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Lactose hydrolysis by sonicated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842

by

Mary Ellen Kreft 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring 2001

University of Alberta

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Title of Thesis: Lactose hydrolysis by sonicated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842.

Degree: Master of Science

Year this Degree Granted: 2001

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 'Lactose hydrolysis by sonicated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842', submitted by Mary Ellen Kreft in partial fulfillment of the requirements for the degree of Master of Science in Food Science and Technology.

Dr. P. Jelen

Dr. P. Sporns

Dr. L. McMullen

Dr. L. McGann

Date: January 8, 2001

QUOTE

"QUAECUMQUE VERA"

"Finally, brethern, whatever things are true,

whatever things are noble, whatever things are just,

whatever things are pure, whatever things are lovely,

whatever things are of good report,

if there is any virtue, and if there is anything praiseworthy

—meditate on these things."

Philippians 4:8

(NKJV)

DEDICATION

This thesis is dedicated to my supervisor, Dr. Paul Jelen. During the course of this work he was an encourager, confidante, teacher, and gracious host. In addition he became a good friend. We had many a productive meeting at his second office, the Faculty Club. Unfortunately, I did not learn much Czech while under his direction, but I did gain an appreciation of classical music. Thank-you Dr. J. for your patience in the face of unavoidable circumstances.

ABSTRACT

Growth and lactose hydrolysing characteristics of a common dairy micro-organism, *Lactobacillus delbrueckii* subsp. *bulgaricus* strain 11842 (LB 11842) were determined to enable potential use in a proposed method of lactose hydrolysis by disrupted dairy cultures. LB 11842 was grown aerobically in MRS medium, sonicated to disrupt the organisms, then added to various lactose-containing substrates. The progress of hydrolysis was followed by freezing point changes. Cultures added to 5% lactose test solutions resulted in 20% and 63% of the lactose being hydrolysed after 3 hours at 7° and 51°C, respectively. Enzyme activity was not hindered in 30% lactose solution and the rate was acceptable at 7°C in skim milk. Cultures were sonicated in Na^+ or K^+ buffers at various pH levels, then held at various temperatures, before adding to lactose test solutions. Cultures sonicated in K^+ buffer had higher activity and stability than those in Na^+ buffer; both were the highest at pH 6 and 7. Holding at 61°C for 60 minutes caused 70% loss of activity with K^+ ions; with Na^+ loss of activity was almost complete. As an incidental observation made during the propagation of the organism, there were major differences in growth on two commercial brands of MRS medium, which was also the case with four other strains of *Lactobacillus*. LB 11842 shows promise as a suitable source of β-galactosidase for use in the proposed process as the enzyme showed acceptable activity at a wide temperature range with high thermostability especially in the presence of K^+ ions.

ACKNOWLEDGEMENT

I would like to acknowledge the assistance of many people, without whose help this project would not have been possible. First and foremost, I would like to thank my supervisor, Dr. P. Jelen, who was not always in his office, but who was supportive from start to finish. James Hwang encouraged me to enter Graduate Studies in the first place. Stephanie Aldenrath, Lawrence Roth and the staff at the Food Quality Branch, Alberta Agriculture, introduced me to the "real world" of laboratory research. Dean Bury, a colleague, gave me many good suggestions and insights. Other helpful colleagues were Todor Vasiljevic and Jana Geciova. Jean Bourgois and Dr. George Patocka helped however they could. Also I would like to thank the members of my examining committee, Dr. L. McMullen, Dr. P. Sporns, and Dr. L. McGann whose time and effort in reading my thesis produced suggestions that greatly improved its quality. The support staff of the Department of AFNS, especially Jody Forslund, contributed to making this a gratifying experience.

I express my appreciation to the NSERC Research Network on Lactic Acid Bacteria, administered by the Universite Laval (including Novalait Inc. and Institut Rosell Inc.) and also the Dairy Farmers of Canada and Agriculture and Agrifood Canada for financial support of the overall project which resulted in this investigation.

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Chapter 1

Introduction

1.1: Rationale for studying lactose hydrolysis

Most infants in the different ethnic groups around the world are able to digest lactose. Jenness and Holt (1987) surveyed milk from 31 mammalian species and of all the species studied, human milk had the highest level of lactose at 6.7%, as compared to 4.5% in cow's milk. Even though most human infants can digest a higher level of lactose than other mammals, production of the lactose hydrolysing enzyme, β-galactosidase (β-gal) usually decreases with age so that a majority of individuals over 2 to 4 years cannot digest lactose due to a deficiency in the required enzyme (Shukla 1975). The lactose that is not digested is passed on to the large intestine where it is fermented by intestinal microbes, giving rise to the condition known as lactose intolerance, with the common symptoms of diarrhea and cramps; and resulting health issues such as tissue dehydration and poor calcium absorption (Shukla 1975). Lactose intolerance is prevalent among the adults in most ethnic groups except those of Caucasian origin. If the lactose in dairy products is at least partially hydrolysed into its constituent molecules, glucose and galactose, such products are much easier for the affected people to digest. An inexpensive and uncomplicated method of lactose hydrolysis might encourage increased sales of dairy products in countries where a majority of adults suffer from lactose intolerance. Increased consumption of dairy products would also improve their overall nutrition.

In addition to lactose intolerance, another characteristic of lactose that causes technological difficulties with production of lactose-containing products is its poor solubility resulting in crystallization in concentrated dairy systems. Yet another

characteristic of lactose that may warrant special consideration in some product formulations is its low sweetness. The hydrolysis products, glucose and galactose, are sweeter and more soluble than lactose. These and other issues relating to lactose hydrolysis were reviewed by Yang and Silva (1995), and earlier by Shukla (1975), and others. A simple, inexpensive method of hydrolysis could help the development of more new products based on lactose and more new uses for whey, the solids of which are mainly lactose.

1.2: Lactose hydrolysis methods

Lactose can be hydrolysed by the application of high temperature to a dilute solution in a strong acid, and by β-gal enzymes. The first method is a harsh treatment with several disadvantages for use in food products: adverse flavor effects, product browning and corrosion of equipment, among others. Most currently available enzymatic methods of lactose hydrolysis for use in food products utilize enzymes produced by yeasts or molds; purification of the enzymes makes them quite expensive. For example, a commercial producer of β-gal enzyme for consumer use employs *Kluyveromyces lactis* and *Aspergillus oryzae*, a yeast and a mold respectively (Anon 1996). Enzymatic methods involve soluble, single use enzymes; systems to recover enzymes based on the use of membranes; and immobilized enzymes, in which the enzyme is physically or chemically attached to a solid matrix (Zadow 1984). Soluble enzymes are usually puri-fied and then used only once, which makes their use economically impractical. Immo-bilized enzymes can be used for more than one batch of hydrolysis but the method is quite complex, and therefore again quite expensive. With the membrane-based method of enzyme recovery, contamination and stability of the enzyme are important considerations.

A potentially simple and inexpensive method of lactose hydrolysis proposed by Jelen (1993) involves growing a bacterial culture commonly used in the manufacture of fermented dairy products, separating the culture from the growth media, and disrupting the cells to release the intracellular β–gal enzyme. The whole disrupted culture would then be added to the lactose-containing product, the only purification being the removal of growth medium, and hydrolysis allowed to proceed. With relatively minor capital outlay for equipment, and no expensive enzymes to purchase, this method of lactose hydrolysis could become economically feasible (Bury and Jelen 2000).

1.3: Research objectives

The general objective of the present work was to determine growth characteristics of a common dairy microorganism as a source of β-gal, and lactose hydrolysing characteristics of the sonicated cultures to enable potential use in the proposed method of lactose hydrolysis by disrupted dairy cultures. In particular, the specific objectives were (1) to confirm freezing point depression as a suitable method for following lactose hydrolysis in complex media, such as milk; (2) to determine optimum growth conditions such as time, temperature, and gaseous atmosphere and process characteristics such as time of sonication, and amount of sonicated cell biomass required to produce maximum rate of lactose hydrolysis by the selected microorganism; (3) to determine the stability and activity of the β–gal enzyme from this organism at various pH levels, temperatures and in various ionic environments and lactose-containing substrates; and (4) to compare two brands of commercially-available MRS medium for suitability as a growth medium for maximizing production of the biomass of enzyme-producing species of *Lactobacillus*.

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Chapter 2

Literature Review

2.1: Lactose

2.1.1: General Characteristics

Lactose is a disaccharide consisting of a single molecule of the monosaccharide galactose joined by a β(1→4) glycosidic linkage to a single molecule of glucose (the full scientific name is 4-O-β-D-galactopyranosyl-D-glucopyranose). The major source of lactose is the milk of most (but not all) mammals where it exists in aqueous solution; the concentration depends on the species. Other sources are very rare. When crystallized, lactose generally exists in 2 forms: α-monohydrate (the ordinary commercial form) or β-anhydride. The forms are based on the orientation of the hydroxyl group on carbon 1 of the glucose moiety. Conversion of one form to the other is referred to as mutarotation (Holsinger 1997). An amorphous mixture of α and β, termed "glass", is formed when a solution is dried rapidly. The most familiar crystalline forms of lactose are the prism and tomahawk shapes of α-hydrate. Anhydrous α-lactose can also be produced but it is generally very hygroscopic. When crystallization of lactose takes place above 93.5°C, anhydrous β crystals are formed. The β form is sweeter and more soluble than the α form. Lactose can undergo chemical reactions in which the β(1→4) bond is not broken: reduction of the anomeric carbon of glucose produces lactitol (galactose-sorbitol), isomerization of the glucose moiety can produce lactulose (galactose-fructose), and oxidation of the anomeric carbon of glucose produces lactobionic acid (galactose-gluconic acid) (Holsinger 1988).

The concentration of lactose in human milk is among the highest of all mammals at approximately 7%, while bovine milk contains about 4.8%. This suggests the most common use for lactose, which is the modification of cow's milk for infant formula (Holsinger 1988). Lactose synthesis takes place in the mammary gland. Glucose in the blood is drawn into the mammary cell against a concentration gradient. The glucose then undergoes rearrangements using at least five different enzymes before lactose is formed; α-lactalbumin is involved in the final step. Vesicles containing the lactose are formed and, along with milk proteins, move to the apical surface of the cell. Water is drawn into the vesicle because of the high osmotic pressure of the lactose and other anions. The vesicles fuse with the apical membrane and discharge their contents into the alveolar lumen, and the milk is excreted (Holsinger 1988).

2.1.2: Solubility and crystallization

The two forms of lactose differ in their solubility in aqueous solution. Initial solubility of the α–hydrate form is about 7 g/100 g water at 15°C, and that of the β form is about 50 g/100 g. After mutarotation when equilibrium is reached, the final lactose solubility is about 17 g/100 g, consisting of 63% β and 37% α, whereas the solubility of sucrose is approximately 200 g/100 g; this makes lactose less than one-tenth as soluble as sucrose (Jelen 1985). The low solubility causes crystallization in concentrated dairy products such as sweetened condensed milk, ice cream, and whey cheese; this can cause the defect known as "sandiness". By using β-galactosidase (β-gal) enzyme to hydrolyse the lactose, the concentration can be reduced to a point where crystallization is no longer a problem, since the hydrolysis products glucose and galactose are much more soluble than lactose (the solubility of glucose is 89.6 g/100 g water) (Shukla 1975). As an example of

the result of partial lactose hydrolysis, a whey based spread that contained 17.5% lactose had a tendency to develop sandiness upon storage. When the lactose was hydrolysed to below 12.5%, no crystals were detectable (Patocka and Jelen 1988).

2.1.3: Sweetness

In 10% solutions, the sweetness of lactose is about 1/6 that of sucrose, while the sweetness of galactose and glucose are about 1/3 and 3/4, respectively (Shukla 1975; Zadow 1992). Therefore hydrolysis of the lactose in milk and other dairy products into glucose and galactose will increase the overall sweetness level of the product. Sweetener syrups can be produced from acid and sweet whey after recovering the protein (Shukla 1975). A model system of unflavored ice cream mix sweetened with sucrose was used by Shah and Nickerson (1978) to investigate the relative sweetness of lactose-hydrolysed syrups. Results showed a synergistic effect with sucrose: replacing 25% of the sucrose with 70% hydrolysed lactose syrup resulted in greater sweetness than when the sucrose was replaced with 100% hydrolysed lactose syrup. Therefore it would not be necessary for the lactose to be 100% hydrolysed to achieve the greatest improvement in sweetness.

2.2: Lactose digestion and lactose intolerance

Lactose intolerance is a common intestinal disorder among a majority of the world's ethnic groups. Low levels of lactase produced in the intestine of most adults cause a reduced ability to digest lactose. The lactose that is not absorbed causes the appearance of a variety of unpleasant gastro-intestinal symptoms that detract from the consumption of milk and milk products (Holsinger 1997). The disorder, which is also called "hypo-lactasia" (Toba *et al.* 1981), lactose malabsorption or lactose maldigestion, is prevalent among the adults in most of the world's people groups except those of

Caucasian origin (Shukla 1975). The highest prevalence of lactose intolerance among various racial and ethnic groups reported by Shukla (1975) was 98% among adult Thai's, and 90% among Chinese. There are many more ethnic groups world-wide in which the adults are intolerant to lactose, than those that are tolerant; the tolerant groups therefore represent a minority.

The magnitude of the lactose intolerance problem among different ethnic groups in Nepal was studied by Shah and Jelen (1989). The incidence among households ranged from 12.5% to 68.7%. The overall incidence of lactose intolerance was lower than reports for other Asian populations. The authors conjectured that this may be due to the universally low level of milk consumed by all groups; many households did not drink milk so they were unaware whether they were lactose intolerant or not.

In the intestine, β-gal is produced in the villi of the jejunum, the second of the three units of the small intestine. Lactose intolerant individuals do not produce enough β-gal to break down the lactose molecule, before it can be absorbed by the intestine. There is no chemical or structural difference in the β-gal produced by lactose-tolerant and intolerant individuals, the difference is only in the quantity produced; intolerant individuals produce approximately 5 - 10% the amount of β-gal produced by tolerant individuals (Mustapha *et al* 1997).

After hydrolysis in the jejunum, the constituent molecules, galactose and glucose, are absorbed into the bloodstream and finally reach the liver cells, where metabolization takes place (Shukla 1975). The lactose that is not hydrolysed passes on to the large intestine where fermentation by the intestinal bacteria causes distension and discomfort, leading in some circumstances to diarrhea. Other health problems related to lactose

malabsorption are tissue dehydration and poor calcium absorption (Zadow 1984, Shukla 1975). Lactose-intolerant individuals can often safely consume cheese, which contains almost no lactose (Zadow 1984). Other fermented milk products such as yogurt can usually also be consumed without distress because the live fermentation bacteria survive passage through the stomach and maintain their lactase activity in the duodenum, resulting in improved lactose absorption (Mustapha *et al.* 1997). Thus, not all dairy products cause symptoms of lactose intolerance.

Gastric discomfort after the consumption of dairy products is not necessarily related to lactose intolerance because milk proteins can also cause an allergic response, resulting in similar symptoms. Surveys suggest an incidence of milk protein allergy of one case in 5000 births (Renner 1992), with β-lactoglobulin being the most common allergen. To confirm the presence of lactose intolerance, the "breath hydrogen" test, or alternatively the blood glucose or galactose determination after lactose challenge may be utilized (Zadow 1992).

The principle of the breath hydrogen test is that the lactose that is not digested passes on to the colon where it is hydrolysed by microorganisms, producing hydrogen which diffuses into the blood and then is exhaled through the lungs. These colonic micro-organisms are the only source of hydrogen in the body.

For the blood glucose or galactose test, the patient consumes milk after an over-night fast. Blood samples are checked at set time intervals. If lactose is being digested and the products absorbed, blood glucose or galactose level will rise quickly, but if the patient is a lactose maldigestor, the rise in level will be very slow (Mustapha *et al.* 1997).

2.3: Lactose hydrolysis

2.3.1: Mechanism and methods of lactose hydrolysis

Hydrolysis is defined as "a chemical reaction in which a compound reacts with water to produce other compounds" (Hanks 1986). With lactose hydrolysis in aqueous solutions the molecule is cleaved into its two constituent molecules, galactose and glucose. Figure 2.1 is a schematic drawing of the lactose molecule indicating the bond that is broken during hydrolysis.

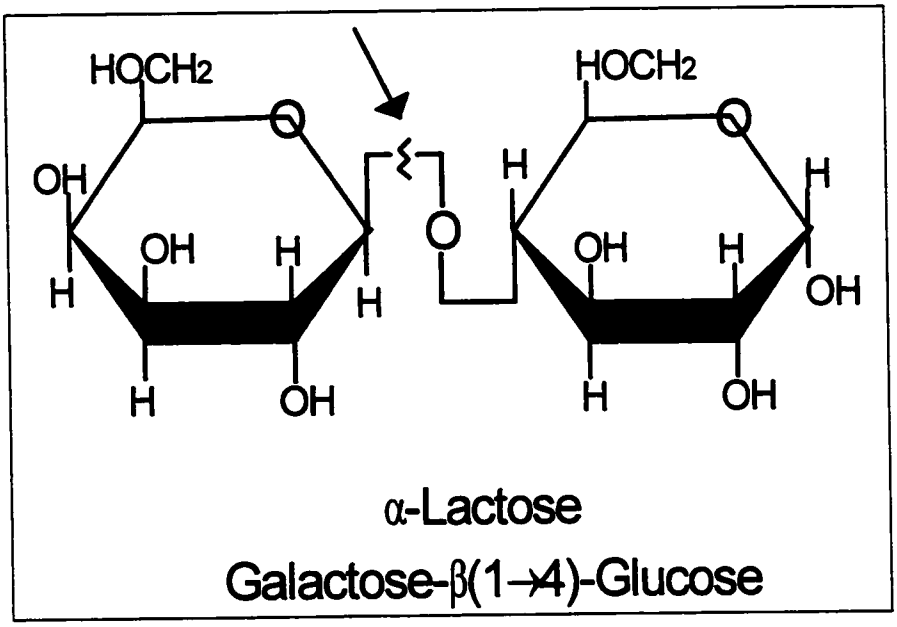

Figure 2.1:
Schematic illustration of the lactose molecule. The bond that is cleaved during hydrolysis is indicated by an arrow.

