# ISOLATION AND CHARACTERIZATION OF THE LACTOSE-HYDROLYZING ENZYME OF *LACTOBACILLUS ACIDOPHILUS*

By

JERRY WAYNE NIELSEN

Bachelor of Science
Northeast Louisiana University
Monroe, Louisiana
1979

Master of Science
Northeast Louisiana University
Monroe, Louisiana
1981

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the degree of
DOCTOR OF PHILOSOPHY
December, 1987

ISOLATION AND CHARACTERIZATION

OF THE LACTOSE-HYDROLYZING

ENZYME OF LACTOBACILLUS

ACIDOPHILUS

Thesis Approved:

Stanley E. Gilliland
Thesis Adviser

John J. Guenther

Mary M. Gruda

Eldon C. Nelson

Norman N. Dunham
Dean of the Graduate College

# PREFACE

The lactose-hydrolyzing enzyme of Lactobacillus acidophilus was purified so its characteristics could be determined. Cells of the lactobacilli were lysed enzymatically to release the enzyme for study.

Characteristics which were determined included kinetic properties and molecular weight. The effect of such factors as temperature, pH, storage, and divalent ions were determined as well as the influence of some sugars on induction of the enzyme.

I wish to express my sincere thanks to Dr. S. E. Gilliland for his patience and guidance as major advisor during my time at Oklahoma State University.

I am also thankful to the other members of my advisory committee, Dr. Mary Grula, Dr. J. J. Guenther, and Dr. E. C. Nelson, for their comments and advice during this work.

A special thanks is extended to the Oklahoma Agricultural Experiment Station and the Animal Science Department for providing financial support during my graduate studies.

To my wife, Ann, daughter, Laura, and son, Jeffrey, I extend my sincerest appreciation for their encouragement, confidence, and devotion during the preparation of this manuscript.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

The inability to digest lactose, caused by reduced lactase activity in the intestine, is a common problem in most populations today (Johnson et al, 1984). Lactose which is not digested passes into the colon where bacteria break it down into acid, gas, and other metabolites. The normal osmotic balance is altered and fluid is accumulated (Kleyn, 1972). The symptoms which occur because of this include abdominal bloating, cramps, flatulance, and diarrhea (Bayless and Rosensweig, 1966). Many people, lacking the ability to adequately digest lactose, avoid milk consumption and thus eliminate a major source of calcium in the diet.

One way proposed for dealing with the problem of lactose intolerance is to reduce the amount of lactose in milk. This can be accomplished by adding lactase to milk and incubating it 12 to 18 hours in the refrigerator. Paige et al (1975) reported that when milk treated in such a manner was consumed, the symptoms of lactose intolerance were diminished but most people found the milk too sweet and therefore acceptance was limited.

The beneficial effects of consuming viable lactic acid

bacteria has been known for many years. One such "beneficial" bacterium is Lactobacillus acidophilus. Originally it was added to milk and allowed to grow and ferment part of the lactose in the milk. The resulting acidophilus milk was not well accepted because of its displeasing flavor (Vadejs, 1954).

Nonfermented acidophilus milk contains viable cells of L. acidophilus but does not have the unpleasant flavor of regular acidophilus milk. A concentrated culture of L. acidophilus is added to cold, pasteurized low fat milk which is kept refrigerated to prevent growth of the culture. The product tastes like regular low fat milk and has experienced a much greater degree of acceptance than did the fermented acidophilus milk (Nielsen, 1976).

As a dietary adjunct, L. acidophilus supplies $\beta$-galactosidase to enable lactose intolerant persons to consume milk (Kim and Gilliland, 1983). Gilliland et al (1978) reported that healthy males who consumed non fermented acidophilus milk exhibited an increase in the number of lactobacilli in the feces but the numbers decreased after they quit consuming the milk. Speck (1980) suggested replenishment of bacteria daily to maintain the level.

The lactase found in L. acidophilus apparently supplements or replaces the human intestinal lactase which is less active or absent in lactose malabsorbers.

Bacteria which are able to metabolize lactose have

different methods of breaking it down. Some species of bacteria phosphorylate lactose prior to hydrolysis. The "lactase" (Phospho-β-galactosidase) present in these bacteria can break down only lactose which has been phosphorylated. This occurs in Staphylococcus aureus (Hengstenberg et al, 1968). Lactic streptococci such as Streptococcus cremoris, S. lactis subspecies diacetilactis, and many other strains of S. lactis also utilize this enzyme system for lactose hydrolysis (Molskness et al, 1973, and Farrow, 1980).

Some lactobacilli also exhibit phospho-β-galactosidase activity. Lactobacillus buchneri and L. casei possess this enzyme only while many other lactobacilli possess both phospho-β-galactosidase and β-galactosidase. The most active "lactase" found in all species of lactobacilli tested was β-galactosidase (Premi et al, 1972).

Many researchers have purified β-galactosidase from various bacteria and fungi and have determined some of the enzyme's characteristics. Lederberg (1950) developed a method for assaying the activity of β-galactosidase from Escherichia coli K-12 using o-nitrophenyl-β-D-galactoside (ONPG) as substrate.

Lester and Bonner (1952) also investigated the β-galactosidase of E. coli and found it to be inducible. Craven et al (1965) determined the molecular weight to be 540,000.

The β-galactosidase of lactobacilli is of great

interest because some of these organisms are recognized as the beneficial bacteria used for dietary adjuncts. Macias et al (1983) isolated β-galactosidase from L. murinus. They reported a molecular weight of 170,000, optimum temperature of 45 C, and optimum pH of 7.0. Macias et al (1983) also isolated β-galactosidase from L. helveticus. Optimum temperature for this enzyme was 42 C with an optimum pH of 6.5, and molecular weight of 250,000.

Premi et al (1972) assayed β-galactosidase activity in several lactobacilli. Characteristics were determined using purified β-galactosidase from L. thermophilus. The molecular weight was estimated at 540,000 with optimum pH of 6.5 and optimum temperature of 54 C. Even though a culture designated as L. acidophilus was included in this study, Miller et al (1970) indicated that it was probably not L. acidophilus. Fisher et al (1985) reported that L. acidophilus contained both β-galactosidase and phospho-β-galactosidase, however, the identity of the cultures studied was not confirmed.

The species of lactobacilli which has been used most often as a dietary adjunct because it has the characteristics necessary to enter and colonize the small intestine is L. acidophilus. So far, no one has purified and characterized the lactose hydrolyzing enzyme of this species.

The purpose of this study was to determine which lactose-hydrolyzing enzyme is present in L. acidophilus and

to determine some of its characteristics. Characteristics studied included molecular weight, kinetic properties, and effect of environmental factors such as temperature, pH, storage, and divalent ions on its activity. The influence of sugars such as lactose, galactose, and glucose on levels of the enzyme in the culture was also evaluated.

# CHAPTER II

# REVIEW OF LITERATURE

## Incidence of Lactose Malabsorption

Many people in the world today experience mild to severe discomfort when they drink milk because they are unable to break down lactose or milk sugar. These people are referred to as being lactose intolerant or as lactose malabsorbers. Symptoms of lactose malabsorption include abdminal bloating, cramps, flatulance, and diarrhea (Bayless and Rosensweig, 1966). This happens because lactase activity is reduced or absent in the small intestine and much of the lactose passes to the colon. The normal osmotic balance is upset which causes an accumulation of fluid. Bacteria present in the colon utilize lactose to produce acid, gas, and other metabolites (Kleyn, 1972).

The incidence of lactose malabsorption is higher in certain races or ethnic groups than in others. Johnson et al (1984) reported as many as 30% of Japanese, Chinese, and Koreans had some difficulty digesting milk while less than 10% of Hawaiians were malabsorbers. In Italy, from 50 to 70% of healthy adults tested were lactose malabsorbers (Burgio et al, 1984). Healthy adult volunteers were tested

for lactose intolerance in north and south India. Two-thirds of those tested in south India were lactose intolerant while less than 30% in north India were intolerant. The population of north Indians were dairymen and descendants of dairymen (Tandon et al, 1981).

Brand et al (1983) reported lactose intolerance in over 80% of Australian Aborigenes as compared to only 20% from the multiracial control group. Populations in the middle east also have large numbers of lactose malabsorbers. Nasrallah (1979) reported the incidence of lactose intolerance at 78% in a sample of Lebanese volunteers and Sadre and Karbasi (1979) reported that 86% of Iranian adults were lactose intolerant.

In the United States, as much as 80% of the black adult population may suffer some degree of lactose intolerance while the white adult population of Scandinavian and Northwestern European heritage consists of from 5 to 15% lactose malabsorbers (Bayless et al, 1975).

Adults are not the only persons who are lactose malabsorbers. Lisker et al (1980) studied two groups of Mexican children and concluded that about 15% had some degree of lactose intolerance. He also noted that the incidence of lactose intolerance was higher in the rural population than in the urban population. The rural children were from lower socioeconomic communities and also had a higher incidence of gastrointestinal infections.

A study conducted to determine the effect of Ascaris

lumbricoides (roundworm) infestation on lactose digestion in children revealed that infected children had a much higher incidence of lactose intolerance than healthy children. After treatment for the parasite, near normal lactose tolerance was regained by the previously infected children (Carrera et al, 1984).

Milk and other dairy products are major sources of calcium and riboflavin. They also supply a portion of the protein requirement for consumers. Persons who are lactose intolerant may cut down or eliminate their consumption of milk which may result in a calcium deficiency in the diet.

A prolonged calcium deficient diet has been linked to osteoporosis. Newcomer et al (1978) reported the incidence of lactose malabsorbance in post menopausal women with osteoporosis to be eight times as high as it was in post menopausal women without osteoporosis.

Lactose is the sugar found in milk. It contributes to the flavor and nutritive value of milk products. Lactose is not as effectively utilized in the human digestive system as are other sugars. It may pass further along the digestive tract as a disaccharide. Its presence favors an acid type fermentation by intestinal bacteria which improves the utilization of calcium and phosphorus. The acid environment also discourages the growth of putrefactive organisms (Kleyn, 1972).

In lactose tolerant people, lactose has long been known to help maintain regularity in the digestive system.

This may be caused by various mechanisms. (a) Lactose may irritate intestinal musculature causing stronger or more frequent contractions. (b) Lactose may remain in the lumen of the intestine longer, raising the osmotic pressure above blood and thus drawing water into the intestine. This subsequent distention causes muscular contractions and flushes the intestines. (c) Lactose fermentation changes the pH of the intestinal content which may stimulate intestinal muscles (Fischer and Sutton, 1949).

For those people who are lactose intolerant, the effect of lactose goes beyond mild laxation and results in both discomfort and inconvenience. These people then avoid or reduce milk intake to avoid the symptoms. The only reported incidence of lactose malabsorbers drinking more milk came from Bayless and Rosensewig (1969). Two former narcotic addicts, now prisoners, reported drinking a quart of milk at one time to overcome constipation caused by opiate usage.

When a lactose tolerant person consumes milk, there is a measurable increase in the blood glucose level since the lactose is broken down into its components of glucose and galactose in the small intestine. These are then absorbed into the blood. A lactose malabsorber who consumes the same amount of milk will experience little or no increase in the blood glucose level due to the absence of a lactose hydrolyzing enzyme in the small intestine. Also, a lactose intolerant person will exhibit a moderate to large increase

in the level of hydrogen in the breath for one to six hours after consuming milk. This happens because the lactose passes into the large intestine where bacteria break it down into lactic acid and other metabolites with the subsequent release of hydrogen gas. The determination of lactose intolerance is based on these phenomena. In earlier tests, blood glucose level was monitored for lactose intolerance but it required unrealistic amounts of lactose to be administered to subjects. In most recent tests, the breath hydrogen levels have been monitored. This test, (Breath Hydrogen Test) is more sensitive to normal levels of lactose intake and is more convenient to perform (Solomons et al, 1980).

## Approaches to Treatment of Lactose Malabsorption

### Reduction of Lactose Content in Milk

One method to relieve the symptoms of lactose intolerance other than eliminating milk from the diet is to reduce the amount of lactose in milk. An enzyme (lactase) which breaks down lactose is added to milk and incubated at refrigeration temperature for 12 to 18 hours (Cheng et al, 1979). This product is called low lactose milk.

Low lactose milk has been fed to lactose malabsorbers to test its effectiveness and to compare its acceptance to unmodified milk. Cheng et al (1979) found no differences for abdominal distention, pain, and diarrhea between

tolerant and intolerant subjects when they consumed low lactose milk. He did report, however, that test subjects noted that low lactose milk was sweeter than unmodified milk.

Paige et al (1975) also noted a sharp decrease in symptoms by lactose malabsorbers when they consumed low lactose milk. Most of the adolescents in the study stated that low lactose milk was sweeter than unmodified milk and some said they did not like it.

