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Growth and disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* for lactose hydrolysis applications.

by

Dean Bury 

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 2000

University of Alberta

Library Release Form

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Title of Thesis: Growth and disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* for lactose hydrolysis applications.

Degree: Master of Science

Year this Degree Granted: 2000

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Abstract

The growth and β-galactosidase production of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 was investigated for the purpose of using disrupted cultures to hydrolyze lactose. The addition of 0.2-1% yeast extract to whey-based media increased the rate of growth, acidification, and β-galactosidase production. Supplementation with various whey protein concentrates or isolates increased the rate of growth and acidification but to a much lesser extent than yeast extract. The cultures were disrupted using sonication, a high-pressure homogenizer, or a bead mill. The release of β-galactosidase was measured using o-nitrophenyl-β–D-galactopyranoside. The maximum activity occurred within 2-3 minutes of milling or after three passes through a high-pressure homogenizer operating at 135 MPa. Sonication was not as effective and its use on an industrial scale appears doubtful. An economic analysis of the process suggested that the production of partially lactose hydrolyzed milk may be feasible for volumes as low as 2000 L/d, whereas, the hydrolysis and concentration of 500 000 L of whey per day for production of a sweetener syrup cannot compete with other sweetener commodities.

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Chapter 1

Introduction

Holsinger (1992) estimated that 70% of the world's population might be classified as lactose maldigesters and experience various degrees of gastrointestinal discomfort due to the consumption of lactose. The enzymatic hydrolysis of lactose in milk or other foodstuffs could alleviate the symptoms of lactose intolerance and increase the demand for dairy-based products for which a potential market of some 50 million Americans has been identified (Sloan, 1999). The enzyme β-galactosidase may be obtained from fungi, yeast, or bacteria and can be used to break down (hydrolyze) lactose to glucose and galactose which are sweeter, more soluble, and readily digestible. Current methods of hydrolysis involve the use of highly purified β-galactosidase which can add considerable expense to production of lactose hydrolyzed milk or whey-based beverages. To reduce costs, Jelen (1993) proposed the possibility of using a sonicated dairy culture to hydrolyze the lactose in dairy products.

A process based on this approach would involve "in-house" fermentations with a suitable dairy organism capable of expressing high levels of β-galactosidase. As the lactic acid bacteria utilize an intracellular β-galactosidase for growth on lactose, the culture would have to be disrupted after collection and concentration. The disrupted culture would then be added to milk, whey, or any other product containing lactose for hydrolysis.

The lactic acid bacterium *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 was selected for this study as it is capable of producing relatively high levels of β-galacto-

sidase (Friend *et al.*, 1983; Shah and Jelen, 1990, 1991) and because it has a long history of safe use in the dairy processing industry for the production of yogurt.

The objectives of this project encompassed 1) investigations with whey-based media supplemented with whey protein concentrates, whey protein isolates, or yeast extract to increase the microbial growth and production of β-galactosidase from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842, 2) a comparison of alternative means for disrupting the culture adaptable to an industrial scale, and 3) an exploratory economic evaluation of producing partially lactose hydrolyzed milk or whey syrups using the direct addition of disrupted cultures.

To aid in the understanding and flow of ideas in this thesis, the general concepts of each chapter are presented as follows. Chapter 2 is a literature review covering the growth and production of β-galactosidase by *L. delbrueckii* ssp. *bulgaricus* 11842, mechanical means of cellular disruption, and industrial methods for the hydrolysis of lactose. Chapters 3 and 4 summarize experiments involving the addition of various whey protein concentrates (WPC's) to sweet whey or ultrafiltered (UF) whey permeate in order to improve the growth of *L. delbrueckii* ssp. *bulgaricus* 11842. Chapter 5 reports on investigations of the production of β-galactosidase by *L. delbrueckii* ssp. *bulgaricus* 11842 in whey-based media supplemented with various amounts of yeast extract. In chapter 6, the release of β-galactosidase from cells of *L. delbrueckii* ssp. *bulgaricus* 11842 disrupted using sonication, a high-pressure homogenizer, or a bead mill is compared. Chapter 7 provides an exploratory economic analysis of the concept of using disrupted cultures of *L. delbrueckii* ssp. *bulgaricus* 11842 to produce partially lactose hydrolyzed milk or partially lactose hydrolyzed whey syrup. Finally, chapter 8 integrates

the outcomes of the studies presented in the preceding chapters into the wider context of the ongoing research program and speculates on future prospects of the proposed approach.