Hydrolysis of lactose can be achieved in dilute solutions of strong acids at high temperatures. The common method for hydrolysis of other sugars such as sucrose, by milder treatments with organic acids such as citric acid is virtually ineffective with lactose. It is therefore necessary to use stronger mineral acids at a pH level below 2 at

temperatures in the vicinity of 150° C (Zadow 1984). A high degree of hydrolysis is obtained in a short period of time under these conditions; a typical example is 80% hydrolysis in three minutes at pH 1.2 and temperature 150°C (Gekas and Lopez-Leiva 1985). However, this severe treatment may produce secondary products with off-flavors (Riel 1985), and discoloration (Zadow 1984). Other disadvantages of acid hydrolysis are that the method cannot be applied to protein-containing solutions because of denaturation of the protein, and cost of the equipment necessary to resist these chemically aggressive conditions is very high (Gekas and Lopez-Leiva 1985).

Lactose can also be hydrolysed into its constituent molecules by the β-galactosidase enzyme (β-galactoside galactohydrolase, EC 3.2.1.23). When produced in the human intestine the common term 'lactase' is generally used. The proposed enzymatic mechanism involves a two step process of hydrolysis, followed by a transferase reaction. According to Shukla (1975) the active site of a β-galactosidase enzyme contains one SH group and one imidazole group. The first important step in lactose hydrolysis is the formation of an enzyme-galactose complex with liberation of glucose. Second, the enzyme transfers the galactose moiety to an acceptor containing a hydroxyl group (Prenosil *et al.* 1987a). During this process, occasionally the glycosidic bond between galactose and glucose changes from 1→ 4 to 1→ 6 with no prior release of glucose. The product then released is allolactose, which has the same molecular arrangement as lactose, but with a 1→ 6, rather than a 1→ 4 bond.

If the acceptor of the galactosyl moiety from the enzyme complex is water, then galactose is formed and liberated from the active site. However the acceptor can also be one of the other mono- or polysaccharides or alcohols that may be in the solution. Thus

the transferase step may cause the formation of various oligosaccharides (OS) in addition to the normal hydrolysis products. Typical products are di-galactose compounds in which 1→ 6 bonds are most common, but 1→ 2 and 1→ 3 bonds are also formed. Among all OS, 1→ 6 glycosidic bonds are the most frequent (Prenosil *et al.* 1987a; Smart 1993).

2.3.2: Sources of β-galactosidase enzyme

The enzyme β-gal occurs in a wide range of sources. For example, bovine liver, bovine testes, and jack beans are used by a major chemical company (Anon 1993) to manufacture β-gal for laboratory purposes. Shukla (1975) also mentions that β-gal is found in peaches, almonds, alfalfa seed and coffee.

Yeasts and molds are the most common sources of the β-gal enzyme preparations that are available for consumer use. For example "Lactaid®" brand β-gal enzyme in liquid form is obtained from *Kluyveromyces lactis*, a yeast, and "Lactaid®" in tablets from *Aspergillus oryzae*, a conidial fungus (Anon 1996). Another yeast, *Saccharomyces fragilis*, is used by Sigma Chemical Co. to produce β-gal (Anon 1993). According to Shukla (1975) β-gal is also found in *Candida pseudotropicalis, Neurospora craussa*, and *Mucor meihi*.

Enzyme preparations from non-dairy bacterial sources available for laboratory purposes are produced from the organisms *E. coli* and *Diplococcus pneumoniae* (Anon 1993). Other non-dairy sources include *Bacillus megaterium*, other *Bacillus* species and *Thermus aquaticus* (Shukla 1975). No β-gal from dairy bacteria was available from Sigma Chemical Company (Anon 1993).

2.3.3: Lactic acid bacteria as β-galactosidase sources

"Dairy cultures" are bacteria that are commonly used in the dairy industry for production of fermented dairy products such as cheese or yogurt and are, as a rule, species of lactic acid bacteria (LAB), such as *Streptococcus* and *Lactobacillus*. Significant amounts of β-gal are produced by most dairy cultures widely used in the dairy industry, and such cultures are generally recognized as safe (GRAS). Therefore they would be suitable for use in the proposed process of lactose hydrolysis by disrupted bacterial cultures.

Numerous authors have shown that various different dairy organisms produce β-gals with notable hydrolysing ability. Wierzbicki and Kosikowski (1972a) surveyed several mold, yeast and bacterial species grown in whey for their lactose-hydrolysing potential. Of the 8 species of LAB studied, strains of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus (L. bulgaricus)*, and *Lactobacillus helveticus* showed the highest β-gal activity. Kilara and Shahani (1975) studied the β-gal activity of composite yogurt cultures consisting of *L. bulgaricus* and *S. thermophilus*. The β-gal activity per gram of the composite culture was about double the sum of the individual cultures grown separately, suggesting a synergistic effect. Greenberg and Mahoney (1982) purified and characterized the β-gal from four strains of *S. thermophilus*. Strain B-3641 from the USDA Northern Regional Research Laboratory showed the highest production of β-gal among the strains tested. When grown on the same medium for the same incubation period, it showed more than 3 times the activity of the strain with the lowest production. Shah and Jelen (1991) reported about 3.5 times higher β-gal activity

in LB 11842 than in LB 7994, using o-nitrophenyl-β-D-galactopyranoside (ONPG) a lactose analogue as a substrate for hydrolysis.

A preliminary study of lactose hydrolysis with disrupted dairy cultures using six dairy organisms was performed by Hwang (1996). Organisms used were *L. bulgaricus* (2 strains), *L. helveticus* (1 strain), *Lactococcus cremoris* (2 strain), and *S. thermophilus* (1 strain). *Lactobacillus delbrueckii* subsp. *bulgaricus* strain 11842 (LB 11842) had the highest level of β-gal activity at 35°C and at 55°C.

In another preliminary study with disrupted dairy cultures, the lactose hydrolysing abilities of 5 other species of *Lactobacillus* plus LB 11842, were determined after growth on a whey-based medium, fortified with a whey protein concentrate and a yeast extract. The highest enzymatic activity was again obtained with LB 11842. The highest cell counts did not necessarily indicate the highest enzymatic activity (Geciova 1999).

2.4: The characteristics and functions of β-galactosidase enzymes

2.4.1: Properties of β-galactosidases according to source

The enzyme β-galactosidase is not a unique entity, with a single structure; rather characteristics vary widely among the sources. In addition the amounts and the conditions under which β-gal is produced by different species also vary widely. Nevertheless the unifying function is the hydrolysis of lactose and other β-galactosides.

Amino acid sequences of the β-gal from different species show major differences and there is considerable diversity at the DNA sequence level. DeMacias *et al.* (1986) studied the immunological relationships between crude extracts of β-gal from several Lactobacilli and from combined results obtained with different methods, phylogenetic

maps were derived. Nine species of *Lactobacillus* out of the 18 tested produced β-gal with 60% or less cross-reaction with an anti-serum prepared from LB 11842, with one species having only 26% cross reactivity.

The 3-D shape of the β-gal molecule produced by *E. coli* was described by Jacobson *et al.* (1993). Structure consisted of 4 sub-units, each of which had 1023 amino acids, and two bound magnesium ions. The alignment of the amino acid sequence of the *E. coli* enzyme with five homologous β–galactosidases showed major variability in the active site region. Smart *et al.* (1993) used β-gal DNA fragments from *Lactococcus cremoris, S. thermophilus*, and *L. bulgaricus* as probes in hybridization studies. Little or no hybridization was detected between these probes and plasmid or genomic DNA isolated from other species, even though the corresponding enzyme activity was present in those species.

The molecular weights of β-gal from different sources vary widely. Huang *et al.* (1995) estimated the molecular weight (MW) of β-gal produced by *Leuconostoc mesenteroides* to be 80,000 as determined by comparison of SDS-PAGE protein profiles for a lactose-producing parental strain and a non-producing mutant. Itoh *et al.* (1980) purified the β-gal from *L. bulgaricus* B-6 and determined many of its properties. Results indicated that a sulphydryl group was involved in the active site. Molecular weight was estimated to be 195,000; crude and purified enzyme showed the same hydrolytic action on lactose. Smart and Richardson (1987) purified β-gal from a strain of *S. thermophilus*. Three native forms were identified by HPLC, which had MW of 204,000, 186,000 and 282,000; a single subunit with a MW of 116,000 was identified by gel electrophoresis. Ramana Rao and Dutta (1981) purified and determined many of the properties of β-gal

produced by *Streptococcus thermophilus*. Gel filtration indicated a molecular weight of 580,000.

2.4.2: Formation of oligosaccharides during lactose hydrolysis

In the past, OS have been seen as unwanted by-products, because human beings are incapable of digesting them (Shukla 1975). However, recently interest has been shown in the production of OS as Bifidus growth factors (Smart 1993). The number and type of oligosaccharides formed are affected by enzyme source, concentration and nature of substrate (Mahoney 1997), pH, temperature, and inorganic ions (Wierzbicki and Kosikowski 1972c).

During hydrolysis of lactose in acid whey by the β-gal from *A. niger*, Wierzbicki and Kosikowski (1972c) found 5 newly formed OS which accounted for 1 to 2 % of the initial lactose. The amount of OS formed was influenced by substrate concentration and reaction time. The highest rate of production occurred in the first 15 min of hydrolysis at all substrate levels, and the rate increased with increased substrate concentration. This is hardly surprising since the increased concentration of sugars in the solution gives an increased probability that the acceptor molecule with the hydroxyl group will be another sugar, rather than water. The OS were not identified in this work, they were merely numbered as OS-1 to OS-5.

Jeon and Mantha (1984) studied the production of OS by β-gal from *Candida pseudotropicalis* and *K. lactis*, 2 yeasts, in lactose solutions of 5% and 20%. Maximum concentration of OS was achieved at between 45 min and 1 h hydrolysis, at which time they represented about 11.3% of the total lactose for 5% lactose solutions and 16.0% for

20% lactose. The concentration of OS decreased to 5.5% and 10.8%, respectively, after 4 hours of hydrolysis.

Prenosil *et al.* (1987a) also found that the maximum concentration of OS was higher in experiments with a high initial lactose concentration, than with a low initial concentration. Regardless of initial lactose concentration, after about 85% hydrolysis had taken place, the levels of OS in the solutions of all concentrations were very similar, less than 2.5% of total sugar (Prenosil *et al.* 1987b). Temperature, pH, and ionic strength did not have a significant influence on OS formation in their experiments, which is not in agreement with Wierzbicki and Kosikowski (1972c). Nevertheless, the reaction solution consisted of approximately 95% monosaccharides when sufficiently long time for the reaction was allowed (Prenosil *et al.* 1987b).

Similar types of OS were formed by the transferase reactions catalysed by β-gal from various strains of LAB, but differences were observed in the amount and rate of product formation (Garman *et al.* 1996). The rate of production of free galactose increased with time, which indicated that, while transgalactosylation occurred early in the reaction, most of the products formed were subsequently hydrolysed, in agreement with Prenosil *et al.* (1987b).

2.4.3: Lactose transport into bacterial cells

Lactose can be transported into the bacterial cell by two different methods. In most Lactobacilli, lactose is taken up by a specific lactose permease and is hydrolysed by β-gal, prior to phosphorylation of the resulting monosaccharides. In most Streptococci and some Lactobacilli such as *L. casei*, lactose is taken up by the phospho-enol-pyruvate-phospho-transferase system (PEP-PTS), which allows for faster transfer than the per-

mease system. Some *S. lactis* strains have both systems. Lactose phosphate is then formed and subsequently hydrolysed by β-D-phosphogalactoside galactohydrolase (P-β-gal., EC 3.2.1.85)(Kandler 1983; Chassy and Thompson 1983).

The data of Hickey *et al.* (1986) suggest that lactose is transported into the cell by a permease system in *L. bulgaricus* rather than a PEP-PTS system. No evidence for a PEP:lactose PTS system was obtained for the 12 species of *L. bulgaricus, L helveticus* and *L. acidophilus* that were studied. P-β-gal was not detected in the 33 strains of different species of *Leuconostoc* investigated by Huang *et al.* (1995), suggesting that generally the PEP-PTS lactose transport system is not functional in this genus.

2.5: Factors that influence β-galactosidase production and activity

2.5.1: Common factors affecting bacterial enzyme production and activity

The amount of β-gal produced by a bacterial culture and the hydrolysing activity of that enzyme are influenced by a great many factors, such as species and strain of a given microorganism, growth media composition, growth temperature, as well as the point in the growth cycle at which cells are harvested. Important hydrolysis conditions that affect activity are type of substrate, pH, temperature, and concentration of enzyme and substrate. The rate of product inhibition by galactose is another factor which depends on the source of β-gal (Gekas and Lopez-Leiva 1985). Galactose was found to be a weak competitive inhibitor of lactose hydrolysis by the β-gal produced by an unspecified strain of *S. thermophilus* (Smart and Richardson 1987).

The rate of hydrolysis by a given β-gal is affected by the mutarotation of lactose. The β-gal from *E. coli* hydrolyses α-lactose 2 times faster than the β form. When muta-rotation is slower than hydrolysis, the α-form will eventually become exhausted and

mutarotation becomes the rate-limiting step of the process (Huber *et al.* 1981). The work of Shah and Jelen (1990) is an example of the effect of pH and temperature: with LB 11842 the β-gal activity was 2 times higher at pH 7 compared to pH 4.5, and 2 times higher at 55°C compared to 35°C.

The effect of substrate concentration was determined by Wierzbicki and Kosikowski (1972b) using a commercial preparation of β-gal obtained from *A. niger* to survey the rate of lactose hydrolysis in acid whey concentrate containing 3.5 to 36% lactose at pH 4.5. The maximum rate was obtained when the lactose concentration was 21%. When lactose concentration was raised higher than this, the rate of hydrolysis decreased (Wierzbicki and Kosikowski 1972b).

2.5.2: Growth media for propagation of cultures

A commonly used, commercially available growth medium (MRS) for the propagation of LAB was originally formulated by deMan *et al.* (1960). MRS is often used in culture growth experiments in the laboratory because of its convenience and proven ability of supporting excellent growth. However, for pilot plant or industrial scale production of bacterial cultures and their products such as β-galactosidase, MRS would be much too expensive.

In many studies, LAB were grown on media based on whey and other milk by-products and subsequent lactose hydrolysis was tested. If a recipe using these relatively inexpensive products would support high bacterial growth and a high production of β-gal, their usage potential would increase. Greenberg and Mahoney (1982) studied the β-gal produced by *S. thermophilus* B-3641, using growth media consisting of solutions of various combinations of deproteinized whey at two concentrations plus corn steep liquor and

peptone and with or without 2% potassium phosphate. Various growth times ranging from 18 to 36 hours were used. The highest hydrolysing activity was obtained with 28 hours growth in 2% whey with 2% potassium phosphate.

Hajsmanova (1998) compared the growth of LB 11842 on whey-based media supplemented with each of six brands of whey protein concentrate (WPC) or whey protein isolate (WPI). The effect of the WPC's was highly variable, with two WPC's from different manufacturers supporting the highest increase in lactic acid production and cell growth over no supplementation. The WPI's had almost no effect.

An unspecified strain of *L. bulgaricus* was grown by Murad (1998) on milk ultrafiltration (UF) permeate supplemented with various proportions of yeast extract, peptone, other nitrogen sources, and amino acids and sugars. All added amino acids tended to lower the β-gal activity, as did added sugars including glucose and galactose. The nitrogen source added to the basic medium which produced the highest activity was $(NH_4)_2HPO_4$ at 0.4%. The best buffering system and phosphorus source was K_2HPO_4 (1g/L) and KH_2PO_4 (0.5 g/L).

The effect of supplementation of a whey-based growth medium with yeast extract (YE) at rates of 0% to 1% was determined by means of cell counts, acid production and β-gal activity as measured by ONPG. Acid production increased with increasing amount of YE supplementation; the increase in β-gal activity as measured by ONPG was similar with 0.2 to 0.8 % YE added, but total cell count seemed relatively insensitive to the amount of YE added (Bury 2000).

2.5.3: Inducibility of β-galactosidase production in bacteria

In his discussion about genetic control of enzyme synthesis using β-gal as an example, Stryer (1988) stated that β-galactosidase is an inducible enzyme; however he was solely discussing that obtained from *E. coli*. Even though inducibility of β-gal production is the case in most microorganisms, many exceptions have been discovered in recent years. In agreement with Stryer, β-gal activity of 31 strains of *Streptococcus thermophilus* was at least doubled and sometimes up to 120-fold higher in the same strains grown in either lactose or galactose medium compared to that found in cells grown in media containing glucose. If cells were grown in a medium with both glucose and lactose the increase in activity was less. In some strains galactose was even slightly better than lactose as an enzyme inducer (Somkuti and Steinberg 1979).

Of the several strains of *L. casei* studied by Chassy and Thompson (1983), about one-half required growth on lactose for expression of the lactose-hydrolysing genes; the remaining strains had constitutive β-gal activity. Smart *et al.* (1993) found that in general, activity was low or absent in cells grown on glucose compared to lactose among 58 species of LAB and bifidobacteria. However, there were two strains of *S. thermophilus* and two species of *Lactobacillus*, including *L. bulgaricus*, that had up to double the β-gal activity when grown on glucose, rather than lactose; thus these particular strains were constitutive for the lactose-hydrolysing enzyme. One strain of *Bifidobacterium bifidum* showed only slightly better activity when grown on lactose rather than glucose. Table 2.1 shows the results of experiments with the 58 strains grown on lactose or glucose, and associated β-gal and P-β-gal activity. When grown on lactose, β-gal activity was found

Table 2.1:
Growth of selected strains of 6 genera of LAB on media with glucose or lactose and correlation with distribution of β-galactosidase (β-gal) and phospho-β-galactosidase (P-β-gal) enzyme activities. Numbers indicate the number of strains displaying a given characteristic. (Adapted from Smart *et al.* 1993.)

	Growth Substrate	Growth	β-gal	P-β-gal
Lactococci (19 strains)	Glucose	19	0	14
	Lactose	17	2	17
Streptococci (7 strains)	Glucose	7	7	0
	Lactose	7	7	0
Lactobacilli (21 strains)	Glucose	21	7	2
	Lactose	20	15	8
Leuconostocs (3 strains)	Glucose	3	1	0
	Lactose	3	3	1
Pediococci (4 strains)	Glucose	4	1	0
	Lactose	4	4	0
Bifidobacteria (4 strains)	Glucose	4	4	0
	Lactose	4	4	0

in all six genera surveyed, and when grown on glucose, activity was found in 5 of 6 genera. No β-gal activity was found in any *Lactococcus* sp. grown on glucose. P-β-gal activity was restricted to Lactococci, Lactobacilli and one *Leuconostoc* species.

Huang *et al.* (1995) studied the principal characteristics of β-galactosidases from 33 strains belonging to various *Leuconostoc* species. Of 17 Lac^+ strains, 5 had 1.5 to 3 times higher activity when grown in the presence of glucose, than when grown in the presence of lactose; 4 strains showed very low β-gal activity when grown on glucose and up to 50 times higher activity when grown in lactose; the other 8 strains showed activity

between only slightly greater to 2 to 3 times greater when grown on lactose. While studying composite yogurt cultures, Kilara and Shahani (1975) found that β-gal from *L. bulgaricus* showed very little difference in β-gal activity whether grown in the presence or absence of lactose. In addition, Shah and Jelen (1990) found that a strain of *L. bulgaricus,* also isolated from a commercial yogurt sample, showed higher β-gal activity when grown in the presence of glucose, compared to lactose.

It is clear that inducibility is strain-specific; although general statements can be made, each strain must be evaluated separately.

2.5.4: Cations in the hydrolysis solution

Cations in the hydrolysis solution have varying effects on the activity of β-gal, depending mainly on the source of the enzyme and on the substrate being hydrolysed. Using ONPG as substrate,the activity of purified β-gal derived from a particular strain of *S. thermophilus* in the presence of Mg^{2+} was over 50% higher than the control without Mg^{2+} (Somkuti and Steinberg, 1979). In milk, the lactose hydrolysing activity of purified β-gal from *K. fragilis* was inhibited by Na^{+} and Ca^{2+}, and activated by K^{+}, Mg^{2+} and Mn^{2+} (Mahoney and Adamchuk 1980). A strain of *S. thermophilus* produced a β-galactosidase in which there was a synergistic activation with either Mg^{2+} and K^{+} or Mg^{2+} and Na^{+}. Na^{+} was the better activator of ONPG hydrolysis, while K^{+} was the better activator of lactose hydrolysis (Smart and Richardson 1987). Six strains of LAB were studied by Garman *et al.* (1996) to determine the effect of cations on the hydrolysis of lactose by partially purified β-gal. Activity was measured by an enzymatic method showing the increase in glucose level, and was in all cases enhanced by Mg^{2+}, while the effect of K^{+} and Na^{+}

differed between strains.

2.6: Culture disruption for release of β-galactosidase enzyme

2.6.1: Rationale and methods

During bacterial culture growth, lactose and other sugars are transported into the cell, and metabolized by intracellular enzymes. However, for studies of such enzymes including β-gal, disruption of cultures is necessary because the enzyme produced inside the cell must be released in order to provide ready access between enzyme and substrate. This is especially necessary if the enzyme is to be purified or if cell-free studies are to be carried out. Sometimes the cell walls can be merely permeabilized, without totally disrupting the cells (Somkuti and Steinberg 1994). Commonly used methods of disruption are ultra-sound (*i.e.* sonication), homogenization, bead mill, French press and autolysis. A less-common method of grinding with a mortar and glass beads, a variant of the bead mill, was used by Murad (1998) to disrupt an unspecified strain of *L. bulgaricus*. Greenberg and Mahoney (1982) disrupted cells of *S. thermophilus* by the use of lysozyme.

Wierzbicki and Kosikowski (1972a) freeze-dried cell preparations of *A. phoenicis*, *S. fragilis*, and *L. helveticus* as a storage method. Cell preparations were then physically disrupted by air drying at 60°C for 4 hours, French press crushing and sonication; for comparison cells were also chemically disrupted by ethanol, toluene, and various other chemical solvents. Lactose hydrolysing activity was measured by the percentage of available glucose liberated after 5 hours in whey. For *L. helveticus*, activity ranged from 80.0% hydrolysis completion after freeze-drying only, to 97.2% after disruption with toluene. Cultures subjected to French press and sonication both produced 93.2% hydrolysis. While studying another enzyme, lactate dehydrogenase, Ragout *et al.* (1989) used a

French press at –30°C and pressure of 200 kg/cm^2 to disrupt cultures of LB 11842 to release cellular contents.

Somkuti and Steinberg (1994) used several detergents and bile acid preparations to permeabilize the cell walls of *S. thermophilus* ST128, allowing lactose influx and hydrolysis by cytoplasmic β-gal in treated cells, but without inducing enzyme leakage or denaturation. It was found that permeabilized cells could be washed repeatedly with distilled water without the loss of β-gal activity. Whole permeabilized, non-growing cells were then used in preparation of low-lactose milk (Somkuti and Steinberg 1995). Similar to the proposed process of lactose hydrolysis by disrupted dairy cultures, the authors recognized that the β-gal of *S. thermophilus* would need no further purification or isolation from producing organisms to qualify for food-grade status (Somkuti and Steinberg 1995).

2.6.2: Sonication for bacterial cell disruption: Theory

Sonication is the application of high frequency ultra-sound; it is often used in the laboratory for the disruption of bacterial cells to release intracellular enzymes. A probe is immersed in the fluid to be sonicated. Vertical mechanical vibration of the probe at an amplitude of 30 - 300 μm, which varies depending on the diameter of the probe, and at a high frequency of 20 KHz and above causes extremely small bubbles to form and then collapse. This causes extremely high shear forces (high local pressure of perhaps 20,000 atmospheres) plus shock waves of compressions and rarefactions in the vicinity of the probe tip and the fluid being sonicated is submitted to extremely high acoustic pressures. These occurrences are responsible for the production of a phenomenon called cavitation within the fluid. The shock waves produce local heating in the probe tip and in the sonicated fluid; therefore it is necessary to efficiently cool the fluid to protect cellular compo-

nents from denaturation which may occur before complete cellular breakage has taken place (Anon 1983; Patel 1985; Anon 1995b).

Davidson and Rosett (1967) described the theory and technique of the use of ultrasound in biological applications, especially referring to the disruption of cells. The critical place for cell disruption is just below the probe tip; therefore the shape of the container is important in the amount of disruption that will take place. A conical shape is the most efficient because it restricts the volume below the probe tip (Figure 2.2).

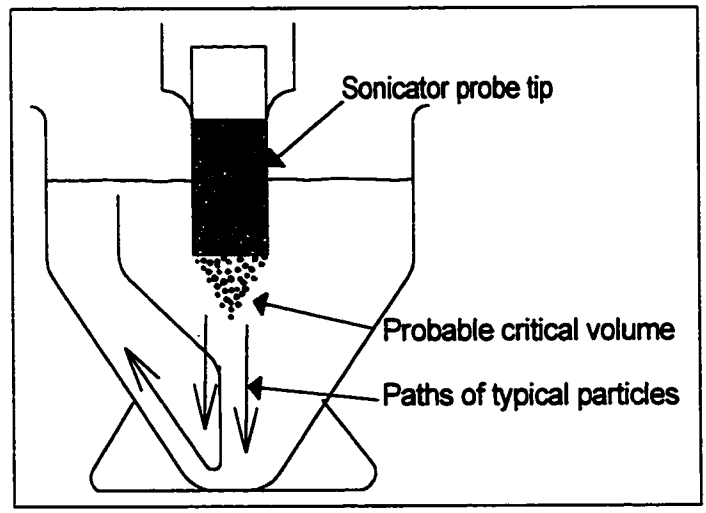

Figure 2.2:
Schematic drawing of sonicator mode of action in a suspension of microorganisms, showing appropriate shape of container. A very small volume, just below the probe tip contains sufficiently high energy to disrupt microorganisms. (Adapted from Davidson and Rosett 1967.)

In addition to disruption of cells, a related effect of sonication is that the cell membrane becomes freely permeable to most of the constituents of the medium in which the cells are suspended (Davidson and Rosett 1967). Thus the activity of intracellular enzymes may increase with sonication even though the cell walls are not totally disrupted, and cells with permeabilized walls may still be viable and may continue to grow after a

prolonged lag phase (Wang *et al.* 1996).

2.6.3: Sonication for bacterial cell disruption: Application

Sonication has been used in various studies for microbial cell disruption. Kilara and Shahani (1975) studied composite yogurt cultures and found that, after sonication, a substantial part of the β-gal was associated with the cell debris; the cell-free medium possessed very little enzyme activity. Sonicated cultures of *L. bulgaricus* showed about 3.8 times the hydrolysing activity of the same cultures that were not sonicated. Shah and Jelen (1990) found that a 4 minute period was the optimum sonication time for cultures of *L. bulgaricus*. Sonicated cultures produced about 5 times greater β-gal activity than un-sonicated ones. Again, a substantial part of the β-gal was associated with the cell debris [which perhaps contained permeabilized cells], rather than the cell free extract for each of 3 LAB. Wang *et al.* (1996) used sonication continuously and intermittently during fermentation runs using *L. bulgaricus* B-5b in milk. High degrees of lactose hydrolysis and high cell viabilities were obtained with intermittent sonication during incubation.

Sonication is suitable for laboratory work (Shah and Jelen 1991), but less so for pilot-plant or commercial scale operations, because the volumes that can be disrupted are too small. Therefore, the efficiency and economic feasibility of other methods of cell disruption including high pressure homogenization and bead milling which can handle larger volumes were evaluated in a recent study (Bury 2000). These two methods appeared equally effective for disruption of LB 11842, whereas sonication was found to be somewhat less effective. Sonication was deemed suitable for bench-top, laboratory

scale work; for commercial, large scale disruption the other two methods appeared preferable.

2.7: Measurement of the hydrolysing ability of β-galactosidase

2.7.1: Measuring lactose hydrolysing ability of β-galactosidase by the use of ONPG

Several substrates in addition to lactose are hydrolysed by β-galactosidase. Among the methods presently available for determining β-gal hydrolysing ability, the most popular is the hydrolysis of the lactose analogue, o-nitrophenyl-β-D-galactoside (ONPG) to o-nitrophenyl (ONP), which causes a color change that can be measured with a spectro-photometer as the product ONP is formed. However, according to Kim *et al.* (1997), if conditions are altered the measured β-gal activity varies markedly according to what substrate is being used and the hydrolysis of lactose does not correlate well with the hydrolysis of other substrates including ONPG, when β-galactosidases from different sources are being compared or when hydrolysis conditions are varied. For example, Toba *et al.* (1981) compared the hydrolysing ability of 14 strains of *Lactobacillus* and 4 strains of *Streptococcus*, using both ONPG and lactose as substrate, while studying OS formation. The ratio of ONPG-hydrolysing activity to lactose-hydrolysing ability of the same β-gal ranged from 4.72 for *S. thermophilus* 510 to 62.56 for *L. helveticus* B-1.

Activity of the purified β-gal from *K. lactis* incubated in lactose solutions was compared with activity when incubated in the presence of other β-galactosides including ONPG (Kim *et al.* 1997). It was found that Co^{2+}, Zn^{2+}, and Ni^{2+} activated the ONPG-hydrolysing activity, whereas the same metals inhibited the lactose-hydrolysing activity; changes in ONPG activity [with change in conditions] were not always consistent with the

changes in lactose activity under the same changes in conditions. This also applied to phosphate concentration and pH level, as well as the above metals (Kim *et al.* 1997). These studies show that the lactose hydrolysing ability cannot be accurately predicted from the ONPG hydrolysing ability.

2.7.2: Measuring lactose hydrolysing ability of β-galactosidase: Other methods

Measurement of enzyme activity can be based on the rate of disappearance of the substrate (*i.*e. decrease in the level of lactose) or appearance of products (*i.e.* increase in the level of glucose or galactose).

High performance liquid chromatography (HPLC) directly measures the amount of sugar in a solution according to size and mobility of the molecule and allows separation of di- from tri-saccharides, but the disaccharides allolactose and galactobiose, formed during hydrolysis, are difficult to distinguish from lactose (Mahoney 1997). This method would be suitable for measuring the lactose level in various lactose solutions. Enzymatic methods usually measure another substance that is in direct proportion to glucose or galactose. For example, with the Glucose HK Assay Kit from Sigma, glucose is phosphorylated, then oxidized to 6-phosphogluconate, during which an equimolar amount of NAD is reduced to NADH, with a consequent increase in absorbance, which is directly proportional to glucose concentration. This method requires prior protein removal (Anon 1995a). Various other methods have been used to determine lactose levels in solution and to follow lactose hydrolysis: chemiluminescence (Rigin 1979), infra-red measurements (Koops *et al.* 1989), reducing sugar determination and a thin-layer chromatographic method (Wierzbicki and Kosikowski 1972a).

2.7.3: Using cryoscopy to measure lactose hydrolysis: Theory

Under atmospheric pressure, pure water freezes at 0°C. If substances are dissolved in water, the freezing point (f.p.) of the solution is lowered in direct proportion to the total concentration of the solution (Chang 1991). Thus, from measurement of f.p. depression (f.p.d.), the total concentration of dissolved ions and small molecules in a solution can be determined. The following formula relates change in temperature of freezing point (ΔT) and molality in a dilute aqueous solution:

$$\Delta T = - 1.858 \times M \times n$$

Formula 2.1

where M = molality, T = temperature (°C), n = # ions into which each molecule dissociates (Jelen 1985; Webster 1985).

The most common industrial use of cryoscopy in dairy laboratories is the determination of whether water has been added to the milk. The f.p. of milk varies within a very small range; the mean is approximately -0.522 ° C (Sherbon 1988). If water is added to the milk the molality will decrease and the f.p. will rise, coming closer to that of pure water. Under normal circumstances, if the f.p. values are lower than the lower limit, abnormal milk is indicated, such as occurs with souring or mastitis. If substances such as lactose and salts which lower the f.p. are added, and at the same time water is added, it is possible to obtain a f.p. equal to the mean (Rattray and Jelen 1996).

In the context of a dairy laboratory, the Hortvet scale rather than the Celsius scale is usually used. In the early 1920's, the milk cryoscope pioneer, Julius Hortvet, determined that 7% and 10% sucrose solutions froze at -0.422 and -0.621° C, respectively

(Sherbon 1988). More recently with more accurate measurement capabilities it has been shown that the figures are closer to -0.408 and -0.600 °C. However the numerical values -0.422 and -0.621 continue to be used as nominal f.p.'s of the above calibrating solutions, with 0 as the f.p. of pure water, giving the Hortvet scale. The scale of a milk cryoscope is usually in minus milli-degrees Hortvet (-m°H), with an error inherent with the machine readings of ± 0.002°H (Anon 1986). The 621 (*i.e.* -0.621°H) milk cryoscope value (Y) equals -0.600°C and since the zeros coincide, the following formula can be derived and used to convert from the scale of the cryoscope to °C, within the limits applicable to milk.

$$Y\ (^{\circ}H) \times 600/621 \times -10^{-3} = {}^{\circ}C. \qquad \text{Formula 2.2}$$

Using cryoscopy as a method to follow lactose hydrolysis relies on the f.p. of the sample being depressed by an amount in proportion to the molal degree of hydrolysis of the lactose (Zadow 1984). Ideally, when one molecule of lactose is hydrolysed, two molecules (1 glucose and 1 galactose) are produced; thus the molal concentration of particles in the solution increases, lowering the f.p. With 100% hydrolysis of an ideal lactose solution, the molality of the solution with respect to lactose would double. Thus if the initial molality is known, the theoretical change in f.p. (Δf.p., °C) for any percent hydrolysis can be calculated using Formula 2.1.

Advantages of the cryoscopic method are that prior removal of protein before estimation of lactose is not required, and it is specific for lactose in the presence of other reducing or nonreducing sugars [assuming no other change in molality is happening at the same time]. Also, it is simple, rapid and requires only a cryoscope (Zarb and Hourigan 1979; Zadow 1984).

2.7.4: Using cryoscopy to measure lactose hydrolysis: Application

In several studies, highly significant linear correlations were found between cryoscopy as a method of following lactose hydrolysis and enzymatic methods. Zarb and Hourigan (1979) measured the f.p. of samples of reconstituted whey and whey with specified amounts of added lactose before and after hydrolysis by β-gal, and found the Δf.p. to be proportional to the initial lactose concentration. Thus a standard graph could be prepared and the initial lactose concentration of a sample ascertained by determining the Δf.p. on hydrolysis.

The cryoscopic method of following lactose hydrolysis was compared with enzymatic methods of following hydrolysis by measurement of glucose and galactose concentrations in two different studies. Both used a commercial preparation of β-gal derived from *K. lactis*. In one study (Nijpels *et al.* 1980), the regression lines of galactose level on Δf.p. for hydrolysis in milk and in UF permeate were very similar, showing highly significant linear relationships with correlation coefficients of 0.983 and 0.994, respectively. The reproducibility of the cryoscopic method was very satisfactory and the precision showed negligible error. Another study (Baer *et al.* 1980) also followed lactose hydrolysis by means of Δf.p. and compared results to those obtained by an enzymatic method. Substrates were acid whey and lactose solutions. Highly significant linear relationships between Δf.p. and % lactose hydrolysed as measured enzymatically were obtained for both substrates ($r=0.985$ and $r=0.998$, respectively).

Jeon and Saunders (1986) compared the cryoscopic method with HPLC while relating oligosaccharide formation to the measurement of lactose hydrolysis in lactose solution, whey permeate, and whole milk. Samples were inoculated with a commercial

source of β-gal derived from *Candida pseudotropicalis* and held at 37 °C for up to 4 h periods. The issue of oligosaccharide formation and cryoscopic measurement of lactose hydrolysis was specifically addressed. Degree of hydrolysis as indicated by Δf.p. was calculated by comparing actual change with the change that would take place with 100% lactose hydrolysiss. Degree of hydrolysis as indicated by HPLC was calculated by comparing the amount of lactose remaining after each time period to the initial amount. Change in f.p. indicated a lower degree of hydrolysis than HPLC. Nevertheless, the degree of linear correlation between the two methods can be calculated from their data; the respective correlation coefficients are 0.9914 (lactose solution), 0.9866 (whey permeate) and 0.9952 (whole milk).

If oligosaccharides are formed during lactose hydrolysis, the relationship of one substrate molecule forming two product molecules will not strictly hold. The formation of other di- and higher oligosaccharides (OS) would counteract the increase in molality that would be caused by two product molecules being formed. Thus the Δf.p. would not be as large as expected. Confirming this theory Jeon and Saunders (1986) found that percent hydrolysis as measured by Δf.p. was less than that as measured by HPLC. Nevertheless, Prenosil *et al.* (1987b) state that at thermodynamic equilibrium, with high initial lactose concentrations, the reaction solution will consist of about 95% monosaccharides glucose and galactose.

The presence of proteolytic and lipolytic activity in a lactose solution being hydrolysed are two factors that could possibly influence the accuracy of f.p. measure-ments as a method of following lactose hydrolysis by disrupted dairy cultures in milk or other complex dairy systems. The actual initial levels of fat, protein or ions are of little conse-

quence to the measurement of lactose hydrolysing activity by cryoscopy, because the important factor is the change in freezing point. If the concentration of these constituents change during hydrolysis such as would occur with the appearance of short peptides or free fatty acids, the method could be compromised. With simple lactose solutions, there would not likely be any change because of the lack of protein or fat in the solution.

With hydrolysis in milk and whey products the changes in f.p. due to change in protein or fat level would be small, compared to the large changes due to lactose hydrolysis. In one study the effect of fat content of milk on f.p.d. of that milk was found to be minimal, since only dissolved solids affect f.p.d. values, and fat is not soluble in water (Chen *et al.* 1996). The percent contribution to the total f.p.d. of farm tank milk was reported as follows: fat, 2.2%; protein, 4.7%; lactose, 53.1%; and ionic level as measured by conductivity, 40.0%. Thus, lactose contributed more than the rest of the factors added together. The f.p.d. of whole milk and that of skim milk made from the same batch were found to be the same (Koops *et al.* 1989). Variations in the f.p.d. of milk were nearly independent of variations in the concentrations of colloidal casein micelles and fat globules (Mitchell 1989).

Another possible influencing factor could be lactic acid production by contaminating bacteria. In simple lactose solutions, there would be very little metabolic activity by viable organisms (e.g. those remaining after culture disruption) or released enzymes other than lactose hydrolysis due to the lack of nutrients and necessary substrates. But with hydrolysis in milk, using the disrupted cultures, as the pH drops due to acid production by the remaining viable organisms, molality would increase. Thus the Δf.p. would be somewhat greater than with lactose hydrolysis alone and the percent hydrolysis may be over-

estimated. However, if hydrolysis conditions, especially temperature, were chosen to minimize the metabolic activity of remaining viable organisms, the effect of acidification could also be minimized. If acidification due to metabolic activities of microorganisms in milk products occurs slowly, the lowering of the f.p. due to acidification and that due to hydrolysis are cumulative. Nijpels *et al.* (1980) suggest following acid production by a titrimetric method, which would be only necessary if hydrolysis conditions permitted a high rate of acid production.

2.8: Utilization of hydrolysed lactose

Solutions in which the lactose is at least partially hydrolysed can be used in the production of a variety of food products for its delicate sweet flavor. Baked goods, candy, ice cream, whey beverages for athletes, soft drinks, and canned fruit are examples of some current or potential uses. Solutions containing hydrolysed lactose can also be further fermented by organisms such as *Saccharomyces cerevisiae* producing various useful by-products. Since lactose is not fermented by a majority of microorganisms, hydrolysis thus increases the range of organisms that can be used (Shukla 1975; Zadow 1983; Gekas and Lopez-Leiva 1985).

The use of hydrolysed lactose in the production of sweetener syrups has been described extensively (Shukla 1975; Zadow 1984; Gekas and Lopez-Leiva 1985). Nutritive syrups can be made by hydrolysing the lactose in whey, which also contains protein. However, presently available lactose hydrolysis processes have poor economic feasibility, which hinders product development; this is the main factor contributing to slow progress, especially because of the strong corn syrup industry in North America (Zadow 1984). Development of an inexpensive hydrolysis method might make lactose-hydrolysed sweet-

ener syrups more economically competitive and thus increase the possible uses for lactose and whey (Bury and Jelen 2000). It might also encourage the development of whole new classes of dairy foods in addition to lactose-hydrolysed milk, especially for consumption by lactose-intolerant individuals, thereby improving their overall nutrition.

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Chapter 3[1]

Lactose hydrolysing ability of sonicated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842

3.1: Introduction

Lactose, the disaccharide 4-O-β-D-galactopyranosyl-D-glucopyranose, is found almost exclusively in milk. The main problem arising from the presence of lactose in food products is the prevalence of "lactose intolerance", common among the adults of many of the world's ethnic groups, which detracts from wider consumption of dairy products. The overall nutrition of the affected groups could be improved with lactose-hydrolysed dairy products.

Sources of the lactose-hydrolysing enzyme, β-galactosidase, (β-D-galactoside galactohydrolase, E.C.3.2.1.23) available commercially include extracts from yeasts, molds and bacterial cultures. The level of purification of enzyme preparations for use in the dairy industry makes them quite expensive. As a result, the cost of lactose-reduced milk is about 80% higher than regular un-hydrolysed milk. If an economically attractive method of hydrolysis were developed, more uses for lactose and dairy products containing lactose could become feasible (Bury and Jelen 2000).

Most bacterial cultures commonly used in the production of fermented dairy products also produce β-galactosidase (β-gal). In an attempt to conceptualize a process

[1] A version of this chapter has been accepted for publication by Le Lait. Kreft ME, Roth L, Jelen P. (Nov. 8, 2000)

for lactose hydrolysis in dairy products which would not require extensive enzyme purification, it was proposed to use a suitable common dairy bacterial culture, disrupt the cells to release the intracellular enzyme and add the disrupted culture to the dairy product with little purification other than removal of the growth medium (Jelen 1993; Shah and Jelen 1991).

The hydrolysis of lactose does not correlate well with the hydrolysis of other related compounds, when working with β-gal from different sources or when data obtained under variable conditions are being compared. Measured β-gal activity can vary markedly according to what substrate is being used (Kim *et al.* 1997; Smart and Richardson 1987). Thus hydrolysis in simple solutions of lactose, the substrate of interest, may be preferable as the indicator of hydrolysing ability rather than the commonly-used o-nitro-phenyl galacto-pyranoside (ONPG).

The progress of hydrolysis in lactose containing solutions may be measured by the rapid, convenient and reliable cryoscopic method. This method has been used in several experimental studies, in which highly significant linear correlations were found between the change in freezing point (Δf.p.) and results from other methods suitable to monitor lactose hydrolysis, including HPLC (Baer *et al.* 1980; Jeon and Saunders 1986; Nijpels *et al.* 1980; Zarb and Hourigan 1979). Substrates used by these authors included milk, ultra-filtration permeate, acid whey, aqueous lactose solutions, and whey with added lactose. More recently, Kreft and Jelen (2000) established highly significant linear correlations between freezing point depression and lactose and glucose levels during lactose hydrolysis using milk as a substrate and disrupted dairy bacterial cultures as a source of β-gal enzyme.

Previous works have shown that *Lactobacillus delbrueckii* subsp. *bulgaricus*, strain 11842 (LB 11842), a common dairy organism, as well as other strains of this species produce β-gal with notable lactose hydrolysing ability (Hickey *et al.* 1986; Shah and Jelen 1990, 1991; Smart *et al.* 1993; Wierzbicki and Kosikowski 1972a). However, optimal conditions for maximization of the β-gal production by LB 11842 or for lactose hydrolysis by the disrupted cultures have not been thoroughly studied.

The specific objectives of this study were (a) to determine the effect of various growth conditions for maximum β-gal production by LB 11842 and (b) to investigate the effectiveness of lactose hydrolysis by β-gal from LB 11842 under various process conditions, after sonication of the cultures, in various lactose-containing test solutions as monitored by f.p. changes. The test solutions included pH 7 buffered 5% lactose solution, 30% lactose, 30% whey permeate solutions and milk.

3.2: Materials and Methods

3.2.1: Organisms and media

Cultures of LB 11842 were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. Cultures were maintained frozen at -20°C, and revived in sterile skim milk. At least 3 successive daily transfers in the basic growth medium, a commercial brand of MRS broth (deMan *et al.* 1960), were made before actual experimental runs were carried out. Since β-gal is constitutively produced in LB 11842 regardless of the presence or absence of lactose in the medium (Kilara and Shahani 1975), the absence of lactose in MRS was not considered a problem. Cultures were grown anaerobically or aerobically, as per the experimental design. The anaerobic atmos-phere was attained by means of BBL GasPak® anaerobic jars with disposable H_2 and CO_2 generator

envelopes (Becton Dickinson and Company, Cockeysville, Maryland). All organisms were grown as static cultures for 18 or 24 hours, or both, at 39° C, as specified.

3.2.2: Culture treatment, cell disruption and viable counts

Growth was stopped by cooling cultures in ice water and growth medium was removed by separating the biomass by centrifugation with an International Clinical Centri-fuge, Model CL 1572C, (International Equipment Co., Boston, Mass.) at 1700 x *g* for 20 minutes, and decanting the growth medium. The harvested cells were not washed, as the basic measure of hydrolysis was the change in the freezing point (Δf.p.) of the test solu-tion, resulting from the hydrolysis. In the initial experiments, the cultures were immed-iately resuspended in chilled 0.1% peptone water or distilled water, equal in volume to the removed growth medium. In later experiments, the cultures were resuspended directly in the specified test solution, in most cases 5% lactose in Fisher brand pH 7 potassium-sodium phosphate buffer (0.05 M).

For every replication in this whole study, the procedure was repeated from the growth of the culture to hydrolysis. Thus, each replication was carried out with freshly grown cells; therefore possible deterioration during storage was not a confounding factor. The number of replicates varied according to the experimental design, but was always at least two. After growth, cell preparations were kept chilled at all stages except during the 20 minutes centrifugation.

Cellular disruption was achieved by sonication with a Braun-Sonic 2000 sonicator (Ultrasonic Power Corp., Freeport, Ill.) using a 19 mm diameter probe and output setting of 75 watts. Cultures resuspended in water or test solutions were sonicated in an ice water bath, initially for times varying between 2 and 12 minutes. A 4 minute treatment

was found to give optimum results, based on the greatest Δf.p. after 1 h hydrolysis in skim milk, and was used for the rest of the study.

Viable plate counts were obtained using standard serial dilution techniques with sterile 0.1% peptone (Bacto-peptone, Difco Laboratories, Detroit, MI) in distilled water. Pour plates were made with MRS broth plus 1.5% agar prepared according to the manufacturer's instructions. Plates were counted after 3 days at 39°C.

3.2.3: Lactose Hydrolysis

The disrupted cultures were added to the buffered lactose test solutions, in a 1:1 ratio unless specified otherwise. Thus, when the sonication liquid was water, the final concentration of the lactose solution was 2.5%. Commercial skim and 2% fat milk were also used in some experiments. Additionally, the effectiveness of hydrolysis in 30% lactose and 30% whey permeate solutions was evaluated. The initial f.p. reading was taken immediately after addition of the sonicated culture, and the mixtures were held at a given temperature, as specified by the experimental design; for most of the experiments 7, 25 or 51°C were used.

Freezing point measurements were taken with an Advanced Cryomatic Milk Cryoscope model 4C2, (Advanced Instruments Inc., Norwood, Mass.). Theoretical percent hydrolysis was calculated from the following formula, derived from Formulas 2.1 and 2.2.

$$\text{Percent hydrolysis} = \frac{f.p._{.t} - f.p._{.o}}{1923 \times M} \times 100\% \qquad \text{Formula 3.1}$$

Where $f.p._{.t}$ = freezing point at time t, $f.p._{.o}$ = initial freezing point, and M = initial molality; freezing point measured in cryoscope readings (-m°H).

(It was assumed that there were no other reactions occuring that were influencing the solution molality.)

Initially, the effectiveness of hydrolysis was followed by measuring the f.p. before and after 1 hour under the given conditions. Later, f.p. was measured at approximately 15 minute intervals for the first hour, then at 30 minute intervals for at least a total three to four hour period. All runs with a specified set of conditions were replicated at least twice (sometimes up to 5 times).

3.2.4: Effect of biomass concentration and hydrolysis temperature

The mass of cells produced with 100 mL of MRS broth was, on average, 1 g on a wet basis. For this series, the concentrated pellet from 100 mL of growth medium was sonicated directly in 100 mL of 5% lactose solution or skim milk (rather than water as previously), giving 1% cell concentration (wet weight /volume). In comparison, the concentration of sonicated cells in previous experiments was approximately 0.5%, with 2.5% final lactose concentration. Appropriate volumes of 5% lactose solution or skim milk containing the sonicated cells were added to volumes of solution or milk with no organisms, to give final dilutions of cells ranging from 0.05% to 1%, and the mixtures were held at 51, 25 and 7 °C. A final dilution of 2% cells was obtained by sonicating the pellet from 100 mL of growth medium in 50 mL of lactose solution or milk. Freezing points were measured for at least 3 hours.

3.2.5: Hydrolysis in concentrated lactose and permeate solutions

As an initial step in determining the lactose concentration producing the optimum rate of hydrolysis with a given amount of culture, the β-gal activity of sonicated cultures of LB 11842 was determined in solutions of 30% lactose in pH 7 buffer, and 30% whey

permeate, reconstituted from an industrial permeate powder (Maple Leaf Foods International, Toronto, Ont.). The usual 5% lactose solution is very near the lactose concentration in milk, which is 4.8% on the average. Since growth and preparation of cultures, including disruption is time consuming, it was speculated that cultures could be concentrated to this ideal concentration and then hydrolysed. Cultures were added to both solutions at a rate of 1%, sonicated in these highly concentrated solutions and held at 25 and 51° C. To bring the solution within the range of the cryoscope, which was calibrated to measure the f.p. of milk, one mL was withdrawn and diluted with 5 mL of distilled water.

The mean and standard deviations were calculated for each set of replicates. The t-test for difference in means (Pellissier 1996) was performed where appropriate. Initial reaction rate was taken as the rate of change of freezing point ($m^{o}H\ min^{-1}$) during the first 15 minutes of hydrolysis.

3.3: Results and discussion

3.3.1: Controls and preliminary tests

To verify the correlation between Δf.p and lactose hydrolysis as well as the variability of the monitoring procedure in our conditions, a commercially available preparation of β–gal enzyme in liquid form (Lactaid® drops, McNeil Consumer Products, Guelph, Ont.) was added at the rate of 0.2 mL of enzyme preparation to 250 mL 2% fat milk, an addition rate similar to the manufacturer's recommended rate of 0.6 mL enzyme for con-version of all the lactose in 1 L milk in 24 hours at 6°C. Mixtures were held at 3, 7, and 25° C. After 3 hours hydrolysis, mixtures held at 3° and 7°C showed 48% hydrolysis, and those incubated at 25°C showed 74% hydrolysis (data not shown). The

Δf.p. values indicated a steady increase in the molality of the solution, with the reproducibility between duplicate readings being within ± 0.002 m°H.

Cultures sonicated in distilled water and added immediately to 5% solutions of sucrose, fructose, and glucose, and held at 7° C and 51° C produced no Δf.p. over 24 hours, whereas the same cultures added to lactose solutions resulted in a Δf.p. equivalent to 60% hydrolysis at 7° C; 85% hydrolysis was reached in lactose solutions within 5 hours at 51° C. According to Bergey's Manual (Kandler and Weiss 1986), *Lactobacillus delbrueckii* subsp. *bulgaricus* does not ferment sucrose, but does ferment glucose and fructose, when the temperature is conducive to its growth. Some strains can tolerate temperatures as high as 52°C but Lactobacilli growing above 55°C are as yet unknown. Thus the Δf.p. due to microbial or enzymatic breakdown of sugars other than lactose was deemed to be negligible for LB 11842.

Cultures sonicated in distilled water and then added to commercially available 2% fat lactose-hydrolysed milk at the ratio of 1:1 showed only relatively small changes in f.p. when held at 7, 25 or 51° C for 3 hours, compared to the same cultures held in regular unhydrolysed 2% fat milk at the same temperatures. For cultures in the lactose-hydrolysed milk the mean Δf.p. for two replicates was 4.9 ± 1.3, 13.0 ± 0.7, and 34.4 ± 7.3 m°H (± 1 st. dev.) respectively, and for unhydrolysed milk the respective changes were 40 ± 4, 69 ± 7, and 145 ± 13 m°H, indicating a theoretical percent hydrolysis of over 99%, for the milk held at 51°C. It is claimed by the manufacturer of the lactose-hydrolysed milk that the level of hydrolysis is 99%. Thus virtually no lactose would be available to be hydrolysed by the free enzyme, but any microbes not disrupted by sonication could possibly grow and/or keep producing lactic acid in the stationary

stage, using the available glucose and other necessary nutrients. This might be the cause of the Δf.p. in the lactose-hydrolysed milk.

The f.p. of plain skim milk and plain lactose solution, with no sonicated cultures added, showed no change over 24 hours when held at 51°C; it can be concluded that there was no change in molality due to evaporation or uncontrolled microbial activity. All these results showed that the Δf.p. due to biochemical changes other than lactose hydrolysis by the free β-gal was negligible compared to that caused by the lactose hydrolysis, when hydrolysis conditions were chosen to inhibit microbial growth.

3.3.2: Determining conditions for optimum hydrolysis

The highest levels of hydrolysis as measured by Δf.p. after 1 hour incubation at various temperatures were reached with either 4 or 8 minutes sonication. However, a decline in level of hydrolysis with more than 4 minutes sonication was noticed in some cases, therefore a 4 minute interval was selected as overall optimum sonication time (data not shown).

Four culture growth times between 18 and 24 hours, tested in order to assess the effect of age of cultures within this time span, indicated that there was little difference in hydrolysing ability of cultures from these growth phases. Thus, subsequent experiments were generally carried out with growth time of 18 hours.

Viable plate counts for cultures sonicated in lactose solutions buffered to pH 7, and then held for 3 hours at 51°C, had no colonies even when only diluted $1:10^3$, indicating at least a 5 log cycle reduction in the number of viable bacteria under these conditions. For cultures sonicated in milk and then held at 51°C, in all cases there was a decrease in viable count of up to 10 fold after 3 hours; in no case did the viable count

increase. Thus, an added advantage of using this relatively high temperature for the hydrolysis was that the growth of remaining, viable cells was effectively eliminated.

The genus *Lactobacillus* is considered to be microaerophilic (Kandler and Weiss 1986). To assess the effect of gaseous atmosphere during growth on subsequent hydrolysing ability, 5 separate cultures were grown aerobically and 7 were grown anaerobically in MRS broth (Table 3.1). After 4 minutes sonication, followed by 1 hour hydrolysis in 2.5 % lactose in pH 7 buffer at 51°C, the mean Δf.p. produced by the aerobically grown cultures was higher, though not significantly different than those grown anaerobically. There was also no significant difference between the means of the plate counts before sonication. Thus, all following experiments were carried out with aerobic growth, because the methodology is simpler, faster and less expensive than anaerobic growth.

Table 3.1:
Effect of sonication and gaseous atmosphere on growth and subsequent hydrolysing ability of cultures of LB 11842 sonicated 4 min, followed by 1 hr in 2.5 % lactose in pH 7 buffer. (Viable count in cfu mL^{-1})

	Aerobic (n=5)	Anaerobic (n=7)	p-value
log (viable count) (Before Sonication)	8.67 ±0.12	8.64 ±0.12	0.369
log (viable count) (After Sonication)	7.69 ±0.21	7.77 ±0.49	0.373
Δf.p. (m°H)	78.8 ±6.7	72.4 ±13.0	0.149

As a measure of the effectiveness of sonication, viable plate counts were also obtained after 4 minutes sonication in the above experimental trials, as shown in Table 3.1. The average reduction in count was 0.97 log cycles for the aerobically grown cultures and 0.90 log cycles for the anaerobically grown cultures. The average log count reduction observed throughout the course of this project, for 44 separate cultures, was 1.62 log cycles. A greater reduction would not measurably change the amount of released enzyme; however, metabolic activities of the high number of bacteria still remaining alive could compromise the monitoring procedure, if conditions conducive to growth of the bacteria remaining viable were to be used.

3.3.3: Effect of biomass concentration and hydrolysis temperature

The effects of concentration of enzyme-containing biomass on the progress of lactose hydrolysis in 5% lactose solution and skim milk are shown in Table 3.2 and Table 3.3, respectively. Initial reaction rate was determined as the difference between the initial f.p. determination at the start of hydrolysis and the f.p. after the first 15 minutes divided by the time. As expected, the level of hydrolysis achieved after 3 h increased with the the sonicated cell concentration (wet weight/volume) in both substrates at the 3 temperatures of 7, 25 and 51 C. For 7 and 51°C, the initial reaction rates appeared roughly proportional to the enzyme concentration except for the 2% addition rate, where the increase over the 1% rate was small. For both substrates at 25°C the initial reaction rates at 2% enzyme addition rate were very close to twice the 1% addition rate.

Table 3.2

Initial reaction rates (initial rate of change of freezing point) and calculated degree of hydrolysis reached after 3 hr with different proportions of sonicated cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 in 5% lactose solution at 3 different hydrolysis temperatures, as measured by change in freezing point. Each data point is the mean of 2 replicates.

5% Lactose solution						
	Cell Concentration					
	2%	1%	0.50%	0.25%	0.10%	0.05%
°C	Initial Reaction Rate, $m^{\circ}H\ min^{-1}$ ± 1 standard deviation					
7	1.19 ±0.61	0.75 ±0.22	0.33 ±0.10	0.15 ±0.05	0.04 ±0.01	0.02 ±0.01
25	1.81 ±0.04	0.99 ±0.03	0.65 ±0.02	0.32 ±0.02	0.12 ±0.05	0.07 ±0.01
51	4.05 ±0.31	3.22 ±0.26	1.90 ±0.15	1.00 ±0.08	0.49 ±0.06	0.24 ±0.01
°C	Degree of hydrolysis at 3 hr (%) ± 1 standard deviation					
7	28.20 ±3.69	20.13 ±3.05	13.24 ±2.21	6.68 ±0.35	2.82 ±0.39	1.41 ±0.54
25	44.83 ±1.57	34.89 ±2.05	26.35 ±0.64	16.17 ±0.44	7.79 ±0.53	4.49 ±0.20
51	74.68 ±2.36	63.00 ±1.76	49.96 ±1.22	36.78 ±1.35	21.81 ±1.02	11.81 ±0.79

Table 3.3:

Initial reaction rates (initial rate of change of freezing point) and calculated degree of hydrolysis reached after 3 hr with different proportions of sonicated cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 in skim milk at 3 different hydrolysis temperatures, as measured by change in freezing point. Each data point is the mean of 2 replicates.

Skim Milk						
	Cell Concentration					
	2%	1%	0.50%	0.25%	0.10%	0.05%
°C	Initial Reaction Rate, $m^oH\ min^{-1}$ ± 1 standard deviation					
7	1.05 ±0.38	0.90 ±0.23	0.40 ±0.05	0.19 ±0.08	0.12 ±0.01	0.05 ±0.07
25	2.39 ±0.30	1.18 ±0.30	0.68 ±0.10	0.34 ±0.01	0.16 ±0.05	0.08 ±0.04
51	5.42 ±0.34	3.58 ±0.45	1.90 ±0.05	0.89 ±0.02	0.44 ±0.10	0.13 ±0.03
°C	Degree of hydrolysis at 3 hr (%) ± 1 standard deviation					
7	32.98 ±3.39	22.16 ±3.29	14.68 ±0.94	7.80 ±0.47	4.75 ±0.98	2.82 ±1.45
25	61.11 ±2.74	43.80 ±0.82	30.57 ±0.03	19.40 ±0.64	9.02 ±0.13	5.20 ±0.68
51	97.00 ±3.03	87.23 ±4.45	69.52 ±4.68	47.12 ±1.22	25.40 ±0.74	12.89 ±0.29

Figure 3.1 illustrates the level of hydrolysis achieved by 1% sonicated culture in skim milk and in 5% lactose at the above mentioned 3 temperatures. The level of hydrolysis reached after 3 hours at 51°C was approximately twice the level reached after 3 hours at 25°C, and 4 times the level reached after 3 hours at 7°C. Changes in f.p. produced by sonicated organisms in skim milk were higher than in 5% lactose solution. This is perhaps due to the stabilizing effect of the other proteins present in milk. For 25° C

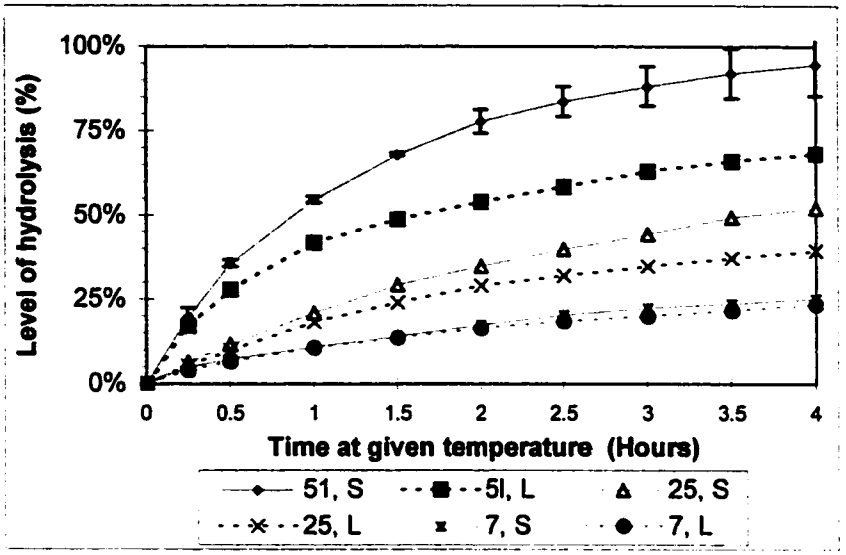

Figure 3.1
Lactose hydrolysis by 1% sonicated cultures of *Lactobacillus delbrueckii*, subsp. *bulgaricus* 11842 in pH 7 buffer containing 5% lactose (L) and in skim milk (S) at 51, 25 and 7° C. Where shown, error bars indicate ± 1 st. dev. (n = 2) for a given set of replicates; otherwise, they are approximately the height of the data marker point.

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and 51°C, both the initial reaction rate and the calculated degree of hydrolysis at 3 hr were up to 40% higher in skim milk, whereas at 7°C the values for skim milk were up to 15% higher. This could indicate some lactic acid production in the skim milk by the remaining viable bacteria not killed by the sonication procedure, thus making the f.p. determination less suitable under these conditions.

3.3.4: Hydrolysis in concentrated lactose and permeate solutions

Results for hydrolysis in a 30% lactose solution at 51 and 25 °C are given in Figure 3.2 in both theoretical percent hydrolysis of the 30% solution, and as a calculated change in cryoscope readings for the undiluted solution. For 30% lactose, 100% hydrolysis would give a theoretical Δf.p. of 1680 m°H. There is a clear upward trend in

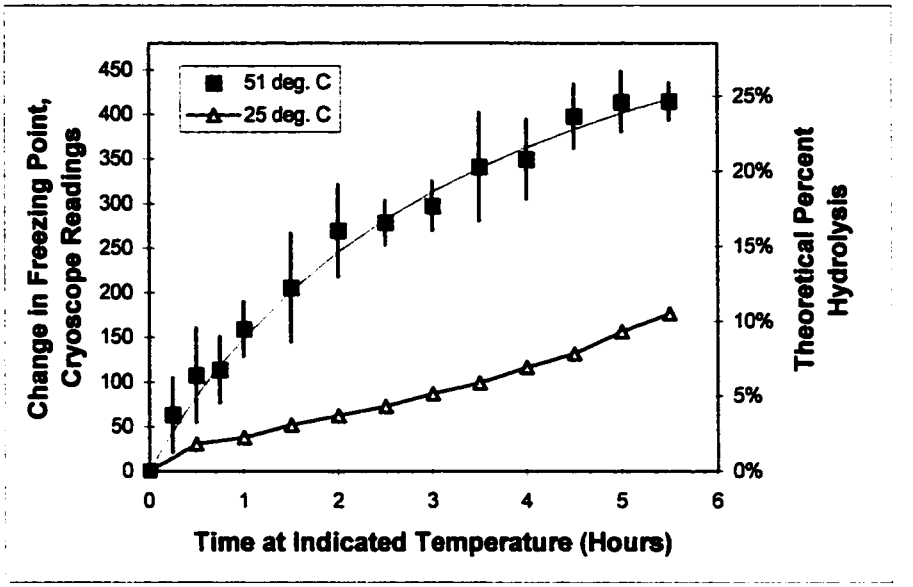

Figure 3.2:
Lactose hydrolysis by sonicated cultures of *Lactobacillus delbrueckii*, subsp. *bulgaricus* 11842 in 30% lactose solution at two temperatures. Where shown, error bars indicate ± 1 st. dev. for each set of 5 replicates; otherwise they are approximately the size of the data marker point.

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theoretical percent hydrolysis of the concentrated lactose solution, reaching 18% after 3 hr. However, the standard deviations between replicates at 51 °C were much greater than seen throughout this work in hydrolysis of a 5% solution, possibly due to interference caused by production of oligosaccharides, which occurs to a greater extent at higher concentrations and at higher temperatures (Prenosil *et al.* 1987). Results for hydrolysis in 30% whey permeate were very similar to hydrolysis in 30% lactose, though slightly higher (data not shown). Enzyme activity did not appear to be hindered by this high lactose concentration.

Comparing the hydrolysis by 1% sonicated cells in 5% and in 30% solutions, the initial reaction rate was very similar at the two concentrations (0.99 and 1.03 $m^{o}H\ min^{-1}$,

respectively) at 25°C, whereas in the 30% lactose solution at 51 °C a higher initial rate of change in f.p. (3.2 vs. 4.2 m°H min^{-1}, respectively) was observed. The average Δf.p. after 3 hr hydrolysis was about 68% higher in the 30% lactose solution than in the 5% solution at 51°C (297.2 and 177.0 m°H, resp). With a 6-fold increase in lactose concentration and only 68% activity increase as measured at 3 h, a lactose concentration of 30% could be near the concentration that produces maximum activity with the present amount of enzyme. This is similar to the maximum rate of hydrolysis in acid whey with β-gal from *Aspergillus niger* obtained when lactose concentration was 21% (Wierzbicki and Kosikowski 1972b).

3.4. Conclusions

Cryoscopy was used to evaluate the effectiveness of the β-gal in sonicated cultures of LB 11842 for lactose hydrolysis using a well defined, simple test system consisting of pH 7 buffered 5% lactose solution. All these results showed that the Δf.p. due to biochemical changes other than lactose hydrolysis by the free β-gal was negligible compared to that caused by the lactose hydrolysis, when hydrolysis conditions were chosen to inhibit microbial growth.

Growth conditions of 18 to 24 hr aerobic growth at 39 °C produced cultures of LB 11842 with good hydrolysing ability in 5% lactose test solutions. Optimum process condi-tions included 4 minutes sonication and a sonicated cell addition rate of 1%. No survival of microorganisms or metabolic activity was detected in the lactose test solutions at the hydrolysis temperature of 51°C. The progress of hydrolysis in milk may have been con-founded by the metabolic activities due to the presence of the viable bacteria surviving sonication. Initial reaction rate of lactose hydrolysis, achieved at the low

temperature of 7°C despite the organism being thermophilic, was about 4 times slower than that at 51°C. Hydrolysis did not appear to be hindered by the high lactose concentration of 30%, but the increased variability of the experimental results might indicate interference by oligo-sac-charide synthesis. Aerobically-grown cultures of LB 11842 after sonication demon-strated good hydrolysing ability on various lactose-containing substrates, and over a wide range of temperatures.

3.5. References

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Chapter 4[1]:

Stability and activity of β-galactosidase in sonicated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 as affected by temperature and ionic environments

4.1: Introduction

The presence of lactose, commonly called milk sugar, in dairy products often leads to the condition known as "lactose intolerance", the main cause of low consumption of dairy products by the adults of many of the world's ethnic groups (Shukla 1975). The products of lactose hydrolysis, the monosaccharides galactose and galactose, are much easier for such people to digest. Currently available lactose hydrolysis methods tend to be rather expensive and prone to technical difficulties. If an inexpensive method of hydrolysis were developed, consumption of lactose and lactose containing dairy products could become more widespread, thus improving the overall nutrition of the affected groups.

A simple and potentially economically feasible approach to lactose hydrolysis (Bury and Jelen 2000) could involve growing and harvesting a suitable dairy bacterial culture, disrupting the cells to release the intra-cellular β-galactosidase (β-gal) enzyme, and using the disrupted culture as the source of the enzyme. With this method based on "in-house" preparation of the crude enzyme, the cost of producing a lactose-hydrolysed dairy product might be reduced.

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[1] A version of this chapter has been published in Journal of Food Science. Kreft ME, Jelen P. 2000. 65, 1364-1368.

Lactobacillus delbrueckii subsp. *bulgaricus* strain 11842 (LB 11842) is known to be a high producer of β–gal, and the lactose hydrolysing ability of its sonicated cultures has been ascertained (Shah and Jelen 1991) . The temperature optimum of LB 11842 β-gal enzyme was determined to be 55°C, using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate (Shah and Jelen 1991). A temperature of hydrolysis higher than the optimum might lead to rapid inactivation of the enzyme.

Various cations present in the solution have a wide range of activating or inhibiting effects on the hydrolysis. In 1957, Cohn reviewed information available regarding what is now called inducibility, using the β-galactosidase (β-gal) from *E. coli* as an example. For hydrolysis of ONPG, Na^+ showed an activating effect, whereas with lactose, the effect of K^+ was more pronounced.

The effect of monovalent cations (K^+ or Na^+) varies with enzymes from different sources. For the enzyme from strains of *S. thermophilus* and ONPG as substrate, no effect was found in one case (Somkuti and Steinberg 1979), and a slight activation in another (Rao and Dutta 1981). Greenberg and Mahoney (1982) found the highest activity with K^+ in the presence of Mn^{2+} . Enzyme from *Bacillus* TA-11 showed no change in activity with addition of K^+ or Na^+, when incubated with ONPG (Choi *et al.* 1995). Mahoney and Adamchuk (1980) and Bernal and Jelen (1985) both found that Na^+ was inhibitory whereas K^+ was activating when lactose was hydrolysed by β-gal from *Kluyveromyces fragilis* or *K. lactis*, resp.

Garman *et al.* (1996) studied 6 species of lactic acid bacteria. In all cases Mg^{2+} had an enhancing effect which varied from slight to pronounced for hydrolysis of lactose. Na^+ and K^+ both enhanced activity; in 3 cases Na^+ was the better activator, and in 3 cases

K^+ had a greater effect. Similarly, the presence or absence of Mg^{2+} in solution with ONPG was shown to have different effects; the β-gal enzyme from *Lactobacillus bulgaricus* was slightly inhibited (Itoh *et al.* 1980), whereas that from *L. acidophilus* was stimulated (Bhowmik *et al.* 1987) and *L. kefiranofaciens* showed no effect (Itoh *et al.* 1992). Mg^{2+} had no effect on β-gal from *Thermus aquaticus* (Ulrich *et al.* 1972), but for *E. coli*, Mg^{2+} was necessary for activity (Strom *et al.* 1971).

Various studies have illustrated the importance of the substrate for the activity enhancement of the individual ions. With lactose, β-gal from *Streptococcus thermophilus* showed greater activation with Mg^{2+} and K^+ than with Mg^{2+} and Na^+, whereas with ONPG, Mg^{2+} and Na^+ showed greater activation than Mg^{2+} and K^+ (Smart and Richardson 1987). β-gal from one strain of *K. lactis* was activated by Mg^{2+} and Mn^{2+} with both substrates but activation with ONPG was much greater (Kim *et al.* 1997). One strain of *E. coli* produced enzyme that required Mg^{2+} for activity when tested on ONPG, whereas with lactose there was no such requirement (Reithal and Kim 1960).

In general, the hydrolysis of lactose does not correlate well with the hydrolysis of other related compounds, such as ONPG, when β-gal enzymes from different sources are being compared or when hydrolysis conditions are varied. Measured β-gal activity itself without any activators or inhibitors varied markedly according to what substrate was used (Kim *et al.* 1997; Smart and Richardson 1987); thus, using model lactose solutions in assessment of β-gal activity may be preferable to the use of ONPG.

Highly significant linear correlations were reported between cryoscopy, *i.e.* measurements of the change in freezing point (Δf.p.) as a result of lactose hydrolysis, and enzymatic methods used for determination of lactose disappearance and/or appearance of

the hydrolysis products glucose and/or galactose. The cryoscopic method was described as simple, fast and reliable (Nijpels *et al.* 1980; Baer *et al.* 1980; Zarb and Hourigan 1979). Various substrates used by these authors included milk, ultra-filtration permeate, acid whey, aqueous lactose solutions, and whey with added lactose. However, the suitability of the cryoscopic method for use with crude enzyme preparations was not ascertained.

The objectives of the present work were: (1) to confirm cryoscopy as a suitable method for monitoring the hydrolysing ability of the disrupted cultures in complex systems such as milk, (2) to determine the effect of the presence of Na^+ and K^+ ions on the stability of the β-gal in whole sonicated cultures of LB 11842 when held at various temperature-pH level combinations before hydrolysis, and (3) to study the effect of these holding conditions on subsequent residual enzymatic activity.

4.2: Materials and methods

4.2.1: Cultures and media

In all experiments, LB 11842, obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, was used. Pure concentrated cultures, maintained frozen at -25° C, were revived in skim milk which had been reconstituted at the rate of 10 g skim milk powder + 95 mL distilled water and autoclaved for 15 min at 121° C. Cultures were inoculated at the rate of 10 μL in 10 mL milk, and grown at 39° C for 18 hr., under aerobic conditions. Growth medium was Lactobacilli MRS broth (Difco Laboratories, Detroit, MI) (deMan, Rogosa and Sharpe 1960). Since *Lactobacillus delbrueckii* subsp. *bulgaricus* species produce the β-gal enzyme whether grown in the presence or absence of lactose (Kilara and Shahani 1975; Smart *et al.* 1993), results were

not compromised by the lack of lactose in the growth medium. Three successive daily transfers into fresh MRS medium at the same rate were made before an actual experiment was carried out.

4.2.2: Culture propagation and sonication techniques

Overnight culture grown in MRS was inoculated at the rate of 0.1 mL into 100 mL of fresh MRS, and allowed to grow at 39° C for 18 hr. Cultures were then cooled in ice water for approximately 30 minutes and centrifuged for 20 minutes at 1700 x g. Spent medium was decanted, and the wet cells were resuspended in 50 mL of 2% fat pasteurized market milk or buffers as subsequently described, according to the experimental design. Each cell suspension was sonicated for 4 minutes in an ice water bath (Shah and Jelen 1991) with a Braun-Sonic 2000 sonicator (Ultrasonic Power Corp., Freeport, Ill.), using a 19 mm probe at output setting of 75 watts, in order to disrupt the cells and release the intra-cellular enzyme. The effectiveness of this treatment has been well established (Kreft *et al.* 2000) and was reconfirmed in some cases by viable plate counts as described below.

4.2.3: Validation of the cryoscopic method for determination of β-galactosidase activity

To confirm the previously proposed suitability of f.p. measurement as a fast and convenient method of following the progress of lactose hydrolysis by the sonicated cultures in our conditions, an experiment was carried out to compare this method with a standard enzymatic method for lactose and glucose determination (Lactose/D Glucose kit, Boehringer-Mannheim, Roche Diagnostics, Laval, Quebec). Cultures were grown, centrifuged, and sonicated as described above. In this case they were resuspended in 50 mL commercially available 2% fat pasteurized milk. After sonication, another 50 mL of milk was added and the cell suspension mixed well. These samples did not receive any pre-

hydrolysis heat treatment. An initial f.p. measurement was taken immediately; as well, a 1 mL sample was removed and clarified by using Carrez Solutions of ferrocyanide and zinc sulphate, to precipitate the protein (Anon 1997). The lactose and glucose contents were then determined by the enzymatic method, by means of UV absorbance measurements at 340 nm (Anon 1997), taken with a Hewlett-Packard model 8452A Diode Array Spectrophotometer.

The cell suspension sonicated in milk was placed in a 51° C water bath for lactose hydrolysis to proceed. Freezing point measurements, taken with a Cryette A Cryoscope, model 5006, (Precision Systems, Natick, MA), and enzymatic determinations were repeated at hourly intervals for four hours. This whole experiment was replicated twice.

4.2.4: Interactive effect of temperature, pH level and ionic environment on β-galactosidase stability and activity

Cultures were grown and centrifuged, as previously described. Buffers of 0.05 molarity and various pH levels ranging from 3.5 to 7.0 were prepared with either sodium or potassium as the cation and phosphate, acetate, or citrate as anion in distilled water, depending on the desired pH. Adjustment was with 2N KOH or 2N NaOH, or 3N HCl, so that buffers contained only one cation. To prevent mold growth in the buffers on storage, 0.02% NaN_3 was added to buffers used at temperatures of 25 to 56° C. Those to be used at 61°C were autoclaved for 15 minutes at 121° C, since viable plate counts could not be done with NaN_3 in the solution. The wet cells from 100 mL growth medium were resuspended in 50 mL of the given buffer, sonicated in an ice water bath, and distributed equally into 4 test tubes (*i.e.* 12.5 mL each) for holding without stirring from 0 to 60 minutes in a water bath at a given temperature, which ranged from 25° C to 61° C. The

0 minute holding time was a built in control in which the substrate solution was added immediately with no pre-hydrolysis holding time. The time for the temperature of the sonicated culture to reach 61° C was approximately 3 minutes. The sonicated cultures held at 61° C were resuspended in pH 6 & 7 buffers only.

As the hydrolysis substrate, a stock solution of 10% w/v lactose in distilled water, buffered to pH 7 with potassium phosphate at approximately 0.25 M was prepared. The much higher molarity of the buffering agent in the substrate solution than that in the holding buffers allowed comparison of the activity under relatively similar ionic conditions (Whitaker 1994). An equal volume of this 10% lactose solution was added to the sonicated cell suspensions in the test tubes after the holding treatment and mixed well with a vortex, resulting in 5% lactose concentration in the final test solution. One 2.5 mL sample of this mixture was removed immediately for f.p. measurement and the rest was put in a 51° C water bath for hydrolysis to proceed. In all cases samples were vortexed for approximately 5 sec every 10 - 15 minutes during treatment. Freezing point was measured over at least 2 hours. Interference of metabolic activity of residual viable organisms on measurements during hydrolysis was not considered to be a problem because of the relatively high temperature of hydrolysis, the lack of nutrients, the presence of sodium azide in the buffer solutions at holding temperatures up to 56° C and the bactericidal effect of the 61° C holding temperature.

The effect of treatment at 61° C on the viability of organisms surviving sonication was determined by viable plate counts. Standard serial dilution techniques with sterile 0.1% peptone (Bacto-peptone, Difco Laboratories, Detroit, MI) in distilled water were used and the plates were poured with MRS plus 1.5% agar prepared according to the

manufacturer's instructions. Plates were counted after 3 days at 39° C. Plate counts were obtained for culture in MRS after 18 hours growth, after resuspension in buffer, after 4 minutes sonication (0 minutes holding), and after each of the 3 holding times. These experiments were replicated twice. Plate counts before and after sonication provided a measure of the effectiveness of sonication. However it is not necessary for the organisms to be completely disrupted for the effectiveness of sonication to be at a maximum. This procedure tends to also permeabilize the cell walls promoting flux of ions, and many organisms recover after a prolonged lag phase (Davidson and Rossett 1967).

4.2.5: Definitions for the purpose of this work

"Activity" (A) was defined as the hydrolysing ability of a disrupted culture, as measured by Δf.p. (-m° Hortvet) of the test solution (*i.e.* 5% lactose solution at pH 7) after a specified time (*i.e.* 30 minutes) of hydrolysis at 51° C.

"Residual activity" was determined as the ratio (percentage) of the activity of a sonicated culture which underwent a pre-hydrolysis holding treatment (A_t) to the activity of the same sonicated culture with no pre-treatment (A_o).

"Stability" was expressed as the loss of activity per minute holding time. Values of $\ln(A_t/A_o)$ determined after each holding time, were plotted vs. the holding time, for each combination of conditions tested. The first order denaturation constant k (determined from the slope of the linear trendline where slope = -k) and half-life were used as the measures of stability (Chang 1991).

4.2.6: Statistical analysis

Significance of differences in activity between Na^+ and K^+ containing solutions at different pH levels was determined by means of two-sample t-test for difference of means,

using Microsoft Excel (Pellissier 1996).

4.3: Results and discussion

4.3.1: Validation of the cryoscopic method for determination of β-galactosidase activity

Figure 4.1 shows the correlation between lactose and glucose concentrations as measured by the enzymatic method vs. f.p. readings during the course of hydrolysis.

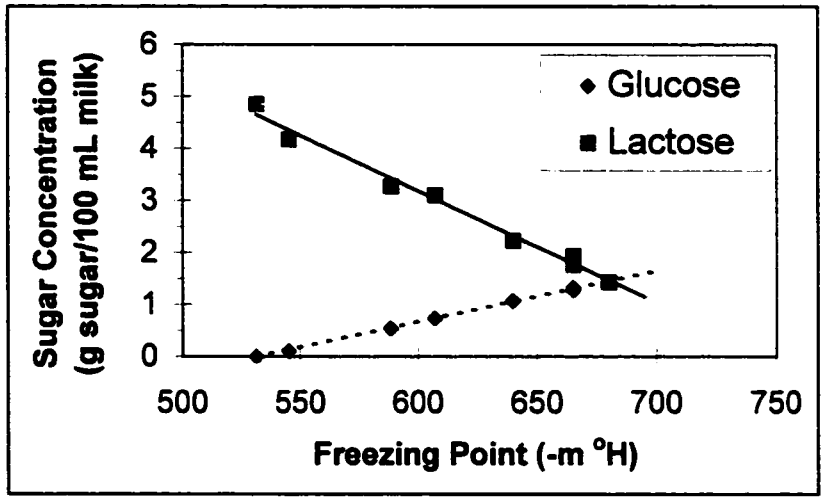

Figure 4.1:
Lactose hydrolysis by sonicated cultures of *Lactobacillus delbrueckii,* subsp. *bulgaricus* 11842 in skim milk, as measured by an enzymatic method of determining sugar concentration and by change in freezing point. Error bars showing ± 1 st. dev. are the size of the data marker point.

In both cases the relationships were linear with very high correlation coefficients, R^2 = 0.9909 for lactose, with a slope of -0.0199; and R^2 = 0.9991 for glucose, with a slope of +0.0098. The slope of the trendline showing decrease in lactose was very close to -2 times that obtained for the increase in glucose, as would be expected, since ideally the hydrolysis of a certain weight of lactose would be expected to produce half that weight of glucose. This confirms previous evidence that cryoscopy is a valid, simple and precise method for following lactose hydrolysis even for a relatively complex system such as used

here including crude enzyme preparation in skim milk. (Data not shown for the much simpler lactose solution model system.)

4.3.2: Interactive effect of temperature, pH level and ionic environment on β-galactosidase stability and activity

Figure 4.2 compares means (number of replicates was at least 2 and sometimes up to 9 for each pH-cation combination) of activities for cultures sonicated in potassium or sodium buffers at each pH level, with no pre-hydrolysis holding. For cultures sonicated

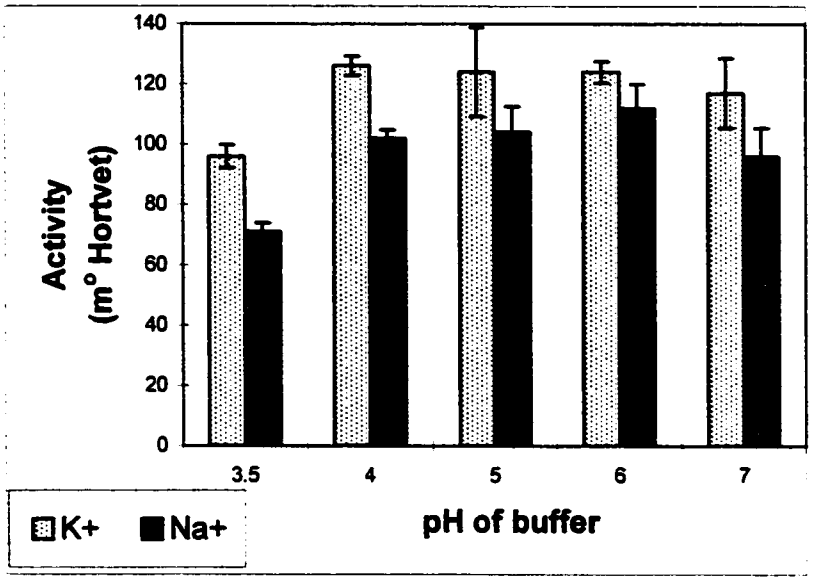

Figure 4.2:
Lactose hydrolysing activity of sonicated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 after sonicating in 0.05 M K^+ or Na^+ buffer, at various pH levels, determined with 5% lactose in 0.25 M K^+ pH 7 buffer at 51°C. Error bars show ± 1 st. dev. for each set of replicates, mean (n = 2 to 9).

at every pH level in the presence of Na^+ ions the subsequent activity in the 5% lactose test solution at pH 7.0 was significantly less than when sonicated in the presence of K^+ ions at the same pH level (except cultures sonicated at pH 5, in which activity was less but not significantly: p-values for pH levels 3.5, 4, 5, 6 and 7 were 0.0083, 0.0059, 0.105, 0.013,

and 0.00015, respectively), even though there was five times more K^+ ions than Na^+ ions in the final lactose test solution. This indicates that sodium inhibits the activity of this β-gal enzyme, when lactose is the substrate. These results appear to contradict those of Garman *et al.* (1996). Both species of *Lactobacillus* that they studied (neither of which was LB 11842) produced β-gal that had greater activity when the solution included Na^+ rather than K^+, with lactose as substrate. Also when β-gal from each of these two species was incubated with both K^+ and Na^+ in the lactose solution there was a slight decrease in activity, indicating K^+ may have an inhibiting effect.

Averaged over the whole pH range, the activity with no pre-hydrolysis holding of all samples sonicated in potassium buffers ($n = 25$) was significantly greater than for all samples sonicated in sodium buffers ($n = 24$; 119 vs. 101 m^o H, resp.; $p < 0.0004$). Cultures sonicated in buffers at pH 3.5 showed significantly lower activity than cultures sonicated at other pH levels ($p < 0.007$). Even though the sonication time in the low pH 3.5 buffer (maximum of 5 minutes) was short, it appeared to be detrimental enough as the enzyme is known to rapidly and permanently lose its activity at low pH (Shah and Jelen 1990).

Table 4.1 shows stabilities for cultures sonicated and held in (a) potassium and (b) sodium buffers. The trends were similar in both cases, except that in general, the presence of potassium resulted in higher stability (*i.e.* lower rate constant) at the same temp-eratures. A denaturation rate constant of ≥ 0.25 min^{-1} indicates a 90% loss of activ-ity within 9.2 min or less holding time under the given conditions, this was taken as the limit of low stability. A constant of ≤ 0.001 min^{-1} indicates no more than 16% loss of activity within 3 h, this was taken as the limit of high stability. For pH 6 and 7 up to

Table 4.1:
Stability (expressed as first order denaturation rate constant (min^{-1})) of the β-galactosidase in sonicated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842, in: (a) K^+ buffer and (b) Na^+ buffer at various pH levels, held at various temperatures before hydrolysis

Table 4.1(a) Potassium Buffer

	pH Level				
Temp. °C	3.5	4	5	6	7
25	0.0297	0.013	0.0059	≤0.001	≤0.001
35	≥0.25	0.0996	0.0018	≤0.001	≤0.001
45	≥0.25	0.0958	0.0709	≤0.001	≤0.001
51	≥0.25	≥0.25	≥0.25	0.0048	≤0.001
56	≥0.25	≥0.25	≥0.25	0.0027	0.002
61	ND	ND	ND	0.026	0.0175

Table 4.1(b) Sodium Buffer

	pH Level				
Temp. °C	3.5	4	5	6	7
25	0.0132	0.0102	≤0.001	≤0.001	0.0021
35	≥0.25	0.1181	0.009	≤0.001	≤0.001
45	≥0.25	≥0.25	0.1294	0.0012	≤0.001
51	≥0.25	≥0.25	0.2468	0.0044	0.0013
56	≥0.25	≥0.25	≥0.25	0.0066	0.0054
61	ND	ND	ND	0.0982	0.0468

56°C, the differences in stability were very small; average k was 0.00165 min^{-1} for K^+ and 0.0025 min^{-1} for Na^+. When sonicated cell suspensions were held at pH 6 and 7 at all temperatures up to and including 51° C in K^+ and Na^+ buffers, there was virtually no loss of activity. At 56°C and pH 6 and 7, the enzyme was more stable with potassium (half-

lives were 257 and 346 min, respectively) than with sodium, (half-lives 105 and 128 min, resp.). Chang and Mahoney (1989) determined half-lives of the β-gal produced by *S. thermophilus* in 0.1 M potassium phosphate pH 7 buffer with Mg^{2+} at 56, 60 and 62°C to be 10.3, 2.1 and 1.1 minutes, respectively, using ONPG as substrate. The above results with LB 11842 show much greater stability of its enzyme even though the hydrolysis conditions were somewhat different and the substrate in the present work was lactose rather than ONPG.

Generally, as pH was decreased below 6, enzyme stability decreased at higher than ambient temperatures; however, at the holding temperature of 25°C, there was no loss in stability at pH levels as low as 5, and there was only a small loss at pH 4 and even 3.5. At pH 5, stability sharply declined as temperature increased from 35° C upward. At pH 3.5 and 4, the enzyme was almost totally denatured after holding at 35° C (half-lives were 7.2 and 5.9 min for holding in K^+ and Na^+ at 35° C, respectively).

The effect of holding the sonicated cultures at 61°C on the subsequent residual β-gal activity is shown in Figure 4.3. Results for K^+ buffer were very similar at pH 6 or 7, as about 30% activity remained after 60 min, whereas with Na^+ in pH 7 buffer only approx. 10% activity remained after 60 min and at pH 6 only 10% activity remained after 30 min. The corresponding half-lives of enzyme activity were 31.3 and 42.4 min for K^+, and 16.6 and 7.2 min for Na^+. Results for colony counts on 11 separate cultures before and after sonication had a mean reduction of 85.3 ± 9.7 % (± st dev), somewhat lower than what was observed previously, when the average reduction was 97.6% for 44 sepa-

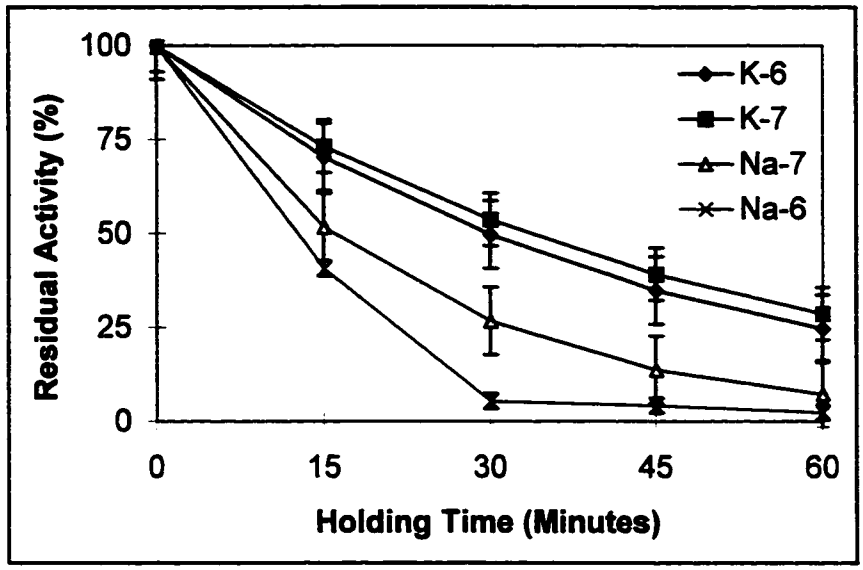

Figure 4.3:
Effect of holding time on residual activity for sonicated cultures of *Lactobacillus delbrueckii*, subsp. *bulgaricus* strain 11842 held at 61° C in K^+ and Na^+ buffers at pH 6 and 7 prior to hydrolysis. Na-6, Na-7, K-6, K-7 indicate the cation and pH of holding buffers. Error bars show ± 1 st. dev. (n = 2, except K-7, where n = 3).

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rate cultures (Kreft *et al.* 2000). However, the reduction in viable bacteria does not necessarily indicate the effectiveness of the sonication treatment, because cell walls can be permeabilized but the organism remains viable, as shown by Wang *et al.* (1996) in their study on ultra-sound enhanced lactose hydrolysis.

Colony counts decreased from over 10^8 cfu/mL to between 10^2 and 10^5 cfu/mL after the 60 min of incubation at 61° C. However, there did not appear to be any recognizable pattern relating organism survival to ionic content and pH holding conditions (Figure 4.4). In relative terms, the decrease in the numbers of viable organisms was much greater than the corresponding decrease in enzyme stability, which indicates that the stability of the free β-gal enzyme released by disruption was relatively higher than the viability of the residual bacteria.

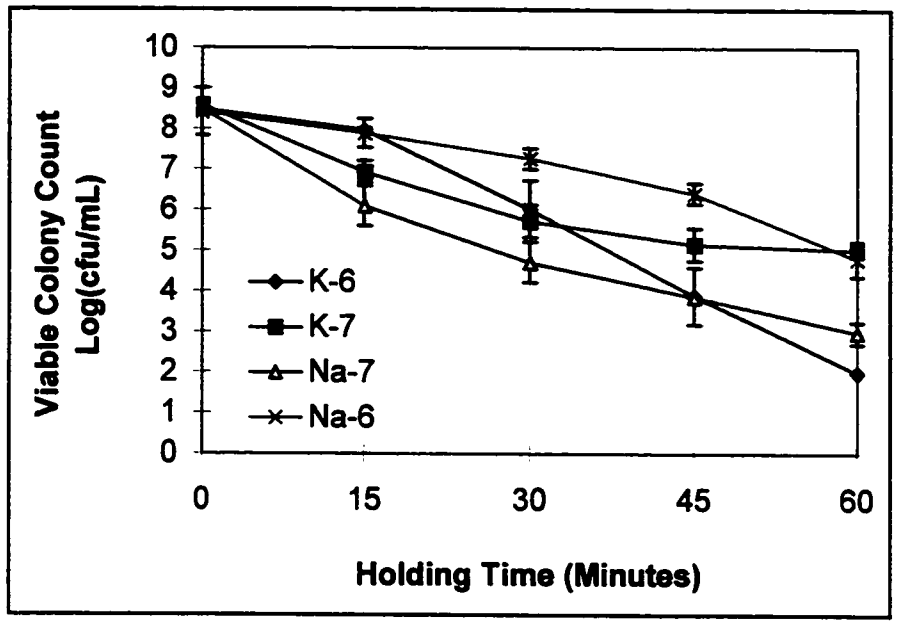

Figure 4.4:
Effect of holding time on viable colony count for sonicated cultures of *Lactobacillus delbrueckii*, subsp. *bulgaricus* strain 11842 held at 61° C in K^+ and Na^+ buffers at pH 6 and 7 prior to hydrolysis. Na-6, Na-7, K-6, K-7 indicate cation-pH level of holding buffers. (n = 2, except K-7, where n = 3). Error bars indicate ± 1 st. dev. Some error bars have been removed for clarity.

In general, the β-gal activity in freshly sonicated cultures of LB 11842 was higher when sonicated in K^+ buffer than when sonicated in Na^+ buffer at all pH levels, indicating Na^+ is an inhibitor of this enzyme, even in the presence of excess K^+ ions in the test solution. Stability of the β-gal was generally higher when sonicated cultures were held in K^+ buffer rather than Na^+ buffer; however, the differences at pH 6 and 7 were small and the enzyme was virtually stable up to 56° C. At 61° C, pH 6 and 7, cultures sonicated in K^+ buffer were much more stable than those sonicated in Na^+ buffers.

4.4: Conclusions

In this study cryoscopy was confirmed to be a simple, fast, and reliable method for determining the progress of lactose hydrolysis, even with complex systems such as crude sonicated bacterial cultures in skim milk. There are very few studies regarding the influence of cations on the enzymatic activity of β-gal from *Lactobacillus* species, especially using lactose rather than ONPG as a substrate. The stability and subsequent residual enzymatic activity of β-gal in freshly sonicated cultures of LB 11842 was higher in K^+ buffer rather than Na^+ buffer. At 25° C the enzyme was relatively stable at all pH levels, while at higher temperatures (51 and 56° C), it was stable at pH 6 and 7. At 61°C there was little difference in stability at pH 6 or 7 for K^+ but a significant loss of activity with Na^+ at pH 6 in comparison to pH 7. Strains of *Streptococcus thermophilus* have been proposed as sources for β-gal for use in dairy products, because of their food grade status and other favorable characteristics (Rao and Dutta 1981, Somkuti and Steinberg 1994, Chang and Mahoney 1989). However, the present study indicates that LB 11842 appears to be a more suitable source as the enzyme showed much higher thermostability.

A lactose hydrolysis process utilizing these sonicated or otherwise disrupted cultures, executed above the temperature optimum for the enzyme could be feasible with reasonably fast hydrolysis rates and no interference of the residual bacteria.

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Chapter 5[1]

Growth of *Lactobacillus* dairy cultures on two different brands of MRS medium

5.1: Introduction

The well-known type of medium especially formulated for growth of *Lactobacillus* species, "MRS", originally proposed by deMan *et al.* (1960), is produced by different suppliers of microbiological media. However, the formulas used by each supplier may be different from the original "MRS", due to economic or standardization reasons (Millsap *et al.* 1996). During the course of a related project (Kreft *et al.* 2000), it was noticed that a recently purchased new brand of MRS was giving significantly lower viable counts compared to the previously used brand. The work reported here was therefore undertaken to determine the reproducibility, the magnitude and the statistical significance of the observed effect and its occurrence with various *Lactobacillus* species.

5.2: Materials and methods

The Lactobacillus species used were *Lactobacillus delbrueckii*, subsp. *bulgaricus*, strains 11842 and 12 (LB 11842 and LB 12), *Lactobacillus helveticus* strain 32K (LH 32K), *Lactobacillus plantarum* strain 8014 (LP 8014), and *Lactobacillus acidophilus* (LB-AC). LB 11842, LB 12 and LP 8014 were from the American Type Culture Collection (ATCC), Rockville, Maryland; LH 32K was supplied by Chr. Hansen's Laboratory, Inc. of Milwaukee, Wisconsin; and LB-AC was isolated from a commercial acidophilus-containing 1% fat milk.

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[1] A version of this chapter has been accepted for publication by Milchwissenschaft. Kreft ME, Champagne CC, Jelen P. (Oct. 5, 2000)

Two different brands of MRS medium, commonly used for cultivation of Lactobacilli, were used and prepared according to the instructions of the manufacturers; both suppliers are well known and their products are readily accepted for routine laboratory work. In this paper they will be identified as "Brand A" and "Brand B".

Organisms from ATCC and Chr. Hansen's were maintained frozen at -25° C and revived in skim milk sterilized by autoclaving for 15 min at 121 °C. All organisms, except LP 8014, were grown at 43° C for 18 hours in broth, under aerobic conditions, whereas LP 8014 was grown at 25° C. Cultures of LB 11842 were grown in 27 separate replicates in Brand A and in 20 replicates in Brand B. Each of the other organisms was grown 6 times in each medium brand. Three successive daily transfers of culture into fresh medium of the given brand at the rate of 0.1% were made. In each case the third sub-culture was successively diluted with 0.1% peptone water sterilized by autoclaving for 15 min at 121 °C and the final dilutions plated on the given MRS agar. Plates were counted after 48 hours incubation at the same temperatures as used for propagation. This method was also used to establish growth curves for LB 11842 on the two media. Samples of the third sub-culture were plated every two hours over an 18 hour period.

To determine the suitability of each brand as an enumeration medium, each of the strains was cultured for the above times and temperatures in skim milk sterilized by autoclaving for 15 min at 121 °C. One set of dilutions was plated on both brands of the medium and plates were counted as above. This procedure was replicated twice.

To determine the relationship between final pH and viable count for LB 11842, the pH of the spent medium at conclusion of growth of the 27 and 20 replicates mentioned

above was measured with a Model Φ 71 pH meter (Beckman Instruments Inc., Irvine, California) and correlated to the viable counts of those cultures.

Means and standard deviations were determined and the t-test for difference of the means (Pellissier 1996) was carried out for each species.

5.3: Results and discussion

As shown in Table 5.1, 3 of the 5 tested Lactobacilli produced significantly higher counts on Brand A medium. For LB 11842 and LB-AC, the differences of the mean log viable counts were highly significant ($p < 0.001$ and < 0.01,respectively).

Table 5.1:
Means and standard deviations of viable counts for 5 *Lactobacillus* strains, grown in two brands of commercially-prepared "MRS" medium. For organism abbreviations see text. LB 11842 was grown 27 separate times in Brand 'A' and 20 times in Brand 'B', the other 4 organisms were grown 6 times each in both brands.

	BRAND 'A' MEAN	SD	BRAND 'B' MEAN	SD	P-VALUE
	log(cfu mL^{-1})				
LP 8014	9.727	0.126	9.561	0.091	0.0279
LB-AC	8.542	0.249	7.144	0.812	0.00687
LB 11842	8.242	0.403	7.551	0.423	1.43E-06
LH 32K	8.238	0.202	8.376	0.381	0.455
LB 12	8.079	0.101	8.312	0.118	0.00438

The actual means of the viable counts for LB 11842 and LB-AC were respectively approx-imately five and twenty five times greater on Brand A. LP 8014 also showed better growth on Brand A, but the difference between the means was not nearly so

significant ($p = 0.0279$); actual growth was about 50% higher. For LH 32K, the mean of the viable count was slightly higher on Medium B, but the difference was not significant ($p = 0.455$). LB 12 showed significantly better growth on Medium B; the p-value was 0.00438, and the actual growth about 70% higher. The observed differences are much greater than may be sometimes observed due to batch-to-batch variations using the same media supplier.

Table 5.2 shows that the viable counts for each organism after growth in skim milk, as enumerated on the two media, were almost exactly the same ($p = 0.92$). However, four of the five species showed larger colonies on Brand A; the only exception was LB-AC which showed slightly larger colonies on Brand B. This indicates that both media can be considered equally suitable as enumeration media, although enumeration on Brand A medium was easier.

Table 5.2:
Viable count and size of colonies for 5 *Lactobacillus* strains grown in milk, and enumerated on two brands of commercially prepared "MRS" medium (means of 2 replicates). For organism abbreviations see text.

	BRAND 'A'		BRAND 'B'	
	COUNT	SIZE	COUNT	SIZE
	($cfu.mL^{-1}$) (10^8)	(mm)	($cfu.mL^{-1}$) (10^8)	(mm)
LP 8014	1.08	1.0 - 3.0	0.962	0.7 - 1.0
LB-AC	6.25	<0.1 - 0.3	6.67	0.3 - 1.5
LB 11842	7.10	1.0 - 2.0	6.99	0.7 - 1.0
LH 32K	10.8	1.0 - 1.5	11.0	0.3 - 0.8
LB 12	1.97	0.8 - 3.0	2.09	0.6 - 1.8

Components that are different in either brand as compared to the original recipe are shown in Table 5.3. Amounts of peptone, glucose, Tween 80, K_2HPO_4, sodium acetate and ammonium citrate are the same in all three recipes. The only major noticeable differences are somewhat lower amounts of meat and yeast extract in Brand B.

Table 5.3:
Differences in components of 2 Brands of MRS medium vs. original formula. Amounts given are per litre of medium as prepared according to manufacturer's instructions.

	Meat Extract	Yeast Extract	$MgSO_4$	$MnSO_4$
Medium	----------(g)----------			
Original	10	5	0.2	0.05
Brand "A"	10	5	0.1	0.05
Brand "B"	8	4	0.2	0.04

The concentration of yeast extracts (YE) in a growth medium has been shown to influence biomass levels with *Lactobacillus plantarum* (Potvin *et al.* 1997); the lower content of YE in Brand B could be partially responsible for the lower populations observed. The YE also contributes to the buffering capacity of the medium; thus a lower YE content not only reduces the amount of growth factors but also allows a more rapid drop in pH. The type and quality of yeast extract, meat extract and peptone could vary greatly between the two brands. In addition, the source of YE is an underestimated factor in the variability of growth media properties. The YE composition varies as a function of yeast strain as well as of the processing parameters used during autolysis (Hough and

Maddox 1970, Peppler 1982), and lot-to-lot variability has been reported (Potvin *et al.* 1997). Furthermore, in a comparative study of 26 commercial YE, it was found that responses to the growth-promoting properties of YE varied between bacterial species and strains (Champagne *et al.* 1999). This could explain the fact that Brand A was better for three strains, while Brand B gave higher biomass with LB 12, as indicated in Table 5.1.

According to Bergey's Manual (Kandler and Weiss 1986), Lactobacilli are acid-tolerant bacteria, showing maximum growth between pH 6.4 and 4.6; growth slows and tends to cease between pH 4.0 and 3.6. The initial pH of the prepared medium was 6.41 for Brand A, and 5.76 for Brand B. A lower initial pH could possibly contribute to a shorter growth time, and therefore lower over-all count. However measurements of pH of the spent media after growth of cultures of LB 11842 (Figure 5.1) showed lower final pH with higher viable counts for Brand A than Brand B. This may indicate that the organisms ran out of some nutrient in Brand B before the low pH became an inhibitory factor.

Since 18 hours, used in many of the replicate runs, is a somewhat long growth period, growth curves were established for LB 11842 (data not shown) to determine whether the lower viable counts for Brand B were due to slower growth or death of the organisms after the stationary phase had been reached. After a shorter lag phase, organisms in Brand A reached the stationary phase at 12 hours. There was no decrease in viable count from 12 to 18 hours. After a longer lag phase, Brand B did in fact show slower growth which continued up to 18 hours. This observation also points to a potential role of YE in explaining the differences between the two brands. Indeed, YE concentration and source influence not only the maximum biomass reached in a medium, but

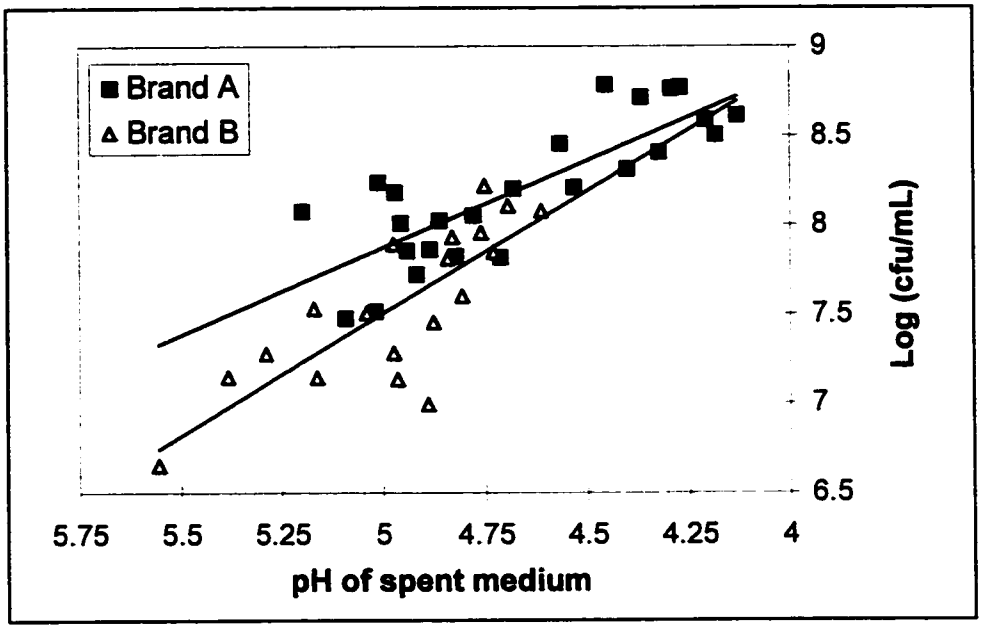

Figure 5.1:
Final pH of two brands of spent MRS medium vs. corresponding viable count of LB 11842. Brand A: $R^2 = 0.6633$, Brand B: $R^2 = 0.6264$.

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also the specific growth rate of lactic cultures (Champagne *et al.* 1999).

From the 27 separate replicates of LB 11842 in Brand A and 20 replicates in Brand B, the means of log viable count for 18, 20, 22, and 24 hours growth were calculated. There was no significant difference between the highest and the lowest mean for each brand ($p = 0.1452$ for Brand A and $p = 0.2874$ for Brand B), which indicates that this growth time period is stationary for this organism grown on either brand. These results do not indicate any decrease in counts due to death of the organisms.

Although there are reports of the effect of ingredient lot or source on its growth-promoting property (Potvin *et al.* 1997), the fact that media designed for general microbiological purposes show such marked differences is unexpected. Indeed, MRS is designed specifically for the growth of Lactobacilli, and many starter-producing companies test the biological value of the ingredients they purchase. It is also a common

practice for dairy processors to test the skim milk powders aimed at propagating starters. The strains used for such quality control must obviously provide an adequate evaluation of the media requirements. The significant differences in Brands A and B thus not only raise questions on the quality of ingredients, but also question the means by which biological value of microbiological media designed for LAB growth is evaluated.

5.4: Conclusions

Reproducibility and magnitude of the effect of the brand of MRS medium on growth of *Lactobacillus* varied according to species: from LB-AC for which the increase in growth on Brand A as compared to Brand B was even greater for than LB 11842; to LB-12 which showed better growth on Brand B. Statistical significance also varied according to species: from not significant (LB-32K) to a p-value < 0.00001 (LB 11842). For LB 11842, results did not indicate that the greater growth on Brand A was due to death of the organisms in Brand B toward the end of the growth cycle. Neither did results for LB 11842 indicate that the lower initial pH of Brand B contributed to a shorter growth time, and therefore lower overall growth. However, these results do raise questions as to differences in sources of the ingredients, since source has an effect on growth-promoting qualites; there did not appear to be differences in the recipes of the two brands great enough to explain the observed differences in growth.

There are various instances where biomass is an important feature in a fermentation process (production of enzymes or other metabolites, production of starter or probiotic cultures). Therefore if high cell growth is required, it is important to consider the brand of medium and its constituents in relation to the microbial species to be used. These results also point to the importance of conducting lab-scale fermentation trials in

the selection of ingredients for the critical step of scaling-up of fermentation processes. Although this study examined media used for laboratory growth of LAB, it raises a concern for potential variations in starter media as well. Champagne *et al.* (1995) have shown that differences between brands of commercial starter growth media occur, and that there are strain-media interactions. Therefore, dairy plants should carefully select media for large-scale as well as lab-scale propagation of starter cultures, and not automatically assume that similar media formulations between brands will provide the same results.

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Chapter 6

Conclusions and suggestions for further research

6.1: Conclusions

As a potential source of β-galactosidase for use in the proposed method of lactose hydrolysis by disrupted dairy cultures, growth characteristics of a common dairy micro-organism, *Lactobacillus delbrueckii* subsp. *bulgaricus* strain 11842 (LB 11842) and lactose-hydrolysing characteristics of its sonicated cultures were determined.

Freezing point depression as a method of following lactose hydrolysis in the complex medium milk was shown to have a highly significant linear relationship to an enzymatic method of measuring sugar concentration. However theoretical percent hydrolysis in milk appeared to be over-estimated by this method, likely because of metabolic activities including acid production by organisms remaining viable after disruption, which would increase the molality of the solution.

Growth conditions of gaseous atmosphere and time of culture incubation were investigated. Growth under aerobic conditions, which is much simpler to provide, produced cultures with as high enzyme activity as growth under anaerobic conditions, even though LB 11842 is considered to be micro-aerophilic. Growth of cultures for periods of time from 18 to 24 hours produced little difference in hydrolysing activity. The effect of varying growth temperature was not investigated. Regarding process characteristics, optimum time of sonication was determined to be 4 min, and the optimum rate of sonicated cell biomass addition to the lactose-containing substrate was 1%. A hydrolysis temperature of 51°C produced the highest rate of β-gal activity in lactose solutions. Reasonable

hydrolysis was achieved in the two substrates, skim milk and lactose solutions, at 7°C. At this temperature, there would be little metabolic activity due to growth of viable organisms remaining after disruption. However, any survivors could begin to grow if the temperature were raised.

Temperature, ionic and pH conditions producing the greatest stability of sonicated cultures of LB 11842 in the period of time between sonication and hydrolysis and subsequent optimum activity after this time were determined, for the reason that in the commercial application of the proposed process, there would likely be a lapse of time between disruption of the bacterial culture and the actual beginning of hydrolysis of the lactose-containing substrate. In addition, according to many studies, the cations K^+ and Na^+ have activating or inhibiting effects on the progress of lactose hydrolysis, depending especially on the source of the β-galactosidase enzyme (Mahoney and Adamchuk 1980; Bernal and Jelen 1985; Smart and Richardson 1987; Garman *et al.* 1996). Of the conditions used in this study, cultures sonicated and held in K^+ buffers, at pH 7, and at 51 °C and lower achieved higher stability and activity than those held in Na^+ buffers, at pH levels lower than 7, and temperatures higher than 51 °C. Enzyme activity even after 1 h at 61°C in K^+ buffers at pH 6 and 7 was good, being approximately 25% of that after no holding time between sonication and hydrolysis. Thus the β-galactosidase (β-gal) enzyme in sonicated cultures of LB 11842 is relatively thermostable. Under the same conditions, colony counts indicated a drop in viable organisms from 10^8 cfu mL^{-1} to between 10^5 to 10^2 cfu.mL^{-1}; the presence of these viable organisms could present a problem in food product quality if temperature conditions allowed their growth.

As an incidental observation made during the propagation of LB 11842, major differences were noted in growth on two commercial brands of MRS medium. Cultures of LB 11842 were grown 27 separate times in Brand A and 20 times in Brand B; the mean colony count for growth in Brand A was approximately five times greater than in Brand B. The growth of four other species of *Lactobacillus* in addition to LB 11842 was then compared on the two brands of MRS medium. It is well-known that there are differences between brands of media designed for the growth of bacterial cultures and even differences between batch numbers of the same brand, producing different rates of growth of bacterial cultures. However, the magnitudes of the differences as found in this study were much greater than would be expected between two media considered to be the same type. The differences were shown to vary according to species and strain of organism. Thus growth medium should be carefully matched to the species under consideration.

The present study indicates that LB 11842 is a suitable source of β-gal for use in the proposed process, as the enzyme showed high thermostability in the presence of K^+ ions at neutral pH. Sonicated cultures showed good lactose hydrolysing ability in a range of conditions: at low temperatures of 4-7°C; and in milk and in 2½ %, 5% and 30% lactose solutions. The use of a commercially prepared growth medium such as MRS would not be economically feasible; growth on a medium based on whey and whey derivatives would be more advantageous. For a commercially-viable process, it will be necessary to use another method of cell disruption rather than sonication because the volumes that can be disrupted with sonication are too small. A major concern for the proposed process is the prevalence of organisms surviving disruption. A lactose hydrolysis process utilizing sonicated or otherwise disrupted cultures of LB 11842, executed

above the temperature optimum for the enzyme or below the growth temperature for the organism could be feasible with reasonably fast hydrolysis rates and minimal interference of the residual bacteria.

6.2: Suggestions for further research

There are many further areas of research which could be undertaken directly related to the present work. Cultures of LB 11842 could be grown for a shorter period of time than in the present work, for example 8 - 10 hr. and the relationship between growth phase, enzyme production and heat stability determined. In addition there may be differences in susceptibility of the cultures to disruption depending on the growth phase.

In 1967, Davidson and Rosett suggested that sonication increased the permeabilization of bacterial cell membranes which then increased the activity of intra-cellular enzymes. Thus it would not be necessary for cells to be totally disrupted for improvement in hydrolysing ability to occur. More recently, Somkuti and Steinberg (1995) used whole, permeabilized non-growing cells of *Streptococcus thermophilus* as a source of β-gal in the preparation of low-lactose milk. Permeabilization was accomplished by the use of detergents and bile acid preparations. Similarly, it would be useful to determine whether cultures of LB 11842 could be permeabilized by sonication or another method, and used as a source of β-gal, and what level of subsequent lactose hydrolysing ability would occur.

In the present work the sonication effectiveness amounted to approximately 90% reduction in viable bacteria. A greater reduction would not likely lead to much greater hydrolysing effectiveness, but interference by the metabolic activities of remaining viable organisms would likely be decreased.

The β-gal from LB 11842 could be purified to a higher extent than simply removal of growth medium, and then the hydrolysing ability of this more purified enzyme compared to that of disrupted cultures. There does not appear to be any reports in the literature in which β-gal from LB 11842 has been purified. Use of the partially purified β-gal in milk would be especially helpful to determine its hydrolysing ability in the absence of viable bacteria. The remaining whole bacteria cannot be merely removed along with the cell debris by micro-filtration because a large portion of the active enzyme is associated with the debris (Kilara and Shahani 1975; Shah and Jelen 1990).

In this work, cultures sonicated in buffers with K^+ ions were found to have greater heat stability and higher subsequent lactose hydrolysing activity than those sonicated in the presence of Na^+ ions. However, the effect of the concentration of these cations on stability and activity was not determined; a single concentration of cation was used. Perhaps there is a concentration of K^+ cation which produces optimum stability and activity. The effect of sonicating cultures in the presence of another monovalent cation, namely NH_4^+, could also be investigated. Many studies have been carried out regarding the effect of cations on hydrolysis; work on the effect of anions might also be useful (Mahoney and Adamchuk 1980; Bernal and Jelen 1985; Smart and Richardson 1987; Garman *et al.* 1996).

The lactose hydrolysing ability of other suitable micro-organisms in addition to LB 11842 could be determined at low temperatures, for example 4 to 7°C, since it is not necessarily true that the organism with the best hydrolysing ability close to its optimum temperature, also has the best ability at a low temperature. A sequence of temperatures

from 56°C and higher could be used to determine the maximum temperature at which hydrolysis occurs before the enzyme is denatured. The establishment of optimum storage conditions for disrupted cell suspensions could be assisted by determining their stability over various periods of time at temperatures of 25° C and lower, including frozen storage at –20 and –70° C.

Knowledge of the hydrolysing ability of sonicated cultures of LB 11842 in solutions of lactose at concentrations lower than 5% and up to 30% and over would have two beneficial effects. Firstly, it would enable the K_m and V_{max} values of the sonicated cultures to be calculated, allowing the present results to be more widely compared with other studies. Secondly, the maximum rate of hydrolysis with a given amount of sonicated cell biomass could be determined. With a given amount of enzyme, as the lactose concentration in a solution is raised to a certain point, the rate of hydrolysis also rises to a maximum; as the concentration is raised farther, the rate of hydrolysis tends to drop (Wierzbicki and Kosikowski 1972). In the present work, hydrolysis by 1% sonicated cultures in 30% lactose solution and 30% whey permeate solution did not appear to be inhibited, but this concentration may not be that which produces the maximum hydrolysing activity. If the lactose concentration for optimum hydrolysis rate with 1% culture were determined, the lactose could be first concentrated to this optimum concentration, then hydrolysed. Thus the optimum amount of lactose could be hydrolysed with a given amount of culture.

If the cryoscopic method of following lactose hydrolysis were employed in a future study, an antimicrobial agent such as sodium azide could be used to stop bacterial growth and consequent production of acid when substrates such as milk which support bacterial growth are used. Obviously this would not be used in an actual food product. It

cannot be assumed that no proteolysis would take place in lactose solutions; for example released proteases could act on other cellular proteins. Whether this is taking place could be determined by the use of a protease inhibitor at temperatures conducible to proteolytic activity. In addition a protease inhibitor could be used to help determine the level of proteolysis taking place in milk or whey. The progress of proteolysis can be followed by cryoscopy; 2.7% proteolysis produces a change in freezing point of 21 $m^{o}C$ (Althouse *et al.* 1995).

Full scale sensory evaluation with potential products will need to be carried out at later stages of this research program, including characterization of off-flavors that could occur due to residual medium and the production of short peptides and free fatty acids.

Up to the present, the focus of the study of lactose hydrolysis by disrupted dairy cultures has been mainly the production of lactose-hydrolysed dairy foods, the main one being milk. Perhaps it should be widened to producing a lactose solution with the lactose at least partially hydrolysed to be used in new non-dairy products such as pet foods and economically competitive sweetener syrups. A wider focus for this process could also include non-food applications such as use of lactose-hydrolysed solutions as substrates in industrial fermentations by a wider variety of organisms which produce useful by-products. In addition, the process could be employed for hydrolysis of the lactose in concentrated whey systems such as whey cheese or possibly to avoid crystallization during transport of liquid whey concentrates. With the appearance of entirely new consumer food items, bias in favor of the taste of traditional products would decline in importance. For example, it is commonly known that many people have a distate for lactose-hydro-

lysed milk because of the increased sweetness compared to regular milk, whereas milk shakes are consumed for the very reason of pleasant sweetness.

Thus the proposed method of lactose hydrolysis by disrupted dairy cultures could encourage the development of new food products especially for consumption by lactose-intolerant individuals, and the development of new non-food products using whey of which there is an abundant supply.

Lactobacillus delbrueckii subsp. *bulgaricus* strain 11842 appears to be suitable for use in the proposed method of lactose hydrolysis by disrupted dairy cultures because of desirable characteristics such as thermostability of its β-gal, very good hydrolysing ability under a wide range of conditions in various lactose-containing substrates and its long record of traditional safe use by the dairy industry.

6.3: References

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