The lactase enzyme or $\beta$-galactosidase, as it is commonly known, has been isolated from a variety of sources. The most common commercial sources are Escherichia coli, Aspergillus niger, Kluyveromyces fragilis, and K. lactis. Applications vary but each preparation is expensive. The cost of a quart of lactose hydrolyzed milk was about ten cents higher than unmodified milk in 1981 (Richmond et al, 1981). The combination of higher cost and sweeter taste limit the acceptance of low lactose milk by the general public.

## Reduction in Amount of Milk Consumed

Another method of coping with lactose malabsorption is to reduce the amount of milk consumed at one sitting. A group of malnourished children were fed an adequate diet of vegetables supplemented with small amounts of milk with each meal. All children receiving milk supplements gained more weight than those not receiving milk. The lactose

malabsorbers showed no significant differences in response to the milk than those who were tolerant (Brown et al, 1980)

## Consumption of Viable Lactic Acid Bacteria

Acidophilus Milk. For many years the possible benefits of consuming viable lactic acid bacteria has been recognized. The most common "beneficial" bacterium considered for such purpose is Lactobacillus acidophilus. In early products, L. acidophilus was added to milk and allowed to grow and ferment the milk. The resulting acidophilus milk was acidic and had a displeasing flavor for many consumers (Vadejs, 1954).

Acidophilus milk was promoted as having many benefits for consumers. It was proposed as a cure for diarrhea and constipation and was thought to eliminate putrefactive bacteria from the intestinal tract. Normal dosage was from one pint to one quart per day (Vadejs, 1954).

Humans were not the only ones to benefit from consumption of acidophilus milk. Shaw and Muth (1937) reported that feeding acidophilus milk to calves, lambs, colts, and pups substantially reduced the severity of the symptoms of dysentary. They pointed out that the cultured product must be produced and maintained properly to be effective.

In spite of all the benefits of acidophilus milk, it

failed to gain much acceptance by the public. Many people did not care for cultured dairy products and many of those who did were not fond of the appearance and acid taste of acidophilus milk. Many proponents of acidophilus milk were slow to accept the fate of their product. Butterworth (1938) suggested that the name was not appealing to consumers. He proposed eliminating the acidophilus name thereby eliminating the association with a medicinal product. This, however, never came about.

Nonfermented Acidophilus milk. The importance of adding viable bacteria to the intestinal tract led researchers to develop nonfermented acidophilus milk. Myers (1931) was the first to suggest the use of unfermented milk containing cells of L. acidophilus. However, he did not develop procedures for producing such a product on a commercial scale. According to an anonymous report (1976), a group of researchers at Oregon State University added a frozen, concentrated culture of L. acidophilus to fresh pasteurized milk to produce a palatable product which still contained large numbers of viable bacteria. The Oregon State group recommended adding a small quantity of a frozen concentrated cell suspension of L. acidophilus just prior to consuming it (Duggan et al, 1959). An improved strain of L. acidophilus was later developed at North Carolina State University (Anon., 1976). This group developed a process for the commercial production of nonfermented acidophilus milk (Speck, 1975).

Nonfermented acidophilus milk was marketed in the next few years by many companies under various names. Marketing the new product did not pose any new problems. A concentrated culture of L. acidophilus was added to cold, pasteurized low fat milk which was then kept refrigerated so no bacterial growth occurred. The product tasted like regular low fat milk so the beneficial effects were stressed to encourage consumers to try it (Nielsen, 1976).

Young and Nelson (1978) tested three commercial brands of nonfermented acidophilus milk for viable bacteria after storage at different intervals. They found that most samples had more than one million viable cells per milliliter after 16 days storage at 5 C.

## Role of Lactobacilli in the Digestive Tract

The role of lactobacilli in the digestive tract has been well documented. Lactobacilli are not the most numerous bacteria in the intestinal tract, however, some lactobacilli can produce antibiotic-like substances which inhibit many enteric bacteria, some of which are pathogenic (Speck, 1976). Some enzymes produced by lactobacilli suppress carcinogens while, on the other hand, other enzymes from other organisms can activate carcinogens (Sandine, 1979; Goldin and Gorbach, 1984). As dietary adjuncts, lactobacilli supply $\beta$-galactosidase to enable lactose intolerant persons to consume milk (Kim and

Gilliland, 1983).

Shahani and Ayebo (1980) reported that lactobacilli produced B-complex vitamins in cultured products. They also noted that lactobacilli made cultured products more digestible by predigesting some milk components.

Gilliland (1979) stated that any bacterium which is expected to establish and grow when it reaches the intestinal tract must survive the low pH of hydrochloric acid in the stomach. Other factors that can limit bacterial growth in the digestive system include enzymes such as lysozyme in the intestine as well as bile salts. A bacterial culture used as a desirable dietary adjunct which is expected to grow and function in the intestine also must be able to overcome these factors.

Microbial cultures used as dietary adjuncts can be important to lactose malabsorbers as a source of $\beta$-galactosidase. In cultured products, the amount of lactose is reduced (Alm, 1982) but there is also some benefit derived from $\beta$-galactosidase contained in the starter culture bacterial cells which remains active in the intestines after consumption of the product (Savaiano et al, 1984; Gilliland and Kim, 1984).

Gilliland and Kim (1984) found that cultured yogurt which had been heated had less lactase activity than unheated cultured yogurt. They further showed that yogurt containing viable starter culture cells improved lactose utilization in lactose malabsorbers. The starter culture

(Lactobacillus bulgaricus and Streptococcus thermophilus) normally used for yogurt will not survive and grow in the intestines. This indicates that cells which may not survive to multiply in the intestine may release lactase there to provide an additional benefit. In a similar study, Kilara and Shahani (1976) found high lactase activity in cultured yogurt but none in yogurt made by direct acidification. The benefit to lactose malabsorbers then can come only from cultured yogurt which has not been heated to kill the starter culture bacteria.

Gilliland and Speck (1977) attempted to add cells of L. acidophilus to cultured yogurt for use as a dietary adjunct. This proved unsuccessful because hydrogen peroxide produced by L. bulgaricus in the yogurt caused the cells of L. acidophilus to become nonviable after a short time.

Consuming cells of L. acidophilus which survive and grow in the digestive tract can provide benefits for longer periods of time. Speck (1980) suggested replenishment of bacteria daily to maintain balance in the digestive tract. Gilliland et al (1978) reported that healthy males who consumed nonfermented acidophilus milk increased the number of lactobacilli in their feces but the numbers decreased when they quit consuming the milk. However, some of the subjects who originally had low numbers of lactobacilli retained higher numbers after the test ended.

## Benefits From Consuming Nonfermented Acidophilus Milk

Letters received from consumers have indicated that many who had in the past had trouble digesting milk could consume nonfermented acidophilus milk without suffering from symptoms associated with lactose malabsorption. Kim and Gilliland (1983) reported results to substantiate this claim. They found that significant benefits (decreased breath hydrogen levels) were immediate when nonfermented acidophilus milk containing cells of L. acidophilus with active β-galactosidase was consumed. In another study, Payne et al (1981) reported that consuming nonfermented acidophilus milk did not reduce breath hydrogen levels. However, acidophilus milk used in the latter study was a commercial brand and the viable numbers, identity, and β-galactosidase activity of the culture were not determined by the researchers.

The lactase in L. acidophilus apparently supplements or replaces the human intestinal lactase which is less active or absent in the lactose malabsorber. Human lactase is found in epithelial cells lining the small intestine. When active, this enzyme breaks lactose down into glucose and galactose which are then absorbed into the bloodstream as monosaccharides (Rosensweig, 1969).

Gray and Santiago (1969) isolated three β-galactosidases from human intestines. Enzyme I had a molecular weight of 280,000 and pH optimum of 6.0. Enzyme II had a

lower pH optimum of 4.5 and occurred in two species of molecular weights 156,000 and 660,000. Enzyme III had the same optimum pH as enzyme I (6.0) and a molecular weight of 80,000. It showed a specificity only for synthetic β-galactosides. Enzymes I and III were peculiar to the intestine while enzyme II was also found in the liver and kidney. Enzyme I which exhibited a higher specificity for lactose was considered to be the lactase involved with digestion of dietary lactose.

## Microbial Lactose-Hydrolyzing Enzymes

Bacteria which are able to metabolize lactose have different methods of breaking it down. Some species of bacteria phosphorylate lactose prior to hydrolysis. The "lactase" (Phospho-β-galactosidase) present in these bacteria can hydrolyze only lactose which has been phosphorylated. This is the enzyme system for lactose hydrolysis used by Staphylococcus aureus (Hengstenberg et al, 1968). Lactic streptococci such as Streptococcus cremoris, S. lactis subspecies diacetilactis, and many other strains of S. lactis also rely on this method of lactose hydrolysis (Molskness et al, 1973; Farrow, 1980).

Some species of lactobacilli also exhibit phospho-β-galactosidase activity. Lactobacillus buchneri and L. casei possessed this enzyme only, while many other lactobacilli possessed both phospho-β-galactosidase and β-galactosidase. The most active "lactase" found in all

other species of lactobacilli tested was β-galactosidase (Premi et al, 1972).

Many researchers have purified β-galactosidase from various bacteria and fungi and have determined some of the characteristics of the enzyme. Lederberg (1950) developed a method for assaying the activity of β-galactosidase from Escherichia coli K-12. He was the first to use o-nitrophenyl-β-D-galactoside (ONPG) as a substrate which is still the substrate of choice for most researchers.

Lester and Bonner (1952) also investigated the E. coli K-12 β-galactosidase. They found that it was present only when the cells were grown in a medium containing lactose as the only sugar. They called this de novo formation of an enzyme, now known as enzyme induction. Craven et al (1965) purified this same enzyme and determined the molecular weight to be approximately 540,000.

Rohlfing and Crawford (1966) purified β-galactosidase from Aeromonas formicans. Its characteristics were compared to the β-galactosidase of E. coli. The two enzymes showed structural similarities but the enzyme from E. coli was more heat stable. There were also many differences in amino acid composition. In a later study, Rohlfing and Crawford (1966) purified β-galactosidase from Bacillus megaterium and found the enzyme to be much smaller than that of both E. coli and A. formicans.

Landman (1957) also characterized β-galactosidase from B. megaterium. He reported an optimum pH range from 7.3 to

8.0. He also pointed out that this enzyme was inducible.

One strain of Streptococcus lactis which possessed β-galactosidase was S. lactis 7962. The enzyme was extracted and characterized (McFeeters et al, 1967). It was found to be an inducible enzyme with lactose being the most effective inducer. Optimum pH was reported at 7.0 and optimum temperature at 37 C.

Streptococcus thermophilus differs from the other lactic streptococci in that it has a limited habitat because of the high temperature requirement. The optimum temperature for β-galactosidase from this species is 57 C. Optimum pH was from 6.6 to 7.1 (Garvie, 1978; Ramana Rao and Dutta, 1981; Greenberg and Mahoney, 1982). Ramana Rao and Dutta (1981) estimated the molecular weight at 600,000 but Greenberg and Mahoney (1982) reported a molecular weight of 530,000.

The β-galactosidase of lactobacilli is of great interest because some of these organisms are recognized as the beneficial bacteria used for dietary adjuncts. Macias et al (1983) isolated β-galactosidase from L. murinus. They reported a molecular weight of 170,000, optimum temperature of 45 C, and an optimum pH of 7.0. The same team of researchers isolated β-galactosidase from L. helveticus. Optimum temperature for this enzyme was 42 C with an optimum pH of 6.5. The molecular weight was estimated at 250,000 with four subunits of equal weight making up the protein (Macias et al, 1983).

Premi et al (1972) assayed β-galactosidase activity in several lactobacilli. Characterization studies were done on purified β-galactosidase from L. thermophilus.. The molecular weight was estimated at 540,000 with an optimum pH of 6.5 and optimum temperature of 54 C. A strain of L. acidophilus was included in this study but Miller et al (1970) indicated that it was probably not L. acidophilus.

Itoh et al (1980) purified β-galactosidase from L. bulgaricus. They reported an optimum temperature of 50 C and pH optimum of 7.0. The enzyme was completely inactivated by heating at 55 C. A molecular weight of 159,000 was also reported for the enzyme.

Fisher et al (1985) examined five strains of L. acidophilus under various growth conditions. They reported that, when grown in lactose at 37 C, each had β-galactosidase activity as well as phospho-β-galactosidase activity with β-galactosidase activity being at least 100 times as high as that of phospho-β-galactosidase. They also reported that β-galactosidase was induced by lactose and galactose but not glucose in three of the strains. In three of the strains, lactose did not induce phospho-β-galactosidase when grown at 45 C. In this study, the identity of the cultures was not confirmed.

Toba et al (1981) determined some enzymatic properties from cell-free extracts of L. helveticus, L. plantarum, L. lactis, and L. bulgaricus. They reported an optimum pH range of from 6.0 to 7.0 for the enzymes from the four

species. The optimum temperature for L. bulgaricus, L. lactis, and L. plantarum was 50 C and for L. helveticus it was 55 C.

While all the previously mentioned enzymes have a common function, there are variations in size and optimum conditions. These variations are increased when properties of β-galactosidase from fungi are compared to those of bacteria. Borglum and Sternberg (1972) found an optimum pH of 4.0 and 6.0 for the β-galactosidase isolated from Aspergillus foevilus. Optimum pH for β-galactosidase from Kluyveromyces lactis was reported at 7.1 to 7.3 (Dickson et al, 1979).

In all previously reviewed papers, the authors stated that β-galactosidase is an inducible enzyme. Citti et al (1967) reported that β-galactosidase in S. lactis was induced by lactose but synthesis was repressed when simpler sugars were present. Thompson et al (1978) observed that S. lactis metabolized glucose, galactose, and lactose in that order. Okamura et al (1983) found β-galactosidase to be inducible in 45 of 50 species of lactose utilizing bacteria tested while it was constituitive in only five species.

The species of bacteria which has been used most often as a dietary adjunct because it has the characteristics necessary to enter and colonize the small intestine is L. acidophilus. So far, no one has purified and characterized the lactose-hydrolyzing enzyme of this species. The

purpose of this study was to determine which enzyme is present and determine some of its physical characteristics.

# CHAPTER III

# MATERIALS AND METHODS

## Source and Maintenance of Cultures

Lactobacillus acidophilus NCFM, isolated from a human intestinal tract was obtained originally from the Food Science Department of North Carolina State University. Lactobacillus acidophilus 1, also of human intestinal origin, was obtained from Dr. M. L. Speck, Raleigh, North Carolina. Lactobacillus acidophilus RAM-1 was isolated from a commercial non-fermented acidophilus milk in another study in our laboratory.

## Media and Growth Conditions

Cultures were maintained by weekly subcultures (1% inoculum) in sterile 10% non fat milk solids and incubated 14 to 18 hours at 37 C. Between transfers, cultures were held at 1 C. The identity of the cultures of L. acidophilus was confirmed periodically using a miniaturized identification system as described by Gilliland and Speck (1977).

## Production of Cultures for Enzyme Isolation

Cultures used for enzyme study were transferred from milk to PMN broth which contained lactose as the only sugar source. Two liters of PMN broth was inoculated (1%, v/v) with a freshly prepared PMN broth culture and incubated 12 to 14 hours at 37 C. PMN broth contained the following ingredients: Peptonized Milk Nutrient (Humko Sheffield Chemical, Memphis, TN), 5%; Tween 80 (Sigmal Chemical Company), 0.1%; yeast extract (Becton Dickinson and Co.), 0.1%; lactose (Mallinckrodt Chemical Works), 2%; and Primatone (Humko Sheffield Chemical), 2%. After all ingredients were dissolved, it was autoclaved at 121 C for 15 minutes.

Cells were harvested from the PMN broth by centrifugation at 4,000 x g for 10 minutes at 1 C in an RC-5 Sorvall refrigerated centrifuge. Cells were washed three times in 100 ml volumes of cold, sterile, distilled water.

## Extraction and Purification of $\beta$-galactosidase

Washed cells harvested from two liters of PMN broth were lysed enzymatically using a modification of the methods described by Metcalf and Diebel (1969). Washed cells were resuspended in 80 ml of sterile distilled water. Ten ml of a 0.2% (w/v) lysozyme solution (Sigma Chemical

Company) was added to the suspended cells. This mixture was incubated one hour at 37 C followed by the addition of 10 ml of 4 M sodium chloride. The mixture was inverted slowly 10 to 15 times to insure proper mixing. As the bacterial cells lysed, the solution became more viscous. In initial trials, examination of stained smears prepared at one minute intervals and viewed at 1000X magnification (oil immersion) revealed that lysis was more than 90% complete after five minutes.

The lysed cells were then centrifuged at 15,000 x g for 20 minutes at 1 C and the sediment was discarded. The supernatant fluid was placed in a beaker and six ml of 25% streptomycin sulfate (Sigma Chemical Company) was added to each 50 ml. The resulting mixture was stirred for 30 minutes on a magnetic stirrer. The temperature was maintained at or below 5 C by placing the beaker in an ice-water bath on top of the stirrer.

After stirring was complete, the preparation was centrifuged at 15,000 x g for 30 minutes at 1 C and the sediment was discarded. The supernatant fluid was returned to the stirring apparatus previously described. Reagent grade ammonium sulfate (Mallinckrodt Chemical Works), which had been reduced to powder with a mortar and pestle, was added slowly until the enzyme preparation was 65% saturated. It was then centrifuged at 10,000 x g for 15 minutes at 1 C. The supernatant fluid was discarded and the precipitated protein was resuspended in 5 ml of 0.01 M

tris (hydroxymethyl) aminomethane (tris)-hydrochloride buffer (pH 7.7) containing 5 X $10^{-4}$ M magnesium sulfate (Mallinckrodt Chemical Works) and 0.002 M mercaptoethanol (Sigma Chemical Company). The protein preparation was placed into dialysis tubing (Spectrum Medical Industries) which was tied closely at each end to prevent an appreciable change in volume. It was then dialyzed against three two-liter changes of tris buffer for successive 10 hour periods at 5 C (Premi, Sandine, and Elliker, 1972)

Five milliliters of the dialyzed enzyme was placed on a column (2.5 x 46 cm) of diethylaminoethyl (DEAE) A25 Sephadex (Pharmacia Chemical Company) which had been equilibrated with tris buffer. It was then washed at a flow rate of 20 ml per hour with 200 ml volumes of tris buffer containing 0, 1.0, 1.5, 2.5, and 4.0% sodium chloride. The fractionation procedure was done in a refrigerated room at 3 to 5 C. Active fractions, which were determined by measuring enzyme activity, were combined and saturated to 40% with powdered ammonium sulfate to precipitate the protein. The precipitated protein was recovered by centrifugation at 15,000 x g for 30 minutes at 1 C. It was then dialyzed as before in three two-liter changes of tris buffer at 5 C for three successive 10 hour periods.

## Enzyme Assays

The assay for $\beta$-galactosidase was similar to that

described by Rohlfing and Crawford (1966). Solution A for the assay was 0.1 M sodium phosphate buffer (Mallinckrodt Chemical Works) pH 7.3. Solution B, consisting of 3.36 M 2-mercaptoethanol (Sigma Chemical Company) was prepared by diluting 1.0 ml of 2-mercaptoethanol in 3.25 ml distilled water. Solution C, 0.03 M magnesium chloride, was prepared by dissolving 61 mg $MgCl_2 \cdot 6\ H_2O$ (Mallinckrodt Chemical Works) in distilled water to a volume of 10 ml. Solution D was the solution containing the enzyme. Concentration of the enzyme varied with the stage of purification. The substrate was o-nitrophenyl-β-D-galacto-pyranoside (ONPG) (Sigma Chemical Company). It was prepared at a concentration of 0.068 M by dissolving 20.5 mg ONPG in 1.0 ml solution A and warming in a water bath (55 C) until all ONPG was dissolved.

The assay was conducted by adding 2.6 ml of solution A to a 3 ml cuvette with a 1 cm light path. To this was added 0.1 ml of solution B, 0.1 ml of solution C, and 0.1 ml of enzyme (solution D). The resulting mixture was incubated for three minutes at 37 C to activate the enzyme. The $A_{410}$ was measured in a Spectronic 21 spectrophotometer (Bausch and Lomb) and then 0.1 ml of substrate (ONPG) was added. The cuvette was covered with parafilm and inverted quickly to mix the reagents and the change in $A_{410}$ was measured against time. A unit of enzyme activity was defined as the number of micromoles of o-nitrophenol liberated per milligram of protein per minute

at pH 7.3 and 37 C. The units per milligram protein was calculated by dividing the change in $A_{410}$ by 3.5 times the milligrams protein per milliliter in the mix. The value of 3.5 is the millimolar extinction coefficient of o-nitrophenol.

The assay for phospho-β-galactosidase was performed in the same manner but o-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-6-P) (Sigma Chemical Company) was used as substrate (Molskness et al, 1973).

Protein concentrations were determined by the protein-dye binding method described by Bradford (1976). Bovine serum albumin (Bio-Rad Laboratories) was used as protein standard.

## Molecular Weight Determination

The purified enzyme and standards of known molecular weight were eluted through a gel column with tris buffer as described by Andrews (1964, 1965). The column (2.5 x 46 cm) was packed with Sephadex G-200 gel (Pharmacia Chemical Company) and a flow rate of 15 ml/hour was maintained. The protein standards (Bio-Rad Laboratories) consisted of the following proteins and molecular weights: thyroglobulin (bovine), 670,000; gamma globulin (bovine), 158,000; ovalbumin (chicken), 44,000; myoglobin (horse), 17,000; and vitamin B-12, 1,350. Fractions were collected in five milliliter volumes. Absorbance was measured for all fractions at 280 nm and the curve was prepared by plotting

the log of the molecular weight versus elution volume. The enzyme was located by assaying fractions for enzymatic activity as described in the previous section.

## Determination of Optimum pH

The dialyzed ammonium sulfate precipitate was used to determine the optimum pH of the enzyme. Enzyme activity was assayed as usual but pH of the sodium phosphate buffer was adjusted to the following pH values: 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, and 7.4. Assays were carried out at 37 C.

## Determination of Optimum Temperature

The dialyzed ammonium sulfate precipitate was used to determine the optimum temperature of the enzyme. Reaction vessels containing required volumes of solutions A, B, C, and D (Enzyme Assay Section) were placed into water baths at the desired temperatures for three minutes prior to assay for activity. Assays were conducted as before at pH 7.3 in water baths at 31, 34, 37, 40, 45, 53, and 61 C.

## Enzyme Kinetics

Changes in $A_{410}$ over time were measured using different concentrations of ONPG. In the original assay 0.1 ml of 0.068 M ONPG was used in a total reaction volume of 3.0 ml which gave a final concentration of 2.27 mM. The 0.068 M solution of ONPG was diluted 1:2 succesively five

times to give the following final millimolar concentrations of substrate in the reaction vessel: 1.14, 0.57, 0.28, 0.14, and 0.07. Change in $A_{410}$ was measured for each concentration of substrate.

A plot of velocity/substrate concentration, V/[S], versus velocity (Eadie-Hofstee plot) was made. This plot has a slope of $-1/K_m$ and the intercepts are V and $V/K_m$ on the V and V/[S] axes (Rawn, 1983).

## Enzyme Induction

Cultures of L. acidophilus NCFM were grown in three variations of MRS broth. All were the same as described by the manufacturer (Difco) except for the sugar source. One contained only glucose as the sugar source, one contained galactose only, and the other contained lactose only. The broth was made from individual ingredients according to the manufacturer's formulation except that beef extract and glucose were omitted. The desired sugar was included at 2%. The broths were inoculated (1%, v/v) with the culture and incubated 12 hours at 37 C. Cells were harvested, washed, and lysed as before. The supernatant fluid from the streptomycin sulfate precipitation from cells grown in each broth was assayed for $\beta$-galactosidase activity.

## Effect of Ethylenediamine-tetraacetate (EDTA)

Three 5 ml samples of the dialyzed ammonium sulfate

precipitate containing the enzyme were placed into dialysis tubing and dialyzed for 12 hours at 5 C against one liter volumes of the following concentrations of EDTA: 0.005 mg/ml, 0.05 mg/ml, and 0.5 mg/ml. Samples were removed from the dialysis tubes and β-galactosidase activity was determined as before.

## Effect of Storage

A dialyzed ammonium sulfate precipitated enzyme preparation was placed in a screw cap test tube and stored at 5 C. Enzyme activity was assayed daily to determine the stability of the enzyme during refrigerated storage.

## Comparison of Different Strains of L. acidophilus

Three strains of L. acidophilus (NCFM, 1, and RAM-1) were assayed for specific activity of β-galactosidase. Each culture was inoculated (1%, v/v) into 20 ml of PMN broth and incubated for 12 hours at 37 C. Each culture was then harvested, washed, and lysed as before. The crude enzyme extracts of each strain were treated with streptomycin sulfate and the supernatant fluid of each was assayed for specific activity.

## Statistical Analyses

The data represent the averages of five replications for each test. An analysis of variance was performed on

each set of data to see if any significant differences existed. The differences and confidence levels were determined by calculating the least significant difference as described by Steel and Torrie (1980).

# CHAPTER IV

# RESULTS

## Confirmation of Identity

The L. acidophilus NCFM in this study was a catalase negative, nonspore forming, Gram positive rod. It grew at 45 C but not at 15 C. It fermented amygdalin, cellobiose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, sucrose, and trehalose but did not ferment arabinose, inositol, mannitol, melezitose, rhamnose, sorbitol, and xylose. Ammonia was not produced from arginine and esculin was hydrolyzed.

## Enzymes Present

Enzyme assays conducted by the method previously described using ONPG as substrate revealed that $\beta$-galactosidase was present in all three strains of L. acidophilus. When ONPG-6-P was used as substrate, no change in $A_{410}$ was detected after one hour incubation in any of the enzyme preparations from L. acidophilus NCFM. The streptomycin sulfate precipitated enzyme preparation from L. acidophilus RAM-1 and L. acidophilus 1 were tested using ONPG-6-P as substrate. No change in $A_{410}$ was detected after one hour incubation in either of these strains.

## Enzyme Purification

Data from the purification procedure appears in Table I. Streptomycin sulfate precipitation resulted in increased specific activity of the enzyme. The increase in specific activity gained by ammonium sulfate precipitation was slight, however total activity decreased drastically during this step emphasizing the lability of the enzyme. The greatest increases, although modest, in specific activity were obtained with the DEAE Sephadex and Sephadex G-200 fractionations. These modest increases associated with reduced total activity again reflected the lability of the enzyme. The amount of protein decreased with each step in the procedure suggesting an increase in purity of the enzyme with each step.

## Molecular Weight Determination

Figure 1 shows the molecular weight curve which was deteminined by measuring the elution volumes of standards for which the molecular weights were known in comparison with the $\beta$-galactosidase. The elution volumes for the standards are plotted against the $\log_{10}$ of their molecular weights. Based on the elution volume for $\beta$-galactosidase from L. acidophilus NCFM, the $\log_{10}$ of its molecular weight is 5.76. Thus the molecular weight would be 570,000.

TABLE I

DATA OBTAINED FROM PURIFICATION OF β-GALACTOSIDASE FROM LACTOBACILLUS ACIDOPHILUS NCFM[a]

| Fraction | Vol. (ml) | Total Protein (mg) | Units[b] | Yield (%) | Specific Activity (U/mg) | Purification (-fold) |
|---|---|---|---|---|---|---|
| Crude Extract..... | 92 | 334 | 156 | 100 | .32 | 1.0 |
| Streptomycin Sulfate Supernatant.. | 97 | 273 | 156 | 100 | .57 | 1.8 |
| Ammonium Sulfate Precipitate..... | 8.7 | 134 | 87 | 56 | .65 | 2.0 |
| DEAE Sephadex Eluate..... | 5 | 19.7 | 53 | 34 | 2.7 | 8.4 |
| Sephadex G-200 Eluate | 15 | 9 | 29 | 19 | 3.2 | 10.0 |

[a]Average of five trials.

[b]Defined as micromoles of o-nitrophenol liberated from o-nitrophenyl-β-D-galactopyranoside per milligram of total protein per minute under the assay conditions used.

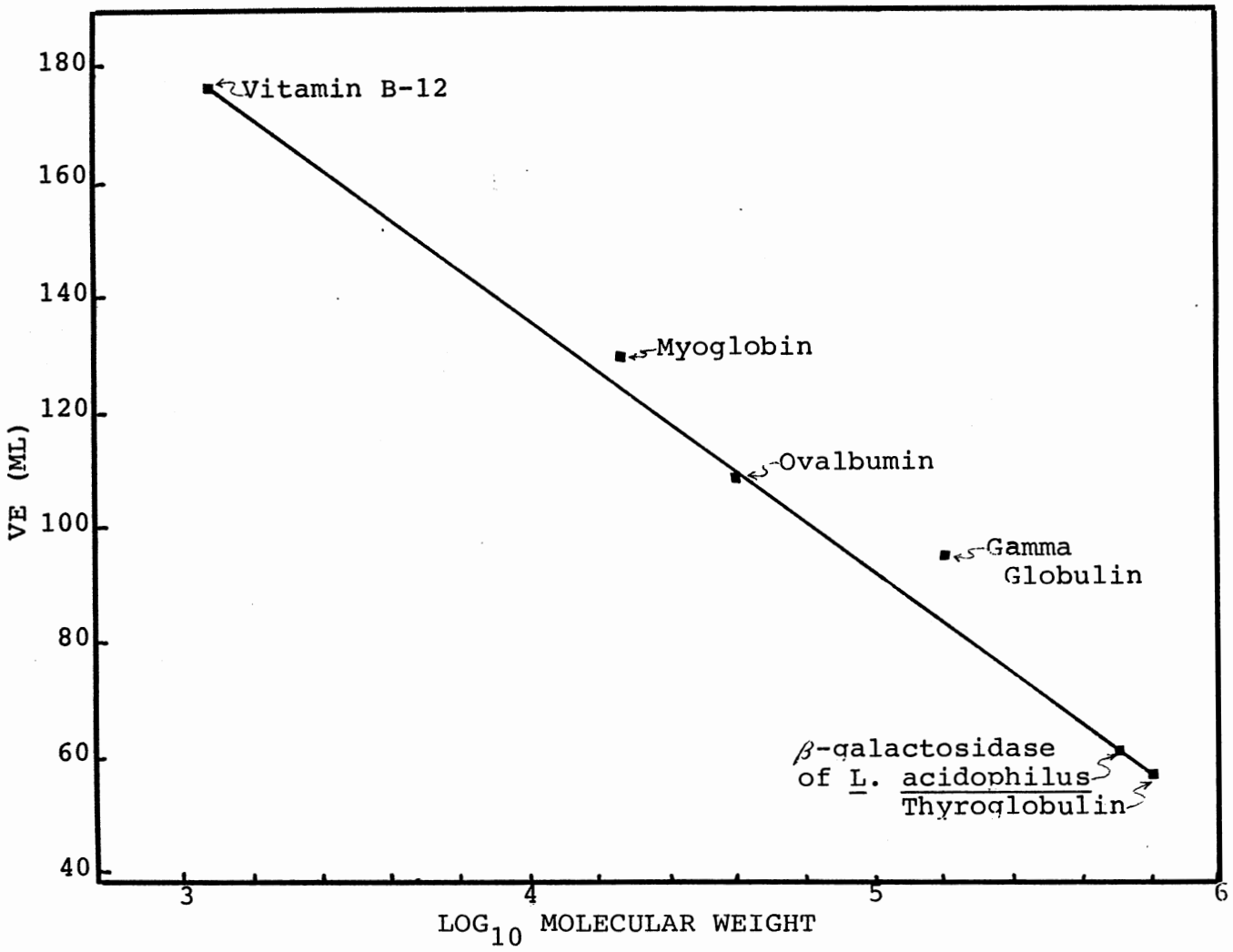


Figure 1. Molecular weight curve determined by measuring the elution volume of known standards in comparison with β-galactosidase from Lactobacillus acidophilus NCFM.

## Effect of pH on Specific Activity

The effect of pH on the specific activity of $\beta$-galactosidase is shown in Figure 2. From pH 6.0, activity increased to a maximum at pH 6.6 and then decreased as the pH increased to reach a minimum activity at pH 7.4. Enzyme activity at pH 6.6 was significantly higher ($P<.05$) than that at all other pH values except pH 6.4.

## Effect of Temperature on Specific Activity

The specific activity of the $\beta$-galactosidase was measured at 31, 34, 37, 40, 45, 53, and 61 C (Figure 3). The greatest activity occurred at 37 C which was significantly higher than the activity at all other temperatures except 40 C ($P<.05$). Temperatures either below or above 37 C reduced the activity of the enzyme. The greatly reduced activity at 61 C suggests the instability of the enzyme at high temperatures. The activity was not regained when the mixture was cooled to 37 C.

## Enzyme Kinetics

To determine enzyme velocity constants, the velocity/substrate concentration was plotted versus velocity (Figure 4). Values obtained for $K_m$ and $V_{max}$ using ONPG as substrate were $2.6 \times 10^{-4}$ M and $1.3 \times 10^{-2}$ micromoles per milligram protein per minute respectively.

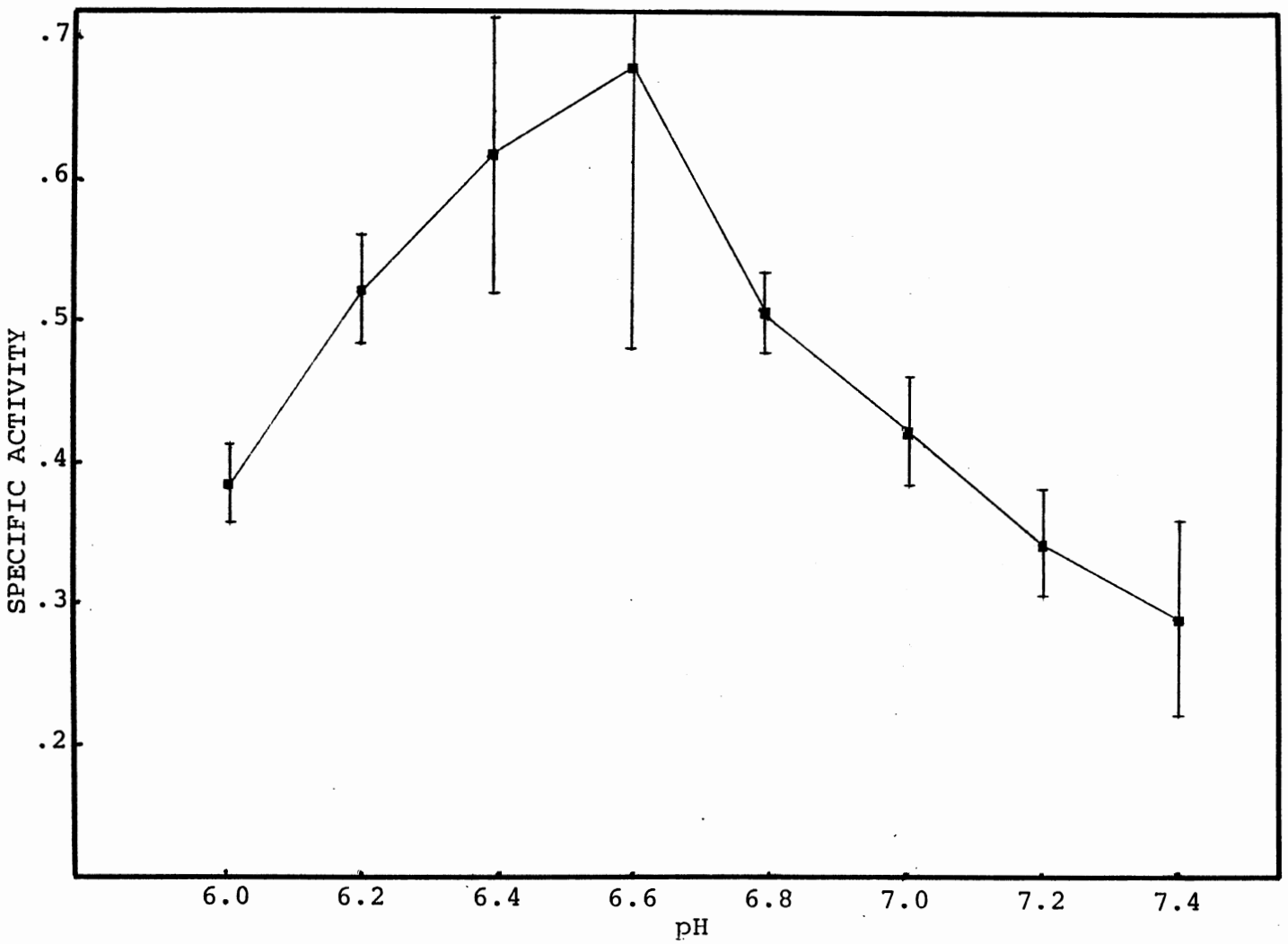


Figure 2. Effect of pH on the specific activity of β-galactosidase from Lactobacillus acidophilus NCFM.

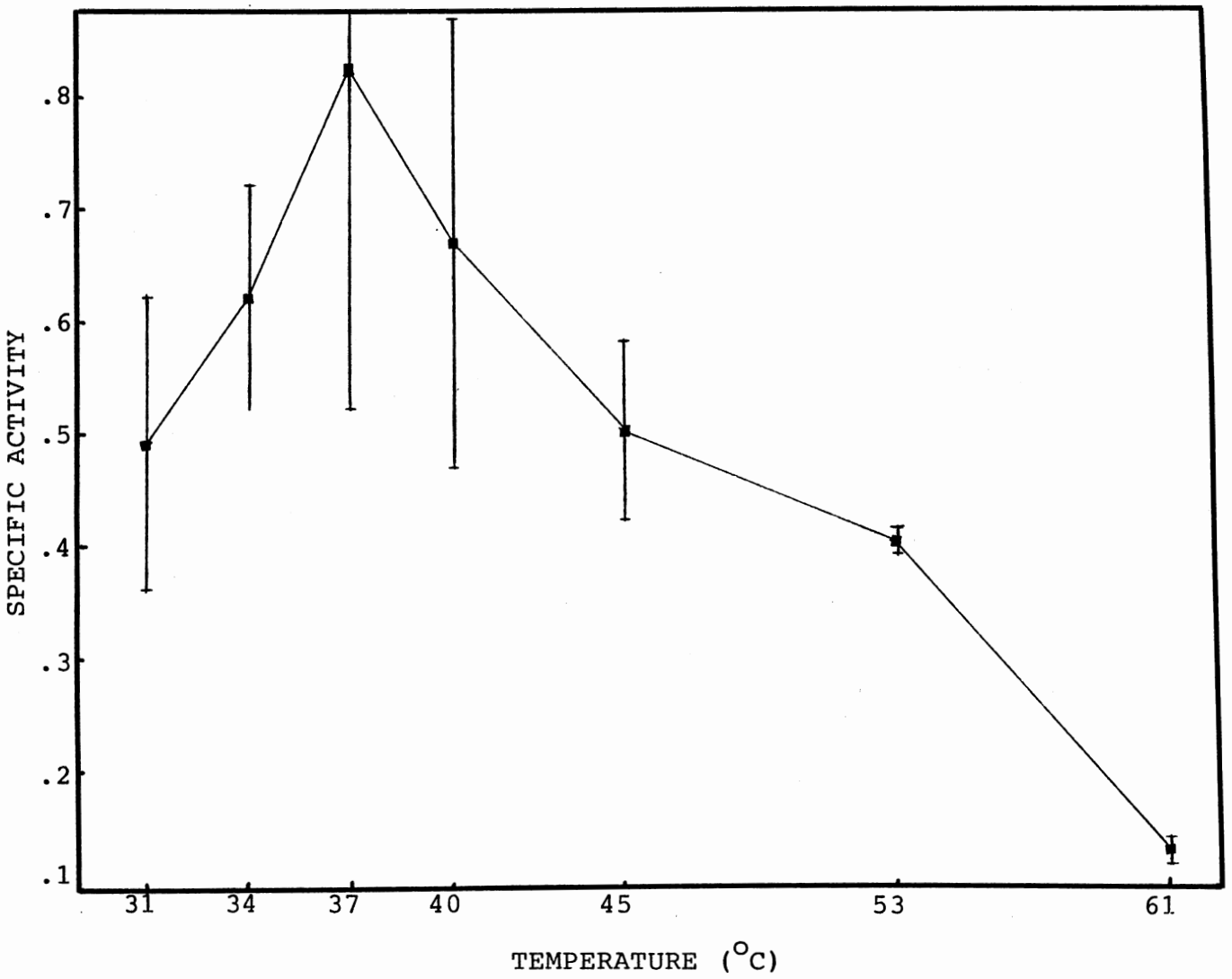


Figure 3. Effect of temperature on the specific activity of β-galactosidase from Lactobacillus acidophilus NCFM.

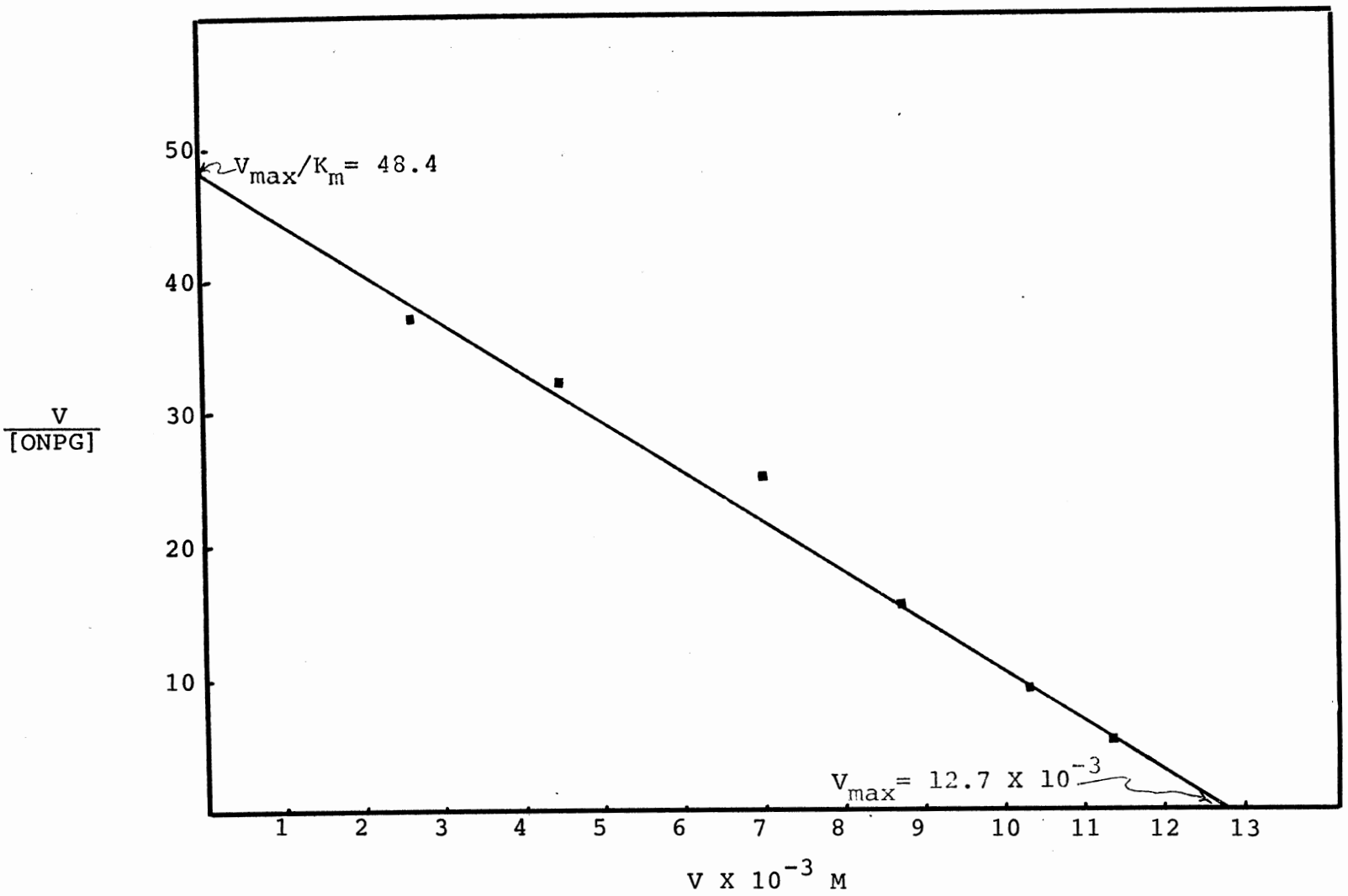


Figure 4. Eadie-Hofstee plot of the effect of substrate concentration on the velocity of the β-galactosidase catalyzed reaction.

## Enzyme Induction

Figure 5 shows the effect of growth of the culture in broth containing lactose, galactose, and glucose on the specific activity of β-galactosidase in the resulting cells. Both lactose and galactose induced high levels of activity. No β-galactosidase activity was detectable in the cells grown on glucose. The level of β-galactosidase activity in cells grown on galactose was significantly higher than that in cells grown on lactose ($P<.05$).

## Effect of EDTA

Activity was not detected after the β-galactosidase was dialyzed against the highest concentration of EDTA (0.5 mg/ml) tested. At an EDTA concentration of 0.05 mg/ml, activity decreased by approximately 60%. Activity was decreased less than 10% when the enzyme was dialyzed against an EDTA concentration of 0.005 mg/ml. Lost activity was not regained when magnesium ions at a concentration of $1 \times 10^{-3}$ M and mercaptoethanol were added to the enzyme preparations.

## Effect of Storage

Activity of β-galactosidase stored at 5 C remained fairly constant for the first four days (Figure 6). Specific activity on day six was significantly lower than that at day zero ($P<.05$). There was a significant decrease in activity at each test interval after day four, however,

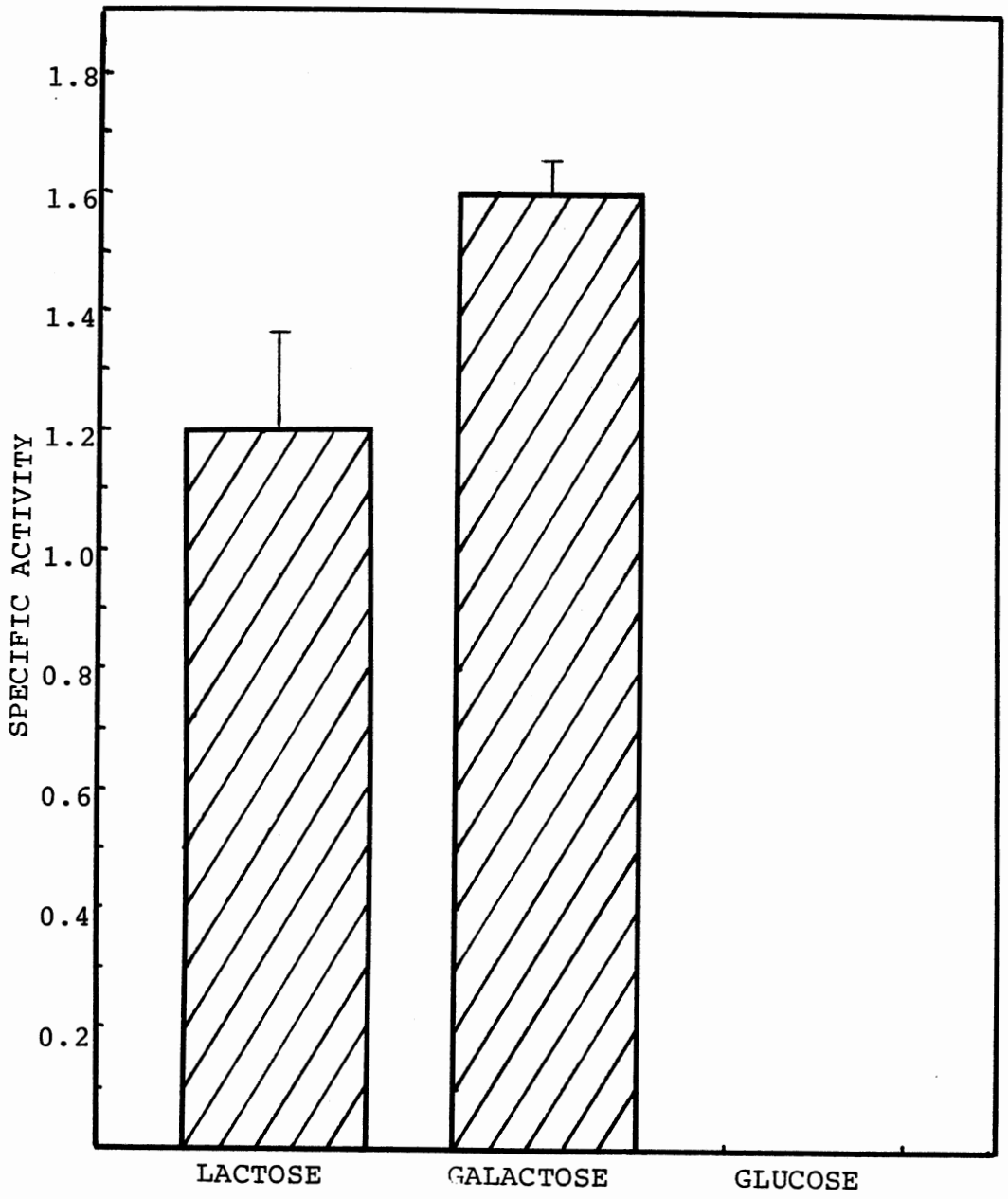


Figure 5. Effect of growth in broths containing lactose, galactose, and glucose as sole sugar sources on the specific activity of β-galactosidase from Lactobacillus acidophilus NCFM. (Average of five trials for each sugar.)

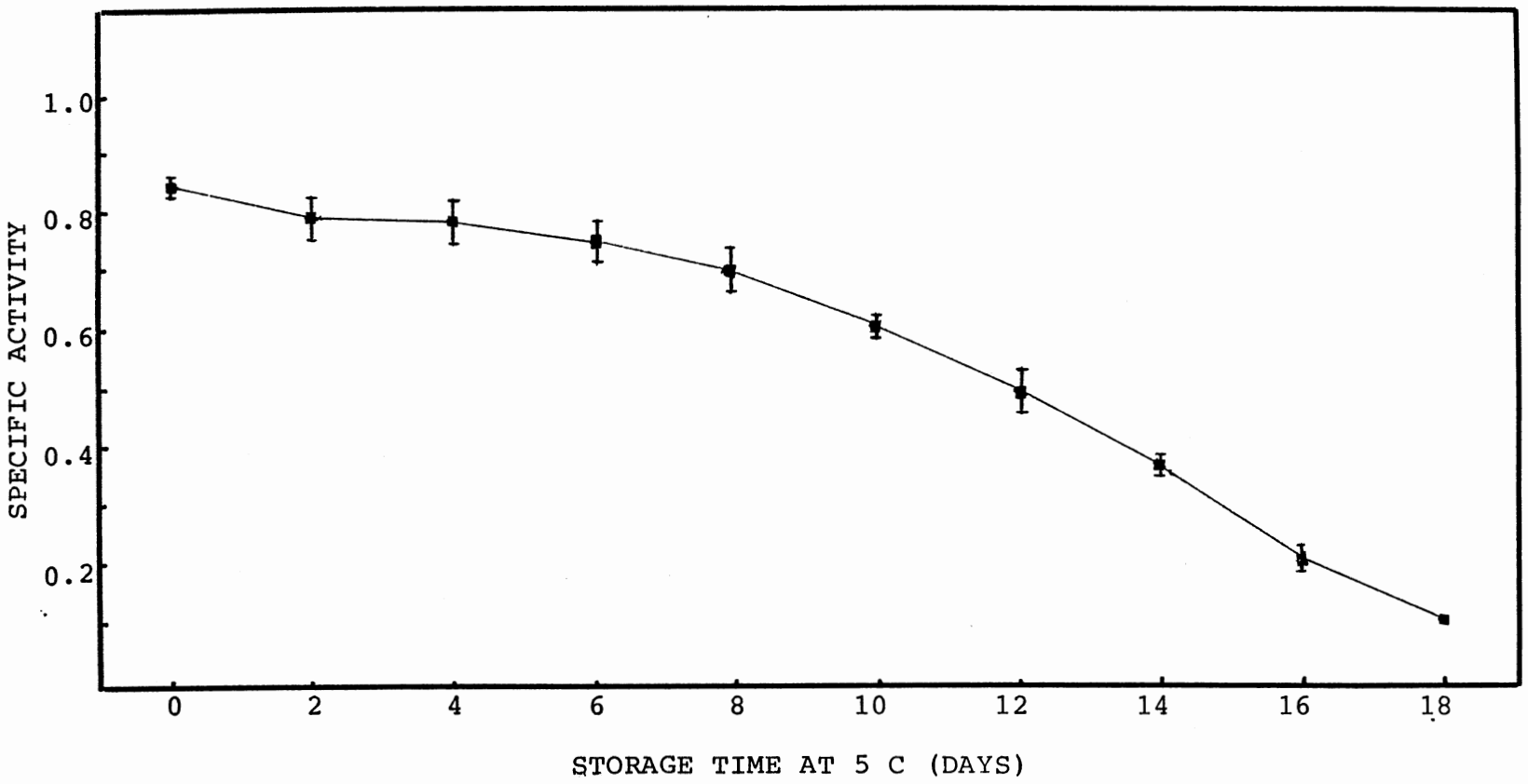


Figure 6. Effect of storage at 5 C on the specific activity of β-galactosidase from Lactobacillus acidophilus NCFM. (Average of five trials.)

more than 50% of the original activity remained after 12 days.

## Comparison of Different Strains of L. acidophilus

Figure 7 shows the comparison of β-galactosidase activity among three strains of L. acidophilus. Lactobacillus acidophilus RAM-1 exhibited a level of activity significantly higher than either L. acidophilus NCFM or L. acidophilus 1 ($P<.05$).

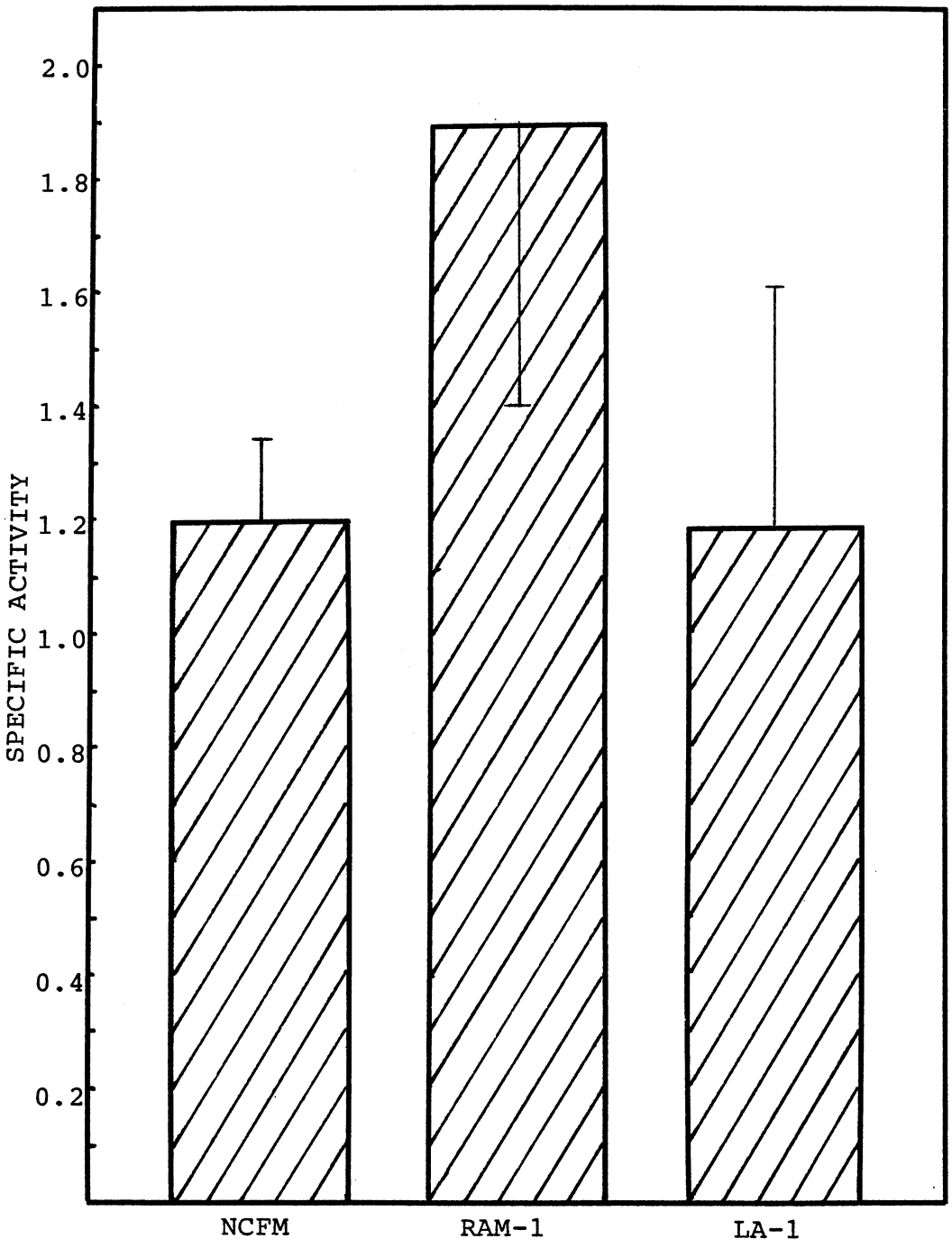


Figure 7. Comparison of the specific activity of $\beta$-galactosidase from three strains of Lactobacillus acidophilus. (Average of five trials per strain.)

# CHAPTER V

## DISCUSSION

The only lactose-hydrolyzing enzyme detected in the three strains of L. acidophilus used in this study was β-galactosidase. Premi et al (1972) reported high levels of β-galactosidase activity and low levels of phospho-β-galactosidase activity in most of the lactobacilli tested except L. casei which exhibited only phospho-β-galactosidase activity. Fisher et al (1985) reported that the five strains of L. acidophilus tested exhibited both β-galactosidase and phospho-β-galactosidase activity when grown at 37 C in broth containing lactose as the only sugar. However, three of these strains exhibited only β-galactosidase activity when grown at 45 C in broth containing lactose as the sole sugar. No explanation was offered for this phenomenon nor did the authors state that the identity of the cultures was confirmed at regular intervals. Itoh et al (1980) observed high levels of β-galactosidase activity in a strain of L. bulgaricus. Phospho-β-galactosidase activity was negligible in a crude enzyme extract and was not detected in the purified enzyme.

Enzyme purification data (Table I) indicated that the enzyme was somewhat unstable. There was a 44% loss in

total activity during ammonium sulfate precipitation. McFeeters et al (1967) experienced a 50% loss in total activity with ammonium sulfate precipitation of β-galactosidase from S. lactis 7962. They also noted an irreversible loss of activity which was greatest in lower concentrations of the enzyme. Citti et al (1965) found β-galactosidase from many strains of S. lactis to be unstable during toluene treatment. The β-galactosidase of one strain (S. lactis C2) was unstable to sonic treatment in the presence or absence of lactose and to disruption in a French press or grinding with alumina or glass beads.

The recovery of 19% of total activity and a ten-fold increase in specific activity during the purification process fit into the wide range of yields reported. Macias et al (1983) reported the recovery of 5.4% of the total activity with a 14.5-fold increase in specific activity for β-galactosidase from L. helveticus. Ramana Rao and Dutta (1981) reported recovery of 25% of total activity with a 56 fold increase in specific activity for β-galactosidase from S. thermophilus while Greenberg and Mahoney (1982) recovered 71% of total activity but gained only a 3.3-fold increase in specific activity for the same organism.

Molecular weight of β-galactosidase from Lactobacillus thermophilus was reported to be 540,000 by Premi et al (1972). Craven et al (1965) reported a molecular weight of 540,000 for β-galactosidase from Escherichia coli K-12. Ramana Rao and Dutta (1981) reported a molecular weight of

600,000 for β-galactosidase from S. thermophilus while Greenberg and Mahoney (1982) reported a molecular weight of 530,000 for the same enzyme. The molecular weight of 570,000 observed for β-galactosidase from L. acidophilus NCFM in this study is consistant with these. Macias et al (1983) reported molecular weights of 257,000 and 170,000 for the β-galactosidases from L. helveticus and L. murinus respectively. There appears to be a large degree of variation in molecular weights of β-galactosidase from similar organisms.

The optimum pH for activity of β-galactosidase in lactic acid producing microorganisms has been found to be in the range from 6.5 to 7.0. Macias et al (1983) reported the optimum pH for β-galactosidase from L. helveticus to be 6.5 and Itoh et al (1980) reported a pH optimum of 7.0 for β-galactosidase from L. bulgaricus.

The optimum temperature of 37 C found in this study would be expected from an enzyme isolated from an inhabitant of the human intestinal tract. The elevation in temperature appeared to increase the rate of denaturation of the enzyme because lost activity could not be regained when the enzyme was returned to 37 C. Enzyme preparations incubated at lower temperatures exhibited an increase in activity when warmed to 37 C. Other lactobacilli have exhibited higher temperature optimums. Macias et al (1983) reported the optimum temperatures of L. murinus and L. helveticus at 45 C and 42 C respectively. Itoh et al

(1980) reported that β-galactosidase from L. bulgaricus had an optimum temperature of 50 C.

Elevated temperatures appear to irreversibly inactivate β-galactosidase in many bacteria. Itoh et al (1980) reported total inactivation of β-galactosidase from L. bulgaricus when incubated at 55 C for 10 minutes. Macias et al (1983) reported a loss in activity of about 50% for β-galactosidase from both L. murinus and L. helveticus when the enzyme was incubated at 50 C for three minutes. Ramana Rao and Dutta (1981) reported that β-galactosidase from S. thermophilus lost 60% of its activity when maintained at 60 C for 10 minutes.

The β-galactosidase of L. acidophilus was found to be inducible as is the case with most lactose hydrolyzing bacteria. Lactose and galactose both induced activity while glucose did not. Fisher et al (1985) also found this pattern with several strains of L. acidophilus. Okamura et al (1983) found β-galactosidase to be inducible in 45 of 50 species of lactose utilyzing bacteria tested.

The lability of the enzyme was demonstrated by the addition of EDTA. Loss of activity increased as concentration of EDTA increased. Lost activity was not regained after the addition of magnesium ions and mercaptoethanol which suggests a denaturing effect of EDTA. Dickson et al (1979) reported that magnesium ions were necessary in the presence of mercaptoethanol to induce maximum activity of β-galactosidase in yeast. Craven et al also reported that

mercaptoethanol improved stability of β-galactosidase from E. coli. Premi et al (1972) reported similar irreversible losses in activity of β-galactosidase from L. thermophilus when it was dialyzed against EDTA.

The enzyme remained fairly stable when stored at 5 C in the presence of magnesium ions and mercaptoethanol. Greatest losses occurred beyond six days of storage. More than 50% of the original activity remained after 12 days storage. McFeeters et al (1967) reported that β-galactosidase from S. lactis 7962 lost 5% of its original activity daily when stored at 5 C. Landman (1957) stored a crude extract of β-galactosidase from Bacillus megaterium at -20 C and found it lost half of its activity in 12 days. Itoh et al (1980) stored a lyopholyzed preparation of β-galactosidase from L. bulgaricus and reported that it was stable after storage for four months at 4 C.

When comparing different strains of L. acidophilus, it was found that some exhibited a higher level of β-galactosidase activity. Those with the highest level of activity should make the best dietary adjuncts to improve lactose utilization provided they were able to function in the small intestine in humans.

# CHAPTER VI

## SUMMARY

The purpose of the study was to isolate the lactose-hydrolyzing enzyme of Lactobacillus acidophilus and determine some of its characteristics. Lactobacillus acidophilus is used as a dietary adjunct, primarily in nonfermented acidophilus milk, to provide possible nutritional and health benefits, one of which is improved lactose utilization by persons who are lactose malabsorbers.

The cells of L. acidophilus were lysed enzymatically to release the enzyme for study. Characteristics which were determined included kinetic properties and molecular weight. The effect of such factors as temperature, pH, storage, and divalent ions were determined as well as the influence of some sugars on induction of the enzyme.

The lactose-hydrolyzing enzyme, $\beta$-galactosidase, exhibited a $V_{max}$ of $1.3 \times 10^{-2}$ micromoles per milligram protein per minute and a $K_m$ of $2.6 \times 10^{-4}$ M using ONPG as substrate. Maximum activity occurred when the enzyme was incubated with magnesium ions and mercaptoethanol in pH 6.6 sodium phosphate buffer at 37 C. Gel filtration indicated a molecular weight of $5.7 \times 10^{5}$.

Lactose and galactose both induced enzyme activity while glucose did not. Phospho-β-galactosidase activity was not detected.

# CHAPTER VII

## CONCLUSIONS

Milk has a high nutritional value and is an important part of the diet. For those consumers who are lactose malabsorbers, milk consumption is reduced or eliminated. Nonfermented acidophilus milk containing a viable L. acidophilus culture, provides many consumers with a source of fresh milk which has all the qualities desired in milk but few or none of the disadvantages associated with lactose malabsorption (Kim and Gilliland, 1983).

Many strains of L. acidophilus are presently being used as dietary adjuncts. The most desirable dietary adjunct is produced from lactobacilli of human origin since they appear to be host species specific (Gilliland, 1979). There are significant differences in the levels of $\beta$-galactosidase activity among these strains. Selection of strains with the highest level of activity should be considered when selecting a dietary adjunct provided it is able to function in the small intestine in humans. Improvement of the product can only increase its benefits so more people can enjoy milk.

A different approach is necessary for milk consumers in developing countries. Due to limited availability of

fresh milk and conditions for rehydration of dry milk, non fermented acidophilus milk is not a logical solution for the problems of lactose malabsorption in these populations. Low lactose milk continues to be the best method of milk preparation for this type of situation.

The β-galactosidase of L. acidophilus is a large enzyme which has optimum activity at the pH of milk (6.6). The cations present in milk may add to stability. This suggests that the enzyme may be suitable for use in an immobilized lactase system. However, further research is needed to evaluate the behavior of the enzyme in that environment.

## LITERATURE CITED


Alm, L. 1982. Effect of fermentation on lactose, glucose, and galactose content in milk and suitability of fermented milk products for lactose intolerant individuals. J. Dairy Sci. 65:346.

Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91:222.

Andrews, P. 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. 96:595.

Anonymous. 1976. Many dairies are selling new sweet acidophilus milk. Am. Dairy Rev. 38:30.

Anonymous. 1976. New milk product was begun in OSU laboratory. Am. Dairy Rev. 38:62.

Bayless, T. M. and N. L. Rosensweig. 1966. A racial difference in incidence of lactase dificiency. JAMA 197:968.

Bayless, T. M., B. Rothfeld, C. Massa, L. Wise, D. Paige, and M. Bedine. 1975. Lactose and milk intolerance: clinical implications. N. Engl. J. Med. 292:1156.

Borglum, G. B. and M. Z. Sternberg. 1972. Properties of a fungal lactase. J. Food Sci. 37:619.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72:248.

Brand, J. C., M. S. Gracey, R. M. Spargo, and S. P. Dutton. 1983. Lactose malabsorption in Australian aborigenes. Am. J. Clin. Nutr. 37:449.

Brown, K. M., M. Khatun, L. Parry, and Md. G. Ahmed. 1980. Nutritional consequences of low dose milk supplements consumed by lactose-malabsorbing children. Am. J. Clin. Nutr. 33:1054.

Burgio, G. R., G. Flatz, C. Barbera, R. Patane, A. Boner, C. Cajozzo, and S. D. Flatz. 1984. Prevalence of primary adult lactose malabsorption and awareness of milk intolerance in Italy. Am. J. Clin. Nutr. 39.100.

Butterworth, T. H. 1938. Lactobacillus acidophilus milk gets psychoanalyzed. Milk Plant Mo. 27:32.

Carrera, E., M. C. Nesheim, and D. W. T. Crompton. 1984. Lactose maldigestion in Ascaris-infected preschool children. Am. J. Clin. Nutr. 39:255.

Cheng, A. H. R., O. Brunser, J. Espinosa, H. L. Fones, F. Monckeberg, C. O. Chichester, G. Rand, and A. G. Hourigan. 1979. Long term acceptance of low-lactose milk. Am. J. Clin. Nutr. 32:1989.

Citti, J. E., W. E. Sandine, and P. R. Elliker. 1965. $\beta$-galactosidase of Streptococcus lactis. J. Bacteriol. 89:937.

Citti, J. E., W. E. Sandine, and P. R. Elliker. 1967. Lactose and maltose uptake by Streptococcus lactis. J. Dairy Sci. 50:485.

Craven, G. R., E. Steers, and C. B. Anfinsen. 1965. Purification, composition, and molecular weight of the $\beta$-galactosidase of Escherichia coli K-12. J. Biol. Chem. 240:2468.

Dickson, R. C., L. R. Dickson, and J. S. Markin. 1979. Purification and properties of an inducible $\beta$-galactosidase isolated from the yeast Kluyveromyces lactis. J. Bacteriol. 137:51.

Duggan, D. E., A. W. Anderson, and P. R. Elliker. 1959. A frozen concentrate of Lactobacillus acidophilus for preparation of a palatable acidophilus milk. Food Tech. 13:465-469.

Farrow, J. A. E. 1980. Lactose hydrolysing enzymes in Streptococcus lactis and Streptococcus cremoris and also in some other species of streptococci. J. Appl. Bacteriol. 49:493.

Fischer, J. E. and T. S. Sutton. 1949. Effects of lactose on gastrointestinal motility: A review. J. Dairy Sci. 32:139.

Fisher, K., M. C. Johnson, and B. Ray. 1985. Lactose hydrolyzing enzymes in Lactobacillus acidophilus strains. Food Microbiol. 2:23.

Garvie, E. I. 1978. Lactate dehydrogenase of Streptococcus thermophilus. J. Dairy Res. 45:515.

Gilliland, S. E. and M. L. Speck. 1977. Instability of Lactobacillus acidophilus in yogurt. J. Dairy Sci. 60:1394.

Gilliland, S. E. and M. L. Speck. 1977. Use of the minitek system for characterizing lactobacilli. Appl. Environ. Microbiol. 33:1289.

Gilliland, S. E., M. L. Speck, G. F. Nauyok, and F. G. Giesbrecht. 1978. Influence of consuming nonfermented milk containing Lactobacillus acidophilus on fecal flora of healthy males. J. Dairy Sci. 61:1.

Gilliland, S. E. 1979. Beneficial interrelationships between certain microorganisms and humans: Candidate microorganisms for use as dietary adjuncts. J. Food Prot. 42:164.

Gilliland, S. E. and H. S. Kim. 1984. Effect of viable starter culture bacteria in yogurt on lactose utilization in humans. J. Dairy Sci. 67:1.

Goldin, B. R. and S. L. Gorbach. 1984. The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. Am. J. Clin. Nutr. 39:756.

Gray, G. M. and N. A. Santiago. 1969. Intestinal $\beta$-galactosidases. I. Separation and characterization of three enzymes in normal human intestine. J. Clin. Invest. 48:716.

Greenberg, N. A. and R. R. Mahoney. 1982. Production and characterization of $\beta$-galactosidase from Streptococcus thermophilus. J. Food Sci. 47:1824.

Hengstenberg, W., J. B. Egan, and M. L. Morse. 1968. Carbohydrate transport in Staphylococcus aureus. J. Biol. Chem. 243:1881.

Itoh, T., M. Ohhashi, T. Toba, and S. Adachi, 1980. Purification and properties of $\beta$-galactosidase from Lactobacillus bulgaricus. Milchwissenschaft 35:593.

Johnson, R. C., K. S. Bowman, S. Y. Schwitters, C. T. Nagoshi, J. R. Wilson, and J. Y. Park. 1984. Ethnic, familial, and environmental influences on lactose tolerance. Human Biol. 56:307.

Kilara, A. and K. M. Shahani. 1976. Lactase activity of cultured and acidified dairy products. J. Dairy Sci. 59:2031.

Kim, H. S. and S. E. Gilliland. 1983. Lactobacillus acidophilus as a dietary adjunct for milk to aid lactose digestion in humans. J. Dairy Sci. 66:959.

Kleyn, D. H. 1972. Lactose intolerance and consumption of dairy products. Am. Dairy Rev. 34:47.

Landman, O. E. 1957. Properties and induction of β-galactosidase in Bacillus megaterium. Biochimica Biophysica Acta 23:558.

Lederberg, J. 1950. The beta-D-galactosidase of Escherichia coli K-12. J. Bacteriol. 60:381.

Lester, G. and D. M. Bonner. 1952. The occurrence of β-galactosidase in Escherichia coli. J. Bacteriol. 63:759.

Lisker, R., L. Aguilar, I. Lares, and J. Cravioto. 1980. Double bllind study of milk lactose intolerance in a group of rural and urban children. Am. J. Clin. Nutr. 33:1049.

Macias, M. E. N., M. C. N. de Nadra, A. M. S. de Saad, A. A. P. de Ruiz Holgado, and G. Oliver. 1983. Isolation and properties of β-galactosidase of a strain of Lactobacillus helveticus isolated from natural whey starter. J. Appl. Biochem. 5:275.

Macias, M. E. N., M. C. M. de Nadra, A. M. S. de Saad, A. A. P. de Ruiz Holgado, and G. Oliver. 1983. Isolation and purification of β-galactosidase of Lactobacillus murinus CNRZ 313. Current Microbiol. 9:99.

McFeeters, G. A., W. E. Sandine, and P. R. Elliker. 1967. Purification and properties of Streptococcus lactis β-galactosidase. J. Bacteriol. 93:914.

Metcalf, R. H. and R. H. Deibel. 1969. Different lytic responses of enterococci associated with addition order of lysozyme and anions. J. Bacteriol. 99:674.

Miller, A., W. E. Sandine, and P. R. Elliker. 1970. Deoxyribonucleic acid base composition of lactobacilli determined by thermal denaturation. J. Bacteriol. 102:278.

Molskness, T. A., D. R. Lee, W. E. Sandine, and P. R. Elliker. 1973. β-D-Phosphogalactoside galacto-hydrolase of lactic streptococci. Appl. Microbiol. 25:373.

Myers, R. P. 1931. Transformation of the intestinal flora through the feeding of unfermented acidophilus milk. Am. J. Pub. Hlth. 21:867-872.

Nasrallah, S. M. 1979. Lactose intolerance in the Lebanese population and in "Mediterranean lymphoma". Am. J. Clin. Nutr. 32:1994.

Newcomer, A. D., S. F. Hodgson, D. B. McGill, and P. J. Thomas. 1978. Lactase deficiency: Prevalence in osteoporosis. Ann. Int. Med. 89:218.

Nielsen, V. H. 1976. Sweet acidophilus milk gains wide public acceptance. Am. Dairy Rev. 38:40.

Okamura, Y., M. Shoda, and S. Udaka. 1983. Activity and synthesis of $\beta$-galactosidase in various lactose utilizing bacteria. Agric. Biol. Chem. 47:1133.

Paige, D. M., T. M. Bayless, S. S. Huang, and R. Wexler. 1975. Lactose hydrolyzed milk. Am. J. Clin. Nutr. 28:818.

Payne, D. L., J. D. Welsh, C. V. Manion, A. Tsegaye, and L. D. Herd. 1981. Effectiveness of milk products in dietary management of lactose malabsorption. Am. J. Clin. Nutr. 34:2711.

Premi, L., W. E. Sandine, and P. R. Elliker. 1972. Lactose hydrolyzing enzymes of Lactobacillus species. Appl. Microbiol. 24:51.

Ramana Rao, M. V. and S. M. Dutta. 1981. Purification and properties of beta-galactosidase from Streptococcus thermophilus. J. Food Sci. 46:1419.

Rawn, J. D. 1983. Biochemistry. Harper and Row, New York, NY.

Richmond, M. L., J. I. Gray, and C. M. Stone. 1981. Beta-galactosidase: Review of recent research related to technological application, nutritional concerns, and immobilization. J. Dairy Sci. 64:1759.

Rohlfing, S. R. and I. P. Crawford. 1966. Partial purification and physical properties of Bacillus megaterium $\beta$-galactosidase. J. Bacteriol. 92:1258.

Rohlfing, S. R. and I. P. Crawford. 1966. Purification and characterization of the $\beta$-galactosidase of Aeromonas formicans. J. Bacteriol. 91:1085.

Rosensweig, N. S. 1969. Adult human milk intolerance and intestinal lactase deficiency. A review. J. Dairy Sci. 52:585.

Sadre, M. and K. Karbosi. 1979. Lactose intolerance in Iran. Am. J. Clin. Nutr. 32:1948.

Sandine, W. E. 1979. Roles of lactobacillus in the intestinal tract. J. Food Prot. 42:259.

Savaiano, D. A., A. A. El Anouar, D. E. Smith, and M. D. Levitt. 1984. Lactose malabsorption from yogurt, pasteurized yogurt, sweet acidophilus milk, and cultured milk in lactase-deficient individuals. Am. J. Clin. Nutr. 40:1219.

Shahani, K. M. and A. D. Ayebo. 1980. Role of dietary lactobacilli in gastrointestinal microecology. Am. J. Clin. Nutr. 33:2448.

Shaw, J. N. and O. H. Muth. 1937. The use of acidophilus milk in the treatment of dysentary of young animals. Am. Vet. Med. Assn. J. 90:171.

Solomons, N. S., R. Garcia-Ibanez, and F. Viteri. 1980. Hydrogen breath test of lactose absorption in adults: The application of physiological doses of whole cow's milk sources. Am. J. Clin. Nutr. 33:545.

Speck, M. L. 1975. Market outlook for acidophilus food products. Cult. Dairy Prod. J. 10:8-10.

Speck, M. L. 1976. Interactions among lactobacilli and man. J. Dairy Sci. 59:338.

Speck, M. L. 1980. Preparation of lactobacilli for dietary uses. J. Food Prot. 42:65.

Steel, R. G. D. and J. H. Torrie. Principles and Procedures of Statistics, Second Edition. McGraw Hill, New York, NY.

Tandon, R. K., Y. K. Joshi, D. S. Singh, M. Narendranathan, V. Balakrishnan, and K. Lal. 1981. Lactose intolerance in north and south Indians. Am. J. Clin. Nutr. 34:943.

Thompson, J., K. W. Turner, and D. Thomas. 1978. Catabolite inhibition and sequential metabolism of sugars by Streptococcus lactis. J. Bacteriol. 133:1163.

Toba, T., Y. Tomita, T. Itoh, and S. Adachi. 1981. β-galactosidase of lactic acid bacteria: Characterization by oligosaccharides formed during hydrolysis of lactose. J. Dairy Sci. 64:185.

Vedejs, L. 1954. A market for acidophilus milk. Milk Plant Mo. 43:45.

Young, C. K. and F. E. Nelson. 1978. Survival of Lactobacillus acidophilus in "Sweet Acidophilus Milk" during refrigerated storage. J. Food Prot. 41:248.

# APPENDIX A

## DATA FROM TREATMENTS

TABLE II

EFFECT OF pH ON THE SPECIFIC ACTIVITY OF β-GALACTOSIDASE FROM LACTOBACILLUS ACIDOPHILUS NCFM

| pH | | 6.0 | 6.2 | 6.4 | 6.6 | 6.8 | 7.0 | 7.2 | 7.4 |
|---|---|---|---|---|---|---|---|---|---|
| Sample | 1 | .40 | .55 | .49 | .73 | .52 | .47 | .33 | .27 |
| | 2 | .33 | .45 | .54 | .78 | .45 | .36 | .33 | .41 |
| | 3 | .37 | .53 | .79 | .32 | .53 | .40 | .35 | .23 |
| | 4 | .40 | .56 | .64 | .69 | .52 | .43 | .39 | .29 |
| | 5 | .38 | .52 | .62 | .77 | .50 | .46 | .29 | .23 |
| Totals | | 1.88 | 2.61 | 3.08 | 3.29 | 2.52 | 2.12 | 1.69 | 1.43 |
| | $\bar{y}$ | .38 | .52 | .62 | .66 | .50 | .42 | .34 | .29 |

TABLE III

EFFECT OF TEMPERATURE ON THE SPECIFIC ACTIVITY OF β-GALACTOSIDASE FROM LACTOBACILLUS ACIDOPHILUS NCFM

| Temp. | | 31 | 34 | 37 | 40 | 45 | 53 | 61 |
|---|---|---|---|---|---|---|---|---|
| Sample | 1 | .31 | .50 | .35 | .38 | .47 | .24 | .09 |
| | 2 | .50 | .55 | .73 | .62 | .58 | .25 | .10 |
| | 3 | .53 | .80 | 1.05 | .75 | .55 | .25 | .09 |
| | 4 | .68 | .64 | 1.18 | .95 | .40 | .23 | .08 |
| | 5 | .49 | .62 | .83 | .67 | .40 | .23 | .09 |
| Totals | | 2.51 | 3.11 | 4.14 | 3.37 | 2.50 | 1.20 | 0.45 |
| | $\bar{y}$ | .50 | .62 | .83 | .67 | .50 | .24 | .09 |

TABLE IV

EFFECT OF THREE SUGARS ON INDUCTION OF β-GALACTOSIDASE IN LACTOBACILLUS ACIDOPHILUS NCFM

| Sugar | | Lactose | Galactose | Glucose |
|---|---|---|---|---|
| Sample | 1 | 1.16 | 1.58 | 0.0 |
| | 2 | 1.38 | 1.53 | 0.0 |
| | 3 | .92 | 1.67 | 0.0 |
| | 4 | 1.22 | 1.58 | 0.0 |
| | 5 | 1.14 | 1.60 | 0.0 |
| Totals | | 5.82 | 7.96 | 0.0 |
| | $\bar{y}$ | 1.16 | 1.59 | 0.0 |

TABLE V

EFFECT OF STORAGE AT 5 C ON THE SPECIFIC ACTIVITY OF β-GALACTOSIDASE FROM LACTOBACILLUS ACIDOPHILUS NCFM

| Day | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | .85 | .80 | .80 | .76 | .70 | .62 | .50 | .38 | .22 | .11 |
| 2 | .84 | .82 | .80 | .78 | .67 | .60 | .51 | .37 | .23 | .11 |
| 3 | .83 | .81 | .83 | .74 | .69 | .61 | .52 | .40 | .21 | .11 |
| 4 | .87 | .78 | .80 | .78 | .72 | .64 | .48 | .39 | .22 | .11 |
| 5 | .85 | .81 | .77 | .75 | .71 | .62 | .49 | .37 | .22 | .11 |
| | 4.24 | 4.02 | 4.00 | 3.81 | 3.49 | 3.09 | 2.50 | 1.91 | 1.10 | 0.55 |
| $\bar{y}$ | .85 | .80 | .80 | .76 | .70 | .62 | .50 | .38 | .22 | .11 |

TABLE VI

COMPARISON OF THE SPECIFIC ACTIVITY OF β-GALACTOSIDASE FROM THREE STRAINS OF LACTOBACILLUS ACIDOPHILUS

| Strain | | 1 | RAM-1 | NCFM |
|---|---|---|---|---|
| Sample | 1 | 1.40 | 2.77 | 1.16 |
| | 2 | .62 | 1.86 | 1.38 |
| | 3 | 1.36 | 1.29 | .92 |
| | 4 | .77 | 1.83 | 1.22 |
| | 5 | 1.70 | 1.54 | 1.14 |
| Totals | | 5.85 | 9.29 | 5.82 |
| | $\bar{y}$ | 1.17 | 1.86 | 1.16 |

# APPENDIX B

# STATISTICAL ANALYSES

TABLE VII

ANALYSIS OF VARIANCE TABLE - EFFECT OF pH ON THE SPECIFIC ACTIVITY OF $\beta$-GALACTOSIDASE FROM LACTOBACILLUS ACIDOPHILUS NCFM

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Between pH | 7 | .612 | .087 | 10.88 |
| Within pH | 32 | .248 | .008 | |
| Total | 39 | .860 | .022 | |

OSL < .005

$LSD_{.05}$ = .114

| pH | 6.6 | 6.4 | 6.2 | 6.8 | 7.0 | 6.0 | 7.2 | 7.4 |
|---|---|---|---|---|---|---|---|---|
| $\bar{y}$ | .66 | .62 | .52 | .50 | .42 | .38 | .34 | .29 |

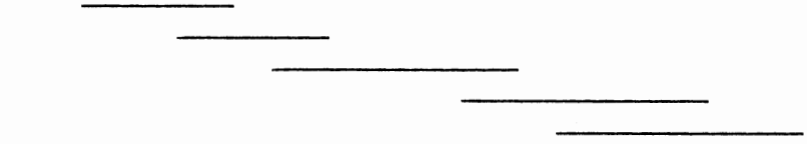

TABLE VIII

ANALYSIS OF VARIANCE TABLE - EFFECT OF TEMPERATURE ON THE SPECIFIC ACTIVITY OF β-GALACTOSIDASE FROM LACTOBACILLUS ACIDOPHILUS NCFM

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Between Temp. | 6 | 1.94 | .324 | 12.46 |
| Within Temp. | 28 | .72 | .026 | |
| Total | 34 | 2.66 | .078 | |

OSL < .005

$LSD_{.05}$ = .209

| Temperatures | 37 | 40 | 34 | 31 | 45 | 53 | 61 |
|---|---|---|---|---|---|---|---|
| $\bar{y}$ | .83 | .67 | .62 | .50 | .50 | .24 | .09 |

TABLE IX

ANALYSIS OF VARIANCE TABLE - EFFECT OF THREE SUGARS ON INDUCTION OF β-GALACTOSIDASE IN *LACTOBACILLUS ACIDOPHILUS* NCFM

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Between Sugars | 2 | 6.788 | 3.394 | 38.135 |
| Within Sugars | 12 | 1.071 | .089 | |
| Total | 14 | 7.859 | .561 | |

OSL < .005

$LSD_{.05}$ = .412

| Sugar | Galactose | Lactose | Glucose |
|---|---|---|---|
| $\bar{y}$ | 1.59 | 1.16 | 0.0 |

TABLE X

ANALYSIS OF VARIANCE TABLE - EFFECT OF STORAGE AT 5 C ON THE SPECIFIC ACTIVITY OF β-GALACTOSIDASE FROM LACTOBACILLUS ACIDOPHILUS NCFM

| Total | Degrees of Freedom | Sum of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Between Days | 9 | 3.073 | .341 | 170.5 |
| Within Days | 40 | .009 | .002 | |
| Total | 49 | 3.082 | .063 | |

OSL < .005

$LSD_{.05}$ = .056

| Day | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|
| $\bar{y}$ | .85 | .80 | .80 | .76 | .70 | .62 | .50 | .38 | .22 | .11 |

TABLE XI

ANALYSIS OF VARIANCE TABLE - COMPARISON OF THE SPECIFIC ACTIVITY OF β-GALACTOSIDASE FROM THREE STRAINS OF LACTOBACILLUS ACIDOPHILUS

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Between Strains | 2 | 1.601 | .800 | 4.04 |
| Within Strains | 12 | 2.381 | .198 | |
| Total | 14 | 3.982 | .284 | |

OSL < .025

$LSD_{.05}$ = .612

| Strain | RAM-1 | 1 | NCFM |
|---|---|---|---|
| $\bar{y}$ | 1.86 | 1.17 | 1.16 |

VITA

Jerry Wayne Nielsen

Candidate for the Degree of

Doctor of Philosophy

Thesis: ISOLATION AND CHARACTERIZATION OF THE LACTOSE-HYDROLYZING ENZYME OF LACTOBACILLUS ACIDOPHILUS

Major Field: Food Science

Biographical:

Personal Data: Born in Delhi, Louisiana, February 12, 1949, the son of Otho J. and Gloria Nielsen. Married to Ann Bornemann on July 30, 1983. Daughter, Laura Ellen, born July 30, 1984. Son, Jeffrey Wayne, born September 21, 1987.

Education: Graduated from Delhi High School, Delhi, Louisiana, in May, 1967; received Bachelor of Science Degree in Biology from Northeast Louisiana University in May 1979; received Master of Science Degree in Biology from Northeast Louisiana University in December, 1981; completed requirements for the Doctor of Philosophy Degree at Oklahoma State University in December, 1987.

Professional Experience: Teaching Assistant, Biology Department, Northeast Louisiana University, August 1979, to December, 1981; Teaching Assistant, Animal Science Department, Oklahoma State University, August, 1982, to August, 1985; Instructor of Biological Science, Western Oklahoma State College, August, 1985, to present.