1.1 References

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Chapter 2

Literature Review

2.1 *Lactobacillus delbrueckii* ssp. *bulgaricus*

L. delbrueckii ssp. *bulgaricus* 11842 was chosen for these studies as it was shown to produce the highest level of β-galactosidase out of the dairy cultures described in literature (Friend *et al.*, 1983; Shah and Jelen, 1990, 1991). *L. delbrueckii* ssp. *bulgaricus* 11842 is considered the type strain for *L. delbrueckii* ssp. *bulgaricus* (Hammes and Vogel, 1992). It is a Gram positive, non-spore forming, non-motile rod 0.5-0.8 μm thick and 2-9 μm long. It grows well at 45°C but is unable to grow at 15°C or below. Although it is an anaerobic bacterium, it is capable of tolerating and even growing in aerobic environments. In the dairy processing industry, *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* are often used for the production of yogurt (Gilliland, 1985).

L. delbrueckii ssp. *bulgaricus* is an obligate homofermentative bacterium, which means that sugars are always converted to a single product such as lactic acid. It ferments hexoses such as glucose via the Embden-Meyerhof-Parnas (EMP) pathway with the production of D-lactic acid, but it is unable to ferment pentoses or gluconate (Hammes and Vogel, 1992). Galactose is not used by *L. delbrueckii* ssp. *bulgaricus* 11842, although some strains of this microorganism are capable of utilizing galactose in addition to glucose (Hickey *et al.*, 1986). This might be one reason for the relatively high β-galactosidase activity as *L. delbrueckii* ssp. *bulgaricus* 11842 would have to hydrolyze twice as much lactose to produce the same amount of energy as those capable of utilizing galactose.

A lactose permease (LP) is responsible for transporting lactose into the cell (Figure 2-1a) where it is hydrolyzed by β-galactosidase (Leong-Morgenthaler *et al.*, 1991). The glucose is metabolized via the EMP pathway and the galactose is transported out of the cell. Other lactic acid bacteria, such as *S. thermophilus*, are also able to transport and hydrolyze lactose using a phosphenolpyruvate - lactose phosphotransferase system (PEP-PTS). The lactose is "activated" as it is transported into the cell (Figure 2-1b) resulting in pyruvate and phospho-lactose. Phospho-β-galactosidase hydrolyzes the

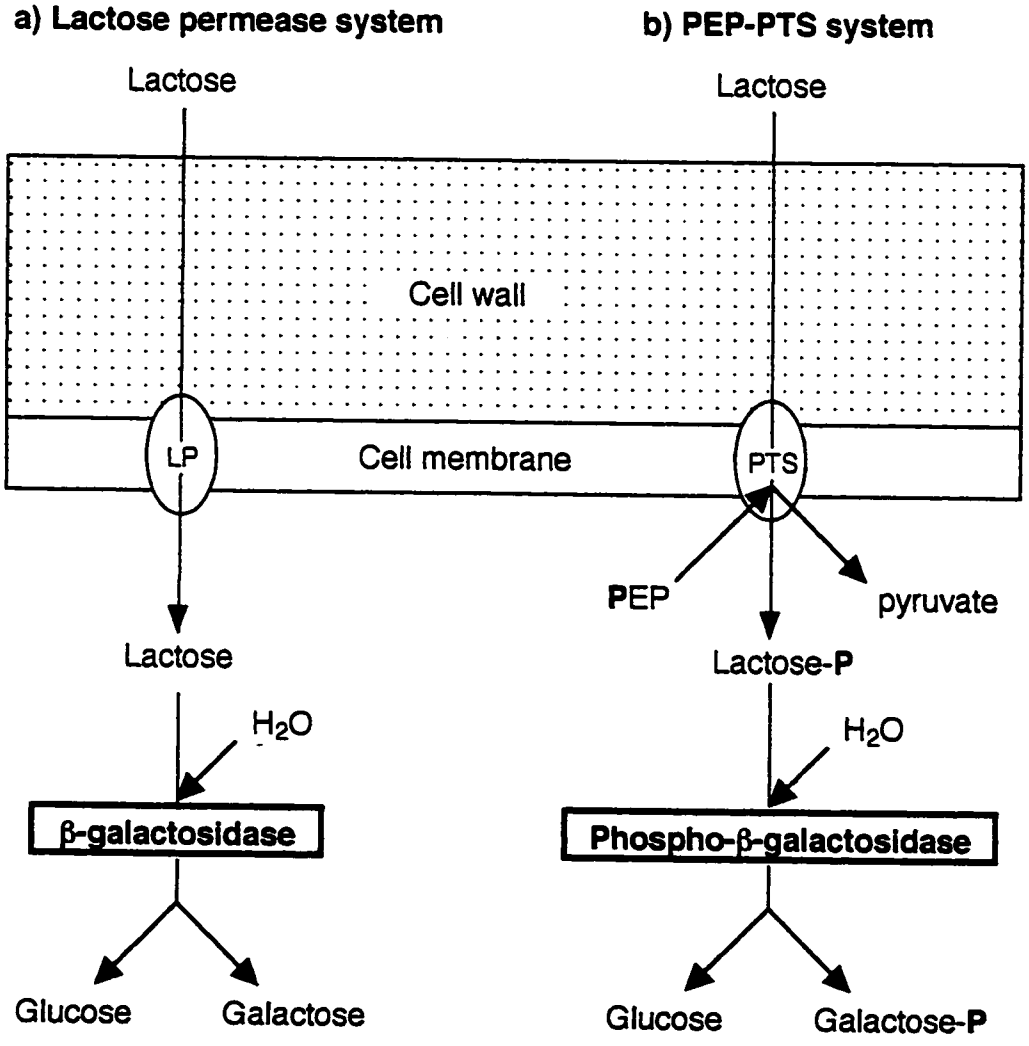

Figure 2-1. Two systems by which lactose is transported and hydrolyzed in lactic acid bacteria. a) Lactose permease system. b) Phosphenolpyruvate-lactose phosphotransferase system (PEP-PTS). Adapted from various sources.

activated lactose to produce glucose and phospho-galactose. The permease system is coded on the chromosomal DNA, whereas the PEP-PTS system is primarily coded on plasmid DNA (M.E. Stiles, 1996, Department of Agricultural Food and Nutritional Science, University of Alberta, personal communication).

The production of β-galactosidase is inducible (Leong-Morgenthaler *et al.*, 1991). For instance, Hickey *et al.* (1986) measured β-galactosidase activity in cultures of *L. delbrueckii* ssp. *bulgaricus* grown on glucose but reported a several fold increase in activity after the addition of lactose. When glucose or galactose was added to cultures growing on lactose, there was a decrease in β-galactosidase activity.

Ventkatesh *et al.* (1993) reported an optimal pH of 5.8 for the growth of *L. delbrueckii* ssp. *bulgaricus* in MRS broth (de Mann *et al.*, 1960) supplemented with lactose. Lactic acid levels greater than 60 g/L were inhibitory. Substrate inhibition was evident as lactose concentrations higher than 80 g/L resulted in slower growth and increased lag times.

L. delbrueckii ssp. *bulgaricus* 11842 is unable to synthesize many of the nutrients it requires and relies on the availability of amino acids, vitamins, purines, pyrimidines, and other factors in the culture medium for growth. The biosynthetic ability of *Lactobacillus* is so limited that even humans have simpler vitamin requirements (Brock and Madigan, 1988). *L. delbrueckii* ssp. *bulgaricus* 11842 is able to grow in whey or ultrafiltered (UF) whey permeate but the addition of complex nutritional supplements such as corn steep liquor (Cox and MacBean, 1977), yeast extract (Cox and MacBean, 1977; Gupta and Gandhi, 1995; Parente and Zottola, 1991), or meat extract (Abd El-Hafez *et al.*, 1994) will markedly increase the rate of growth and acid production.

2.2 Disruption

In some biotechnological applications, a microorganism is cultured under favorable conditions to maximize the production of the desired enzyme or product. The product may be excreted into the growth medium or remain entrapped within the microorganism. As *L. delbrueckii* ssp. *bulgaricus* 11842 utilizes an intracellular β-galactosidase, the cells must be disrupted in order to release the enzyme. Sonication, high-pressure homogenization, microfluidization, and bead milling have all been used for disruption in the laboratory, but only the homogenizer and the bead mill are presently used on an industrial scale.

2.2.1 Sonication

Ultrasonic radiation (20 kHz) is often used to disrupt cells in the laboratory. The device consists of a mechanical transducer (horn) which is placed in the suspension to be disrupted. Doulah (1977) proposed the following relationship for the release of protein S_p as a function of sonication time (t), where k is the protein release constant.

$$1 - S_p = e^{-k \cdot t} \qquad [1]$$

To describe the release of β-galactosidase from *E. coli* cells, Feliu et al. (1998) modified the equation as follows

$$\beta gal = \beta gal_{max} \cdot (1 - e^{-k \cdot t}) \qquad [2]$$

where βgal_{max} is the maximum activity released by sonication and βgal is the activity released after sonicating for time t.

Sound waves are composed of a rarefaction (expansion) phase and a compression phase. Small bubbles formed in the rarefaction phase can collapse in the compression phase producing violent shock waves. Doulah (1977) postulated that the shock waves would interact with each other and the medium, resulting in eddies which in turn would interact to produce smaller and smaller eddies until they were small enough to transfer their energy to the medium as heat. Shear forces arising from eddies larger than the cells are more likely to move the cells rather than disrupt them, whereas, eddies smaller than the cells are capable of generating disruptive shear stresses. Thus, larger cells will experience more eddies than smaller cells. Increasing the power (intensity) will shift the size distribution towards smaller eddies, which in turn, will increase the number of disruptive eddies acting on the cells resulting in greater disruption. James et at (1972) showed that the protein release constant, k, increased linearly with acoustic power when sonicating 200 mL of a 20% yeast suspension in the range of 67 to 187 W, as did Feliu et al. (1998) who sonicated *E. coli* suspensions (5 - 50 mL) using 35 to 95 watts of acoustic power.

To prevent the deactivation of heat sensitive enzymes, Shah and Jelen (1990) cooled their samples in ice-water throughout the sonication. Feliu and Villaverde (1994) used a buffer (pH 7.2) containing 2-mercaptoethanol to reduce oxidation of the β-galactosidase from E. coli and $MgCl_2$ to reduce its denaturation by heating.

Feliu et al. (1998) noticed that the release of protein decreased as the volume of the sample being sonicated increased and derived an expression for the protein release constant, k, based on their results and Doulah's (1977) model of cell disruption.

$$k = h \cdot P \cdot V^{-1} \qquad [3]$$

where P is the acoustic power, V is the sample volume, and h is an empirical constant that varied with the strain of E. coli. In terms of eddies, increasing the sample volume would reduce the power dispersed per unit volume. This would favor the formation of larger eddies and reduce the number of eddies per unit volume which would result in decreased disruption. Although flow-through cells are available for larger volumes (James et al., 1972), sonication is still inefficient and ineffective for pilot scale or industrial use.

2.2.2 Bead Mill

The bead mill, originally used in the paint industry, has been successfully adapted for cell disruption both in the laboratory and in industry. It consists of a horizontal chamber filled with small glass beads (Figure 2-2). As the impeller spins, the beads are

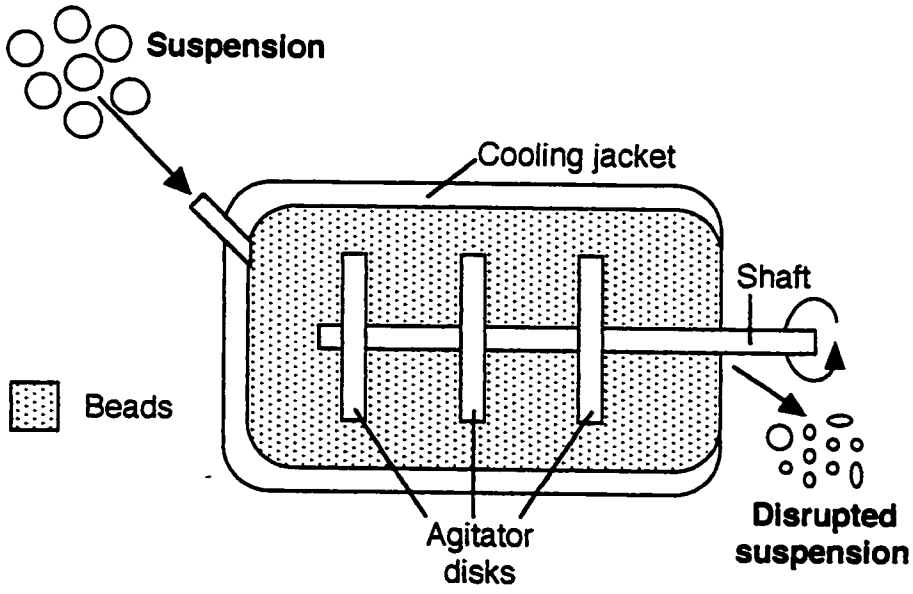

Figure 2-2. Schematic illustration of a bead mill. Based on manufacturer's literature (Glen Mills Inc., Clifton, NJ, USA).

"activated" and the cells are torn apart by the shear forces generated between the beads. Laboratory models are capable of milling smaller volumes (~100 mL) in batch

configuration or larger volumes in a continuous operation. Industrial models operate continuously and are readily available with 275 L, stainless steel grinding chambers.

The degree of disruption depends on several factors such as bead loading, bead size, impeller tip speed, and mean residence time. In general, the following equation can be used to predict the release of protein from microbial cells using a bead mill (Kula and Shütte, 1987)

$$\ln(Rm/(Rm-R)) = k \cdot t \qquad [4]$$

in which k is a first order rate constant, Rm is the maximum protein concentration released by milling, and R is the protein concentration at time t. Rearrangement of equation 4 results in

$$R = Rm\ (1 - e^{-k \cdot t}) \qquad [5]$$

which is similar to equation 2 used to describe the release of β-galactosidase by sonication. Although the release of enzymes can be expressed using equations 4 or 5, these do not account for enzyme deactivation due to factors such as heat or oxidation (Kula and Schütte, 1987) which often results in a decrease of activity with milling time once the rate of deactivation exceeds the rate of solubilization.

Bead loading is the amount of beads loaded into the chamber. For example, with 80% loading, 120 mL of beads would be added to a 150 mL grinding chamber. The degree of disruption increases with bead loading due to increased bead to bead interaction. Heating also increases, because of increased bead to bead interaction, as does the power consumption. A bead loading of 80 - 85% is generally considered optimal (Kula and Schütte, 1987) since the minimal increase in disruption is often outweighed by the heating and power consumption for bead loads greater than 90%.

Bead size can have a profound influence on the disruption of yeasts, fungi, algae, or bacteria. The optimal bead size is dependent on the size of the microorganism. Beads 0.10-0.15 mm in diameter are considered optimal for the disruption of bacteria while beads 0.25-0.75 mm in diameter are used for the disruption of yeasts. The location of the desired protein can also influence the bead size chosen for the disruption. For example, disrupting with larger beads will tend to release periplasmic enzymes in comparison to cytoplasmic proteins (Kula and Schütte, 1987) thus facilitating the purification of periplasmic enzymes. Industrial machines must use beads larger than 0.4-0.6 mm in diameter because of the mechanism for separating the beads from the suspension.

Increasing the impeller tip speed increases the disruption at the expense of power and heat generation. Smaller cells, in general, require higher tip speeds for disruption. A tip speed of 8 m/s is adequate for yeasts while a tip speed of 10 m/s is recommend for the disruption of bacteria (Kula and Schütte, 1987). Similar to the effects of bead loading, tips speeds faster than the optimal may increase heating and power consumption without the additional release of protein.

The longer a cell remains in the chamber, the higher is the probability of its destruction. Therefore, increasing the milling time (batch operation) or mean residence time (continuous operation) will increase the amount of disruption. For continuous operation, increasing the mean residence time by decreasing the flow rate can reduce the effectiveness as the degree of back-mixing increases. To increase the effectiveness and narrow the residence time distribution, two mills in series can be used to increase the mean residence time instead of slowing the flow rate. Thus, if the cell debris are to be removed, the disruption should result in larger cell debris thus facilitating the separation.

The effect of the cell concentration on the release of protein appears to be minimal; but Kula and Schütte (1987) recommended 40-50% cells (wet wt.) to optimize the ratio of power consumption to cell disruption. The bead mill is ideal for heat sensitive products as effective cooling can maintain low temperatures (e.g. 4°C) throughout the disruption. In addition, wet milling virtually eliminates aerosols, it is relatively quiet, reliable, easy to Clean-In-Place (CIP) and well suited for industrial applications.

2.2.3 High-pressure homogenizer

A homogenizer is composed of a piston pump or pumps and a valve assembly. A schematic of a standard homogenizing valve is shown in Figure 2-3a. The pressure generated by the pump is controlled by the spring loaded valve. The fluid flows through the narrow gap between the valve and valve seat only to change direction abruptly at the impact ring. The homogenizer is a vital unit for dairy processing. Milk is often homogenized in two stages (two valve assemblies in series) using pressures around 15 MPa (2500 psi) to break large fat globules (3.3 μm) to smaller ones (0.4 μm) which prevents the separation of cream from the milk. These pressures are much too low to be effective for cell disruption. Instead, pressures ranging from 55-200 MPa (8-29 kpsi) are often utilized for the disruption of cells.

The release of protein can often be expressed using equation 6

$$\ln(Rm/(Rm-R)) = k \cdot N \cdot p^{a} \qquad [6]$$

where Rm is the maximum amount protein of available for release, R is the amount of protein released at time t, k is a rate constant, N is the number of passes, p is the operating pressure, and a is a constant which depends on the microorganism and the conditions of its growth (Kula and Schütte, 1987).

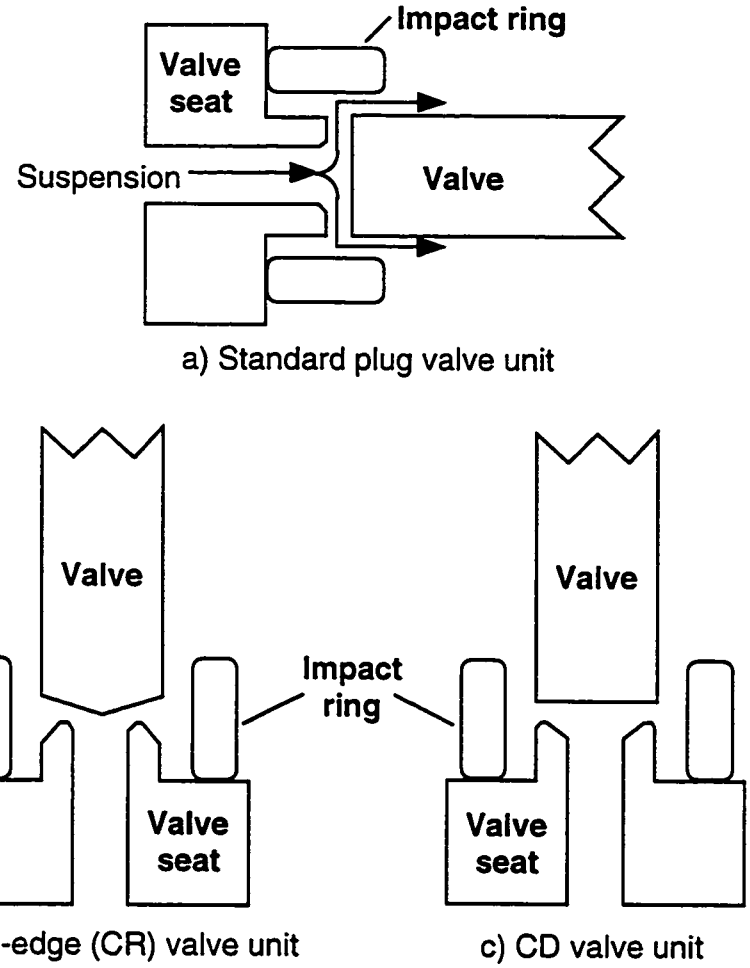

Figure 2-3. Various valve designs for high-pressure homogenization. a) Standard plug valve, b) knife-edge (CR) valve, c) CD valve unit. Based on Kula and Schütte (1987) and Pandolfe (1993).

Considerable amounts of heat are generated, resulting in temperature rises of 0.25-0.26 °C/MPa (Kula and Schütte, 1987). Thus, the temperature of a suspension could increase by 38°C after one pass through a homogenizer operating at 150 MPa. Cooling is required between passages to prevent damage to heat sensitive proteins. Three to five passages are usually required to release 90% of the protein but fewer passes are typically used for practical purposes as the incremental amount of protein released by additional passages may not be economically justified. Increasing the pressure increases the amount of protein liberated per pass, but the additional temperature increase may damage heat

sensitive proteins. In other words, the deactivation of heat sensitive proteins may limit the operating pressure which in turn may increase the number of passages required. Erosion of the valve increases with the operating pressure so the cost of operation at higher pressures may be slightly higher as the valve assembly would have to be replaced more often. High-pressure homogenizers use tungsten carbide or ceramic surfaces to minimize wear. As with the bead mill, biomass concentrations ranging from 20-60% wet wt should have little effect on the release of protein (Kula and Schütte, 1987).

There is some debate as to the mechanism of cell disruption. Some propose that the impact ring has a significant role in disruption (Engler and Robinson, 1981) while others state that the cells are primarily disrupted by cavitation and shear forces generated in the gap between the valve and valve seat (Save *et al.*, 1997); but it is more probable that forces generated in both regions contribute to the disruption of cells (Kula and Schütte, 1987).

The design or configuration of the homogenizing valve can have a considerable influence on the disruption of microorganisms. Kula and Schütte (1987) showed that the knife-edge (Figure 2-3b) or cell rupture (CR) valve design increased the release of enzyme from *Saccharomyces cerevisiae* in recycling experiments at various pressures when compared to that released with a standard configuration (Figure 2-3a). Pandolfe (1993) compared the cell disruption efficiency of the CR and cell disruption (CD) valve using baker's yeast and reported a substantial increase in protein release when using the CD valve design (Figure 2-3c). For the release of protein from microorganisms, a CD valve is superior to a CR design which, in turn, is better than the standard valve configuration.

Laboratory high-pressure homogenizers are suitable for disrupting cell suspensions with volumes as low as 100 mL. High-pressure homogenizers are routinely used in the pharmaceutical and biotechnology industries to disrupt bacteria and yeasts on an industrial scale. Although the use of high-pressure homogenizers may be limited by the temperature increase of the suspension and the potential for aerosols, the technology is dependable, easy to CIP, and familiar to the dairy processing industry.

In terms of operating cost, the high-pressure homogenizer and the bead mill are comparable (Kula and Schütte, 1987; Schütte and Kula, 1993) in that the higher cooling cost of the high-pressure homogenizer is often offset by the power consumption of the bead mill.

2.2.4 Microfluidizer

The Microfluidizer® (Microfluidics Co., Inc., Newton, MA, USA) operates in some respects like a homogenizer. A schematic of the disrupting unit is shown in Figure 2-4. The fluid stream is split and forced through small gaps or orifices to increase fluid velocity. The streams then collide in the reaction chamber where shear forces disrupt the cells. As with the homogenizer, significant amounts of heat are generated and must be removed between passes. The operating pressure is a function of the flow rate, the disruption unit, and the back-pressure unit when used. When operating model M110T (Bury, 1998, unpublished data, Department of Agriculture, Food, and Nutritional Sciences, University of Alberta), the pressure fluctuated from 69 MPa (10 kpsi) to the peak operating pressure of 152 MPa (22 kpsi) because of the single piston air-driven pump. In this instance, the release of protein would fluctuate with the operating pressure making it difficult to relate disruption as a function of pressure. Sauer et al. (1989)

proposed equation 7 (modified for consistency) for the disruption of native and recombinant *E. coli* using model M110T

$$\ln(Rm/(Rm-R)) = k \cdot N^b \cdot p^a \qquad [7]$$

where Rm is the maximum amount of protein released by microfluidization, R is the amount of protein released at time t, k is a rate constant, N is the number of passes, p is the operating pressure, a is a constant which depends on the microorganism and the conditions of its growth, and the empirical exponent b varies with the type of cell, the growth rate of the cell, and the concentration of cells.

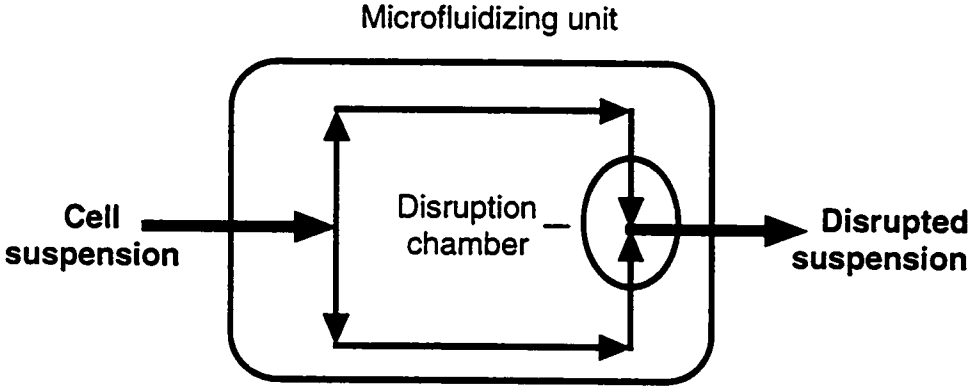

Figure 2-4. Conceptualized flow in a Microfluidizer

Although the microfluidizer was shown to be capable of disrupting *E. coli* (Sauer et al., 1989) the applicability of disrupting *L. delbrueckii* ssp. *bulgaricus* 11842 on an industrial scale is questionable.

2.3 Industrial hydrolysis of lactose

2.3.1 Enzymatic hydrolysis of lactose in milk

There are several processes used for the enzymatic hydrolysis of lactose in milk. The easiest method involves the direct addition of soluble β-galactosidase to milk. The

highly purified enzyme is added to milk and the mixture is held at a specified temperature until the desired degree of hydrolysis is reached, whereupon the milk is pasteurized and packaged. Although easy and effective, the process is somewhat costly as the highly purified, expensive β-galactosidase must be used in a "throw-away mode". Milk produced using this process has 99% of the lactose hydrolyzed and is commercially available in North America at almost double the price of regular milk. The β-galactosidase must be highly purified as contamination with proteases can lead to quality issues. Consumers can also purchase a solution of purified β-galactosidase to add to their milk or ingest tablets of acid stable β-galactosidase to prevent the discomfort of lactose malabsorption while still enjoying the flavour of untreated milk and other dairy products. Unfortunately, the drops and tablets are costly in comparison to milk or other lactose reduced formulations.

Other industrial scale processes, described by Honda et al. (1993) and Mahoney (1997), attempt to recover or reuse the enzyme by entrapment or attachment to a suitable carrier. SNAM Progetti (Centrale del Latte, Italy) utilizes β-galactosidase (*Kluyveromyces lactis*) entrapped in cellulose triacetate fibers. Skim milk is essentially sterilized by heating to 143°C for 3 sec and cooled to 4-7°C for hydrolysis. The milk is circulated through fibers held in a skeen along the reactor axis. The lactose diffuses through the fibers and is hydrolyzed by the entrapped β-galactosidase. One of the limiting constraints is the rate of lactose diffusion into the fiber and the subsequent diffusion of glucose and galactose out of the fiber. The facility was producing 8000 L/day in 1993 (Honda *et al.*, 1993).

The Sumitomo (Sumylact) process (Snow Brand Milk Products, Sapporo, Japan) uses β-galactosidase (*Aspergillus oryzae*) covalently attached to beads of phenol-formaldehyde resin. Reconstituted skim milk (12% solids) is pasteurized at 65°C for 30 min, cooled to 5-7°C and recirculated through a packed bed for 16-18 hours at 45°C (Honda *et al.*, 1993). The pilot scale process was capable of hydrolyzing 200 L per run demonstrating its applicability for larger volumes.

In each case, the milk and the reactor must be kept in a near-sterile condition since the hydrolysis is usually carried out at a temperature and pH favorable for the growth of microorganisms. Gentle washing and sanitizing prolongs the life span and the activity of the immobilized enzyme while reducing microbial build-up (Baret, 1987).

Hydrolysis at low temperature may reduce the rate of microbial growth but increases the time required for hydrolