an active to an inactive configuration as a consequence of prolonged refrigerated storage.

The last mentioned interpretation has been reported for other enzymes, such as the proteinase system of Streptococcus lactis in relation to casein hydrolysis (Cowman and Speck, 1969). The system was said to exist in the bacterial cells in a monomer-dimer equilibrium, for which the monomeric form was favored at 22°C, while the dimer form was favored at 3°C. However, if the enzyme

was stored sufficiently long at 3°C, an inactive trimer structure was formed. Formation of trimer was associated with the onset of terminal stages of cell damage. Cold-labile phosphofructokinase (an oligomeric enzyme) has been found in chicken liver (Kono and Uyeda, 1973), and rabbit skeletal muscle (Bock and Frieden, 1974). These researchers utilized light scattering or sucrose gradient centrifugation to study the effect of temperature on the enzyme and the results were consistent with the view that lowering the temperature caused dissociation of the enzyme into inactive sub-units. More recently, cold lability of phosphofructokinase from potato tubers was investigated by Dixon et al. (1981) using differential scanning calorimetry. Based on the fact that the oligomeric enzyme complex was exothermic, they concluded that hydrophobic interactions contributing to the stability of the enzyme at higher temperature were so weakened at low temperature that the enzyme spontaneously dissociated into sub-units and, consequently, lost its catalytic activity.

Temperature-mediated reversible alterations of catalytic activity was reported for phosphofructokinase (MW 130,000-140,000 daltons) from Bacillus licheniformis by Marschke and Bernlohr (1973). This behavior was believed to be the result of an equilibrating molecular system in which two inactive sub-units having MW of about 68,000 daltons associated to form the active enzyme. The authors indicated that dissociation and, consequently, inactivation occurred

in the presence of magnesium, adenosine-triphosphate, or magnesium-phosphoenol-pyruvate. Association of the protomers at 30°C was found to be dependent on the magnesium levels. In a study by Nielsen and Gilliland (in progress) on the characteristics of the lactose-hydrolyzing enzyme of L. acidophilus, maximum activity was observed when the purified enzyme was incubated with magnesium ions and mercaptoethanol in pH 6.6, sodium phosphate buffer at 37°C. The molecular weight of the enzyme was in the order of 570,000 daltons. Such a molecular weight suggests the presence of a β-galactosidase composed of more than one sub-unit. If cold-labile, the enzyme may dissociate when exposed to low temperature then, if placed back to the optimum temperature, the enzyme activity may be only partially recovered in the absence of magnesium ions. It could also be possible that, with prolonged maintenace at 5°C, an increased incubation time at 37°C may be required to allow recovery of the catalytic function of the enzyme (i.e. to allow reassociation of the inactive sub-units). Regarding the last proposed explanation, it is necessary to emphasize that, in the present experiment, the incubation time at 37°C for monitoring the β-galactosidase activity was kept constant for each strain regardless of the time the bacterial cells had been maintained under frozen and subsequent refrigerated storage.

Little is known at the present time about the biochemical changes, if any, occuring in the β-galactosidase of L. acidophilus during refrigerated storage. Most of

the suggested mechanisms have been observed for other enzymes in a purified state. The behavior of β-galactosidase in its natural intracellular environment may vary from that of the same enzyme in a purified state, or may be different from the one observed for other enzymes, or from that reported for the same enzyme from other microorganisms. Therefore, all the above proposed mechanisms await for further investigation. On the other hand, we can only speculate on the effect of storage in liquid nitrogen on the characteristics of the L. acidophilus cultures studied. In view of the better storage stability of the enzyme activity, viability and bile resistance of the cells kept at -196°C compared to those maintained at 5°C, one suspects that only minor changes in both the enzyme configuration and cellular structure may occur at -195°C.

Lactase-containing lactic acid bacteria present in either fermented (Gallagher et al., 1974; Goodenough and Klein, 1976; Gilliland and Kim, 1984) or nonfermented (Kim and Gilliland, 1983) milk products are believed to continue to be active in the small intestine and contribute to the hydrolysis of ingested lactose. Kolars et al. (1984) observed an increase in lactase activity in the duodenal contents of three patients after ingestion of yogurt. In vitro experiments carried out by these researchers on sonicated yogurt samples resulted in 25 units (One unit = one μmole galactose/hr/g yogurt, at pH 7.0 and 37°C) of lactase activity. No indication about the number of lactic

acid bacteria present in the fermented food was given. Speck and Geoffrion (1980) reported β-galactosidase activity estimates varying from 0.08 to 4.15 units (One unit = one μmole ONP/min/g yogurt, at pH 7.0 and 37°C) for different types of commercial yogurt samples following sonication for bacterial cell rupture. Except for only one type of yogurt, exhibiting concomitantly the lowest enzyme activity and bacterial population (5.66 $\log_{10}$ CFU/ml), the rest of the samples contained populations of bacteria ranging from 8.82 to 9.18 $\log_{10}$ CFU/ml. We have estimated for milk supplemented with cells (2.0×10^7 cells/ml) of L. acidophilus NCFM, treated with lyzozyme under conditions to produce bacterial rupture, β-galactosidase activity values of 0.224, 0.195, 0.161 and 0.140 (for samples obtained prior to, and after 7, 14 and 21 days of refrigerated storage). Here the unit of enzyme activity is equivalent to one μmole ONP/min/ml milk, at pH 7.0 and 37°C. Therefore, these values would correspond approximately to 13 (day 0), 12 (day 7), 10 (day 14) and 8 (day 21) units, if the β-galactosidase activity is expressed in μmoles ONP/hr/ml milk. A comparison of enzyme activity values from these three studies is difficult because of the following reasons. First of all, we are considering two kinds of dairy products which vary a in several aspects (e.g. composition, processing procedures, types of microorganisms, etc.). Second, the methods (sonication vs. lyzozyme treatment) utilized for rupture of bacterial cells, type of substrate (lactose vs. ONPG), and/

or incubation time used in conducting the enzymatic assay may influence the enzyme activity values reported by the authors. Furthermore, in the work by Speck and Geoffrion (1980), the β-galactosidase activity was estimated from yogurt samples whose bacterial populations were almost two $\log_{10}$ cycles higher than the number of L. acidophilus cells present in the milk samples we evaluated in this study.

In addition to the effect of cold on β-galactosidase activity, the results also show a decline in the viability of some strains of L. acidophilus during storage in milk at 5°C. Viability losses of higher magnitude were noticed for L. acidophilus NCFM when compared to that observed for L. acidophilus 1. The effect was more pronounced for these microorganisms after 14 and 21 days of refrigerated storage. Reductions in the mean $\log_{10}$ CFU/ml corresponding to such periods were in the order of 93 and 99%, and 72 and 82% for the former and the latter strain, respectively. L. acidophilus RAM-1, on the other hand, exhibited the highest numbers of viable cells during the entire period of storage at 5°C.

The viability losses observed for two of the cultures of L. acidophilus after prolonged refrigerated storage may have been a consequence of changes and/or damages of the bacterial cell structure. Membrane damage with loss of internal solutes has been described as one of the most common manifestation of injury (Silliker et al., 1980).

Leakage of endogenous constituents after chilling a cell suspension of Aerobacter aerogenes was reported by Strange and Dark (1962). They theorized that the lethal effect of chilling might be due to interference with the functioning of a bacterial permeability control mechanism. Additional evidence supporting this theory was obtained by Ring (1965) while studying the active transport of amino acids in cells of Streptomyces hydrogenans. The permeability of this organims was said to rise drastically upon cooling the cell suspension to about $0^{o}C$. Kinetic studies of the described cold effect suggested an alteration of the membrane structure. It was believed that such an alteration was due to a phase transition within the lipid layer of the cell membrane which resulted in widening of the membrane pores. According to Leder (1972), the phase transition of the membrane lipids caused by cold shock creates hydrophilic channels facilitating the escape of pool solutes. The question of whether or not refrigerated storage affects the cells of L. acidophilus due to a particular cold-sensitive membrane structure can not be answered yet. Future research on the influence of low temperature on the cellular permeability of this species may help to elucidate some of the mechanisms associated with the decline in cell viability occurring for certain strains of L. acidophilus during storage at $5^{o}C$.

Some forms of injury may also involve an increased sensitivity to inhibitors (Silliker et al., 1980). An

example of this response was provided by the work of Jackson (1974) who observed that a culture of Staphylococcus aureus in trypticase broth at 5°C became increasingly sensitive to mannitol salt agar. In the present study, information about the sensitivity of cultures of L. acidophilus to bile salts after refrigerated storage is also presented. The results indicate an increased sensitivity to such compounds for two of the cultures with prolonged storage in milk at 5°C. Comparison between PMN and PMNO counts, at each refrigerated storage period, showed variations in the behavior of the microorganisms. For L. acidophilus NCFM, the differences between the counts corresponded to losses of bile resistant organisms of about 41% (day 7), 69% (day 14) and 76% (day 21). Such losses indicate that refrigeration stresses the cells of this microorganism sufficiently to cause injury, as evidenced by an increased sensitivity of the cells to bile salts. Losses of lower magnitude than those estimated for L. acidophilus NCFM were observed for L. acidophilus 1 after 14 days of storage at 5°C. In the case of L. acidophilus RAM-1, the ability of the cells to grow in the presence of bile salts was not affected by refrigeration indicating it was not as susceptible to injury during refrigerated storage as the other two strains.

The apparent different behavior, in relation to both viability and bile resistance, exhibited by the cultures after refrigerated storage may reflect variations in the

chemical composition of their cell membrane. Experiments conducted by Steim et al. (1969) with Mycoplasma laidldawii showed that when the membranes are cooled the lipids change from a liquid to a solid state. They indicated that the temperature of the phase transition varied with the chain length and the degree of unsaturation of the fatty acids in the membrane phospholipids. Thus further investigation is needed to ascertain whether or not the ability of certain strains, such as L. acidophilus RAM-1, to withstand refrigerated storage is associated with the lipid composition of the cell membrane.

While a number of possible food carriers could be used for L. acidophilus, Speck (1980) suggested that milk is the best product for the introduction of this microorganism as a dietary adjunct. He also stated that several million viable bile resistant lactobacilli/ml could be maintained for two to three weeks if the product is kept refrigerated at appropriate temperatures. In fact, some packages of non-fermented acidophilus milk (held in retail stores for about two weeks) have a label indicating that the product contains a minimum of 2.0×10^6 viable cells of L. acidophilus per milliliter. However, in a study by Young and Nelson (1978), in which the survival of L. acidophilus was evaluated for three brands of nonfermented acidophilus milk, it was found that one of the brands was below the indicated number of cells 13 days after obtaining the product from the processor. The products were stored at $4^{\circ}C$ for two to

three weeks before notable flavor defects occurred. Nevertheless, the number of viable L. acidophilus was said to decline markedly, while the levels of spoilage organisms increased considerably. A variation among the brands in the decline rate was also reported by these authors. It was suggested that both the initial level of inoculation as well as the type of strain used influenced the final number of viable L. acidophilus. Our findings are in agreement with those published by Young and Nelson (1978) in that a progressive decrease in cell viability was noticed for two of the strains under investigation, and that the magnitude of the decrease varied between these cultures. However, the differences in viability noticed among the three strains included in this study can not be attributed to the initial level of lactobacilli due to the following reasons. The cultures under study were grown using closely controlled conditions, collected at the same age, and added to the milk at a constant inoculation rate; after which, the inoculated milk was submitted to the corresponding cold storage treatments for subsequent evaluation. Besides, the inoculation level (2.0×10^7 cells/ml) used was higher than the one usually utilized (2.0×10^6 cells/ml) in the preparation of commercial nonfermented acidophilus milk.

In a recent work by Rich and Gilliland (1986), the survival of L. acidophilus NCFM grown at five pH levels (5.0, 5.5., 6.0, 6.5 and 7.0) was monitored after frozen storage in liquid nitrogen and subsequent refrigeration for

1, 7, 14 and 21 days in milk at 5°C. On days 14 and 21, viability losses corresponding to one and one and a half $\log_{10}$ cycles respectively, with respect to the initial population (2.5×10^6 cells/ml) were reported for L. acidophilus NCFM grown at pH 6.0. Using a higher initial population (2.0×10^7 cells/ml) of the same strain grown under the same conditions, we obtained viability losses of one and two tenths $\log_{10}$ cycles (day 14), and two and two tenths $\log_{10}$ cycles (day 21) during refrigerated storage. These results indicate that, even when a higher inoculum is used, refrigeration causes a marked reduction of the populations of L. acidophilus to levels which are below those suggested by Speck (1980) for two to three weeks of refrigerated storage.

An important finding of Rich and Gilliland (1986) was the improved storage stability upon refrigeration shown by L. acidophilus NCFM when it had been previously grown at pH 5.0. Since this was the culture exhibiting the highest values of β-galactosidase activity up to 14 days of refrigerated storage, it would be interesting to find out whether or not the improvement in cell viability at pH 5.0 reported by these authors correlates with a concomitant increase in the storage stability of the enzyme. Unfortunately, we only assessed the β-galactosidase activity for cells of L. acidophilus cultured at pH 6.0. Therefore, this would be another area of considerable interest for additional investigation.

Improvement is needed in the qualities of the L. acidophilus cultures designated for use as dietary adjuncts for milk to aid lactose digestion in humans. Evidence obtained from this study suggests that storage conditions, in particular refrigerated storage of the food carrier, containing the lactobacilli, can stress cells sufficiently to have an adverse effect on certain desirable characteristics of these microorganisms. The practical implication of this research may be significant in the future, particularly in establishing a more adequate shelf life at the retail level for nonfermented acidophilus milk. Limitation of the refrigerated storage time would be required to ensure that the consumer receives milk containing high levels of viable, bile resistant L. acidophilus capable of hydrolyzing lactose efficiently. For such a purpose, however, more work would be required. In addition to the areas thus far mentioned that need further investigation, future research in this field should also include the following aspects:

1. The environmental conditions for growing the cells used in the preparation of cultures need to be improved. Additional experiments in this field should consider: different pH levels, and type of neutralizer used to maintain the pH; temperature; media composition, including the use of additives which may influence the lipid composition of the cell membrane. Such experiments should not be oriented with the only purpose of getting high

numbers of viable bile resistant lactobacilli, but also with the idea of producing cells containing high levels of β-galactosidase activity.

2. Investigation is needed relative to the age of the culture at which maximum synthesis of β-galactosidase occurs, and the correlation of such an event with the expression of other desirable characteristics e.g. cold storage stability, bile resistance, etc.) of the organism. Information from these studies would help in establishing the proper time for harvesting the cell crops.
3. Electron microscopy studies may be useful in detecting any changes and/or damage occurring to the bacterial cell structure during refrigerated storage in milk.
4. Attention must be given to both the metabolic state of the lactobacilli during storage at 5°C, and the influence (if any) of the milk constituents on the stability of the cells. This information could provide a clearer understanding about how the microorganisms retain their viability when maintained under such temperature conditions.
5. The characteristics and properties of the β-galactosidase of L. acidophilus cultures need to be studied in depth. The influence of storage at 5°C on both the structure and catalytic

function of this protein may also lead to a better understanding of the results found in this research.

6. The screening for new strains of L. acidophilus needs to be continued to select those having a more efficient lactose-hydrolyzing system.
7. Finally, attention must also be given to other qualities which would be important to be found in microbial supplements. For instance, colonization factors (such as pili) present in some bacterial strains are cellular structures which permit them to adhere to the wall of the small intestine. The presence of adhesive pili is often plasmid-linked (Hardy, 1981). These colonization factors would perhaps be desirable characteristic for the lactobacilli used as dietary adjuncts. Synthesis of bacteriocins, which are antibacterial proteins produced by some bacteria, is also specified by plasmids (Hardy, 1981). The ability of cultures of L. acidophilus to produce bacteriocins should better enable them to compete with other lactobacilli in the natural intestinal flora. Bacteriocin-like compounds have been described for some strains of L. acidophilus (Barefoot and Klaenhammer, 1983; 1984). Therefore, in the near future, it may be possible through genetic engineering techniques to clone desirable genes

(such as those coding for pili formation and bacteriocin synthesis) in an organism with an efficient β-galactosidase system, or in one like L. acidophilus RAM-1 capable of withstanding refrigerated storage to improve them for use as dietary supplements.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The beneficial effect of viable starter culture bacteria for individuals who can not digest usual amounts of dietary lactose is not limited to those occurring in fermented dairy foods. It has also been demonstrated that nonfermented milk inoculated with L. acidophilus is useful in improving lactose digestion in humans. However, cold treatments (such as frozen storage prior to inoculation and refrigerated storage in the food carrier) of the microorganisms may kill, or injure some of the bacterial cells resulting in a decrease in their β-galactosidase activity. Injury may also be manifested as a loss of the ability of the cells to grow in the presence of bile salts. This investigation was undertaken to evaluate the influence of frozen (-196°C) and subsequent refrigerated (5°C) storage on the β-galactosidase activity, viability and bile resistance of three cultures of L. acidophilus.

Samples of concentrated cultures (grown at pH 6.0 and 37°C, for 12 hr in PMN broth) were evaluated prior to freezing, and after 14 and 28 days of storage in liquid nitrogen (-196°C). The concentrated cultures were added to cold, pasteurized nonfat milk (10% NFMS) to achieve a

population of about 2.0×10^7 cells/ml. Evaluations were carried out on the refrigerated milk samples containing the added lactobacilli at seven days intervals for 21 days. Hydrolysis of the substrate ONPG by the β-galactosidase present in lyzozyme-treated, inoculated milk samples was used as an estimate of the enzyme activity. Numbers of viable and bile resistant cells were determined utilizing PMN agar and PMNO agar, respectively.

In general, refrigerated storage exerted a more severe effect on the lactobacilli than did frozen storage. This was evidenced by small variations in the characteristics studied after maintaining the microorganisms for 28 days at $-196^{\circ}C$ when compared with the deterioration of such characteristics observed after exposing the microorganisms for 21 days at $5^{\circ}C$.

Differences among the cultures were noticed in regard to their β-galactosidase activity. In fact, prior to frozen and/or refrigerated storage, the enzyme activity of L. acidophilus NCFM was almost twice that obtained from L. acidophilus RAM-1; while an intermediate enzymatic activity was estimated for L. acidophilus 1. In addition to the initial differences in β-galactosidase activity, the stability of the enzyme activity during refrigerated storage varied among the strains. For example, L. acidophilus NCFM which showed the greatest values of enzymatic activity up to 14 days at $5^{\circ}C$ was also the culture suffering the highest reduction in β-galactosidase activity when main-

tained at this temperature. The opposite was found for L. acidophilus RAM-1 with somewhat lower, although fairly stable, enzyme activity levels over the entire 21-day period. Cells of L. acidophilus 1, on the other hand, showed a significant, but less pronounced decrease in the enzyme activity during the same period of refrigerated storage.

An apparent relationship between the stability of both the numbers of viable cells and β-galactosidase activity was manifested after storage of the cultures in milk at 5°C. This interpretation is based on the following observations. L. acidophilus NCFM whose β-galactosidase activity suffered the highest decrease during refrigerated storage also showed the greatest reduction in viability. For L. acidophilus 1, intermediate decrease in enzyme activity values corresponded to intermediate losses of viable cells; while L. acidophilus RAM-1 exhibited the greatest stability in both viability and β-galactosidase activity when maintained under the same temperature condition. Such a relationship may reflect the occurrence of a series of structural and biochemical changes in the bacterial cells that may start as injury and then end in the death of the microorganisms. One of the possible biochemical changes may involve the activation of autolytic enzymes (including proteases acting upon β-galactosidase) which may occur when normal growth of the microorganisms is disrupted. It is also conceivable that at temperatures approaching the

minimum growth temperature the β-galactosidase of some strains of L. acidophilus undergoes structural changes (e.g. changes in the tertiary and/or quaternary protein structure) from an active to an inactive configuration.

Storage at 5°C imposed sufficient stress on the cultures as to cause injury. This was evidenced by an increased sensitivity of L. acidophilus NCFM and L. acidophilus 1 to the presence of bile salts with increased time of storage at 5°C. Losses of bile resistant microorganisms were of higher magnitude for the former than for the latter strain. A different behavior was noticed for L. acidophilus RAM-1 whose numbers of bile resistant cells remained fairly stable regardless of the time this microorganism was kept at 5°C. The apparent variations in the response (in relation to both viability and bile resistance) of the cultures to refrigerated storage may be due to differences in the fatty acid composition of the lipids in their cell membrane.

Results from this investigation may be of practical importance in the future in establishing a more adequate shelf life at the retail level for nonfermented acidophilus milk. However, more work would be needed for such a purpose. Future research in this area should include the following aspects: (1) optimum growth conditions for producing lactobacilli with high β-galactosidase activity; (2) age of the culture which corresponds with maximum synthesis and/or activity of the enzyme; (3) metabolic state

of the L. acidophilus cells during storage at 5°C; (4) effect of cold storage on the protein structure and catalytic function of the β-galactosidase from different strains; (5) screening for new cultures of L. acidophilus having a more efficient system for lactose uptake and utilization; (6) searching for cultures of this organism having desirable genetic characteristics (such as bacteriocin production, colonization factors, etc.) to improve its quality as a dietary supplement.

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APPENDIXES

APPENDIX A

GROWTH CURVES

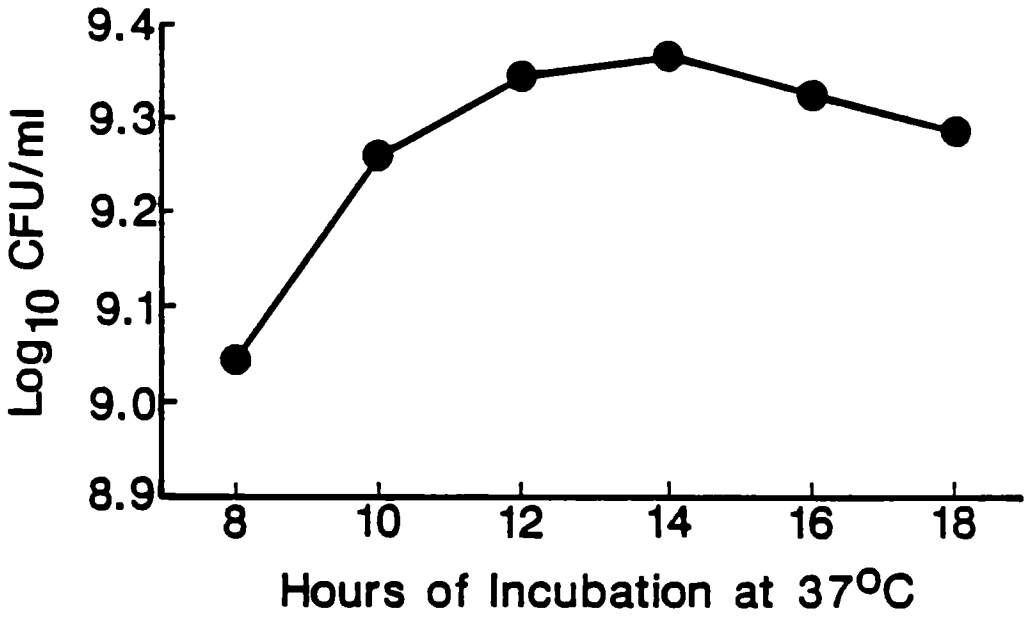

Figure 5. Growth of L. acidophilus 1 in PMN Broth at pH 6.0 and 37°C. Each Point is the Average Log_{10} CFU/ml From Four Trials.

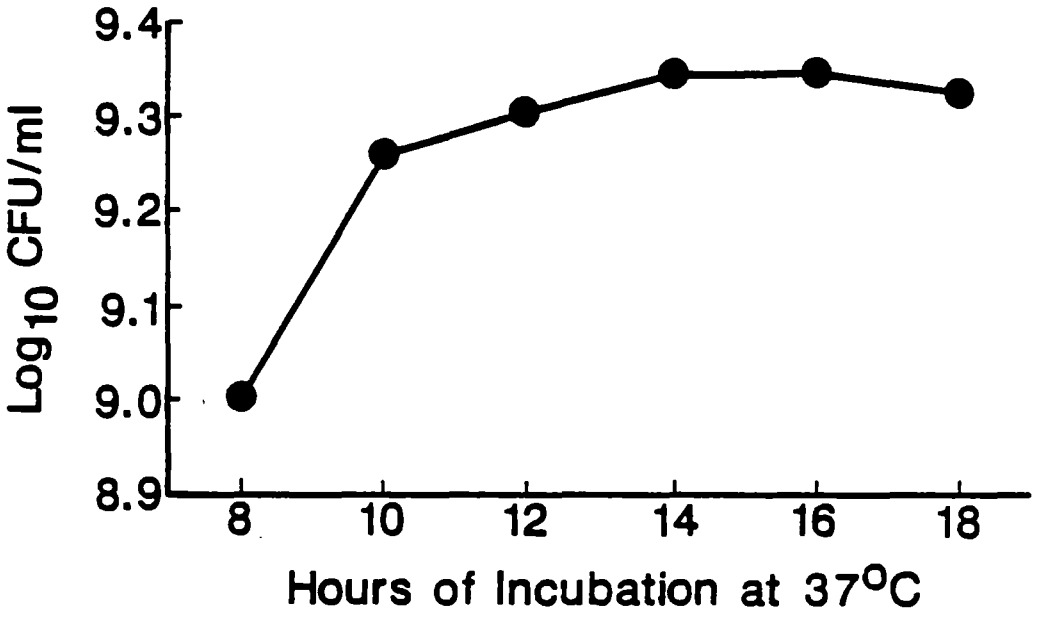

Figure 6. Growth of L. acidophilus RAM-1 in PMN Broth at pH 6.0 and 37°C. Each Point is the Average Log_{10} CFU/ml From Four Trials.

APPENDIX B

DATA FOR CALCULATION OF LINEAR REGRESSION EQUATION USED IN DETERMINING μ MOLES ONP RELEASED FROM ONPG

TABLE VII

DATA FOR CALCULATION OF LINEAR REGRESSION EQUATION USED IN DETERMINING μMOLES ONP RELEASED FROM ONPG

TRIAL #	ABSORBANCE AT 420 nm (A_{420}) (μMOLES ONP/ML)				
	0.06	0.12	0.18	0.24	0.30
1	0.138	0.262	0.415	0.523	0.662
2	0.135	0.273	0.391	0.535	0.620
3	0.138	0.267	0.400	0.530	0.655
4	0.138	0.261	0.400	0.525	0.660
5	0.142	0.271	0.401	0.526	0.662
6	0.141	0.275	0.404	0.521	0.670
7	0.131	0.273	0.400	0.515	0.680
8	0.142	0.258	0.441	0.562	0.690
9	0.132	0.271	0.401	0.530	0.630
10	0.139	0.272	0.390	0.570	0.670
11	0.138	0.271	0.400	0.520	0.645
12	0.139	0.270	0.402	0.510	0.655
13	0.138	0.270	0.402	0.530	0.650
14	0.140	0.275	0.405	0.530	0.660
15	0.141	0.264	0.400	0.540	0.660
16	0.140	0.272	0.400	0.505	0.650
AVERAGE A_{420}	0.138	0.269	0.403	0.530	0.657

LINEAR REGRESSION EQUATION:

$$Y = 0.010 + 2.165X$$

$$Y = A_{420}$$

$$X = \mu\text{moles ONP released/ml} = \frac{Y - 0.010}{2.165}$$

APPENDIX C

TABLES OF MEANS

TABLE VIII

β-GALACTOSIDASE ACTIVITY OF L. acidophilus 1: STABILITY OF THE ENZYMATIC ACTIVITY[a] AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE IN MILK

FREEZING (days)	REFRIGERATION (days)				GREAT MEANS[c]
	0	7	14	21	
0	0.152[b]	0.150	0.130	0.127	0.140
1	0.148	0.142	0.135	0.123	0.137
14	0.165	0.148	0.128	0.120	0.140
28	0.153	0.145	0.128	0.118	0.136
GREAT MEANS[d]	0.155	0.146	0.130	0.122	0.138[e]

[a] Expressed in units of enzymatic activity (EA). One unit of enzymatic activity is equivalent to one μmole of o-nitrophenol released/min/ml milk.

[b] Each value is the average from six trials.

[c] Each value is the average from 24 observations across Refrigeration.

[d] Each value is the average from 24 observations across Freezing.

[e] Overall mean.

TABLE IX

β-GALACTOSIDASE ACTIVITY OF L. acidophilus NCFM: STABILITY OF THE ENZYMATIC ACTIVITY[a] AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE IN MILK

FREEZING (days)	REFRIGERATION (days)				GREAT MEANS[c]
	0	7	14	21	
0	0.245[b]	0.218	0.163	0.148	0.194
1	0.242	0.195	0.160	0.137	0.183
14	0.208	0.178	0.162	0.132	0.170
28	0.200	0.187	0.160	0.142	0.172
GREAT MEANS[d]	0.224	0.195	0.161	0.140	0.180[e]

[a] Expressed in units of enzymatic activity (EA). One unit of enzymatic activity is equivalent to one μ mole of o-nitrophenol released/min/ml milk.

[b] Each value is the average from six trials.

[c] Each value is the average from 24 observations across Refrigeration.

[d] Each value is the average from 24 observations across Freezing.

[e] Overall mean.

TABLE X

β-GALACTOSIDASE ACTIVITY OF L. acidophilus RAM-1: STABILITY OF THE ENZYMATIC ACTIVITY[a] AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE IN MILK

FREEZING (days)	REFRIGERATION (days)				GREAT MEANS[c]
	0	7	14	21	
0	0.140[b]	0.153	0.153	0.153	0.150
1	0.148	0.172	0.162	0.147	0.157
14	0.148	0.160	0.167	0.145	0.155
28	0.157	0.152	0.152	0.132	0.148
GREAT MEANS[d]	0.148	0.160	0.158	0.144	0.153[e]

[a] Expressed in units of enzymatic activity (EA). One unit of enzymatic activity is equivalent to one μmole of o-nitrophenol released/min/ml milk.

[b] Each value is the average from six trials.

[c] Each value is the average from 24 observations across Refrigeration.

[d] Each value is the average from 24 observations across Freezing.

[e] Overall mean.

TABLE XI

VIABILITY OF L. acidophilus 1 IN PMN AND PMNO AGAR AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE IN MILK

FREEZING (days)	MEDIA	REFRIGERATION (days) 0	7	14	21	GREAT MEANS[b]
0	PMN	7.50[a]	7.32	7.00	6.71	7.13
	PMNO	7.44	7.26	6.78	6.34	6.95
1	PMN	7.50	7.27	6.91	6.59	7.06
	PMNO	7.48	7.24	6.65	6.29	6.91
14	PMN	7.46	7.23	6.89	6.58	7.04
	PMNO	7.42	7.16	6.72	6.23	6.88
28	PMN	7.44	7.24	6.89	6.58	7.04
	PMNO	7.39	7.19	6.64	6.30	6.88
GREAT[c] MEANS	PMN	7.47	7.26	6.92	6.61	7.07[d]
	PMNO	7.43	7.21	6.70	6.29	6.91[d]

[a] Each value is the average $\log_{10}$ CFU/ml from six trials.

[b] Each value is the average from 24 observations across Refrigeration.

[c] Each value is the average from 24 observations across Freezing.

[d] Overall mean from 96 observations.

TABLE XII

VIABILITY OF L. acidophilus NCFM IN PMN AND PMNO AGAR AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE IN MILK

FREEZING (days)	MEDIA	REFRIGERATION (days) 0	7	14	21	GREAT MEANS[b]
0	PMN	7.32[a]	7.06	6.41	5.45	6.56
	PMNO	7.32	6.90	5.96	4.73	6.23
1	PMN	7.33	7.01	5.95	4.93	6.31
	PMNO	7.30	6.87	5.66	4.40	6.06
14	PMN	7.33	7.07	5.98	5.22	6.40
	PMNO	7.30	6.83	5.49	4.57	6.05
28	PMN	7.31	6.98	6.26	4.89	6.36
	PMNO	7.26	6.65	5.54	4.34	5.95
GREAT[c] MEANS	PMN	7.32	7.04	6.15	5.12	6.41[d]
	PMNO	7.30	6.81	5.66	4.51	6.07[d]

[a] Each value is the average $\log_{10}$ CFU/ml from six trials.

[b] Each value is the average from 24 observations across Refrigeration.

[c] Each value is the average from 24 observations across Freezing.

[d] Overall mean from 96 observations.

TABLE XIII

VIABILITY OF L. acidophilus RAM-1 in PMN AND PMNO AGAR AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE IN MILK

FREEZING (days)	MEDIA	REFRIGERATION (days) 0	7	14	21	GREAT MEANS[b]
0	PMN	7.60[a]	7.56	7.50	7.34	7.50
	PMNO	7.54	7.50	7.39	7.26	7.42
1	PMN	7.63	7.58	7.52	7.40	7.53
	PMNO	7.57	7.51	7.39	7.27	7.43
14	PMN	7.58	7.53	7.46	7.30	7.47
	PMNO	7.32	7.48	7.41	7.11	7.33
28	PMN	7.55	7.52	7.47	7.15	7.42
	PMNO	7.50	7.48	7.41	7.03	7.35
GREAT[c] MEANS	PMN	7.59	7.55	7.49	7.30	7.48[d]
	PMNO	7.48	7.49	7.40	7.17	7.39[d]

[a] Each value is the average $\log_{10}$ CFU/ml from six trials.

[b] Each value is the average from 24 observations across Refrigeration.

[c] Each value is the average from 24 observations across Freezing.

[d] Overall mean from 96 observations.

APPENDIX D

ANALYSIS OF VARIANCE TABLES

TABLE XIV

ANALYSIS OF VARIANCE ON DATA FROM TRIALS TO EVALUATE THE INFLUENCE OF FREEZING AND SUBSEQUENT REFRIGERATION ON ENZYME ACTIVITY OF *Lactobacillus acidophilus* 1

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	95	0.0419		
Among Freezing				
Trial	5	0.0072	3.37	0.0307
Freezing (F)	3	0.0003	0.22	0.8796
Error (a)	15	0.0064		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	0.0157	28.30	0.0001
F x R	9	0.0013	0.80	0.6169
Error (b)	60	0.0111		

TABLE XV

ANALYSIS OF VARIANCE ON DATA FROM TRIALS TO EVALUATE THE INFLUENCE OF FREEZING AND SUBSEQUENT REFRIGERATION ON ENZYME ACTIVITY OF *Lactobacillus acidophilus* NCFM

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	95	0.2218		
Among Freezing				
Trial	5	0.0449	11.29	0.0001
Freezing (F)	3	0.0087	3.65	0.0371
Error (a)	15	0.0119		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	0.0987	39.02	0.0001
F x R	9	0.0070	0.93	0.5077
Error (b)	60	0.0506		

TABLE XVI

ANALYSIS OF VARIANCE ON DATA FROM TRIALS TO EVALUATE THE INFLUENCE OF FREEZING AND SUBSEQUENT REFRIGERATION ON ENZYME ACTIVITY OF *Lactobacillus acidophilus* RAM-1

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	95	0.0652		
Among Freezing				
Trial	5	0.0200	6.59	0.0020
Freezing (F)	3	0.0012	0.67	0.5828
Error (a)	15	0.0091		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	0.0041	2.98	0.0378
F x R	9	0.0033	0.81	0.6103
Error (b)	60	0.0275		

TABLE XVII

ANALYSIS OF VARIANCE ON DATA FROM TRIALS EVALUATING THE VIABILITY[a] OF L. acidophilus 1 AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	95	15.9357		
Among Freezing				
Trial	5	1.9454	9.23	0.0004
Freezing (F)	3	0.1484	1.17	0.3528
Error (a)	15	0.6323		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	10.3204	72.10	0.0001
F x R	9	0.0264	0.06	1.40
Error (b)	60	2.8628		

[a] Viability of cells in PMN agar

TABLE XVIII

ANALYSIS OF VARIANCE ON DATA FROM TRIALS EVALUATING THE VIABILITY[a] OF *L. acidophilus* NCFM AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	95	89.7068		
Among Freezing				
Trial	5	6.4691	10.67	0.0002
Freezing (F)	3	0.8453	2.32	0.1164
Error (a)	15	1.8195		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	70.8835	169.99	0.0001
F x R	9	1.3494	1.08	0.3918
Error (b)	60	8.3399		

[a] Viability of cells in PMN agar

TABLE XIX

ANALYSIS OF VARIANCE ON DATA FROM TRIALS EVALUATING THE VIABILITY[a] OF *L. acidophilus* RAM-1 AFTER FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	95	2.4501		
Among Freezing				
Trial	5	0.2121	3.88	0.0186
Freezing (F)	3	0.1512	4.61	0.0177
Error (a)	15	0.1639		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	1.2011	37.74	0.0001
F x R	9	0.0852	0.89	0.5383
Error (b)	60	0.6365		

[a] Viability of cells in PMN agar

TABLE XX

ANALYSIS OF VARIANCE FOR COMPARING PMN vs PMNO COUNTS OF L. acidophilus 1 AFTER FROZEN AND REFRIGERATED STORAGE OF THE CULTURE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	191	44.7358		
Among Freezing				
Trial	5	4.9500	8.14	0.0007
Freezing (F)	3	0.2309	0.63	0.6054
Error (a)	15	1.8253		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	28.7643	91.89	0.0001
F x R	9	0.0783	0.08	0.9996
Error (b)	60	6.2608		
Between Media Within Refrigeration				
Media (M)	1	1.2610	160.18	0.0001
F x M	3	0.0058	0.25	0.8643
R x M	3	0.6965	29.49	0.0001
F x R x M	9	0.0331	0.47	0.8928
Error (c)	80	0.6298		

TABLE XXI

ANALYSIS OF VARIANCE FOR COMPARING PMN vs PMNO COUNTS OF L. acidophilus NCFM AFTER FROZEN AND AND REFRIGERATED STORAGE OF THE CULTURE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	191	230.5143		
Among Freezing				
Trial	5	17.4278	16.16	0.0001
Freezing (F)	3	1.6586	2.64	0.0872
Error (a)	15	3.1384		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	180.2434	233.73	0.0001
F x R	9	1.7340	0.75	0.6635
Error (b)	60	15.4231		
Between Media Within Refrigeration				
Media (M)	1	5.5149	179.79	0.0001
F x M	3	0.1494	1.62	0.1889
R x M	3	2.5045	27.22	0.0001
F x R x M	9	0.2665	0.97	0.4754
Error (c)	80	2.4539		

TABLE XXII

ANALYSIS OF VARIANCE FOR COMPARING PMN vs PMNO COUNTS OF L. acidophilus RAM-1 AFTER FROZEN AND REFRIGERATED STORAGE OF THE CULTURE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	191	7.3322		
Among Freezing				
Trial	5	0.3588	2.41	0.0854
Freezing (F)	3	0.3056	3.42	0.0446
Error (a)	15	0.4461		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	2.8111	30.50	0.0001
F x R	9	0.2962	1.07	0.3972
Error (b)	60	1.8435		
Between Media Within Refrigeration				
Media (M)	1	0.4275	50.18	0.0001
F x M	3	0.0320	1.25	0.2967
R x M	3	0.0395	1.54	0.2083
F x R x M	9	0.0904	1.18	0.3199
Error (c)	80	0.6816		

APPENDIX E

NATURAL SUBSTRATE AND ENZYME ASSAY REACTION FOR β-GALACTOSIDASE

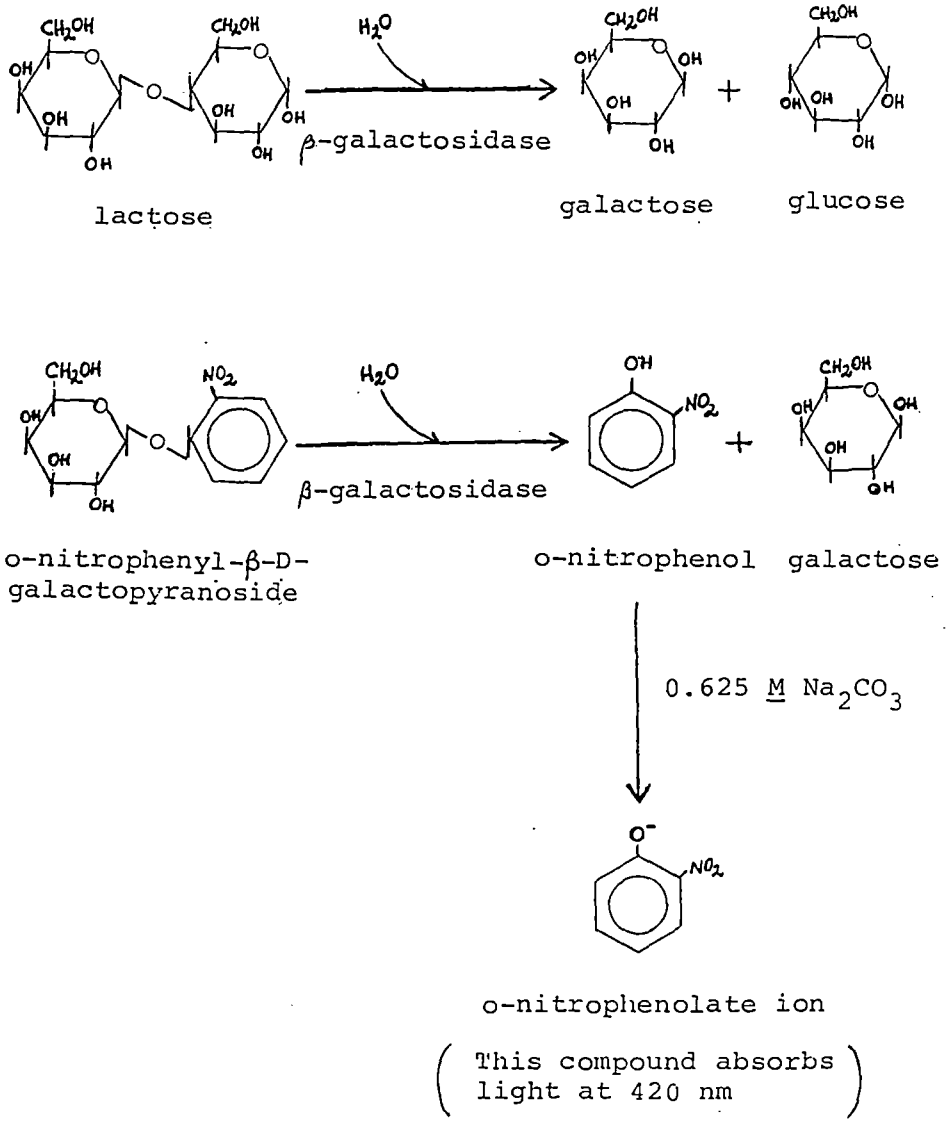

Figure 7. Natural Substrate and Enzyme Assay Reaction for β-galactosidase.

VITA

ROSA CENTENO de LARA

Candidate for the Degree of

Doctor of Philosophy

Thesis: EFFECT OF FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE ON β-GALACTOSIDASE ACTIVITY, VIABILITY AND BILE RESISTANCE OF LACTOBACILLUS ACIDOPHILUS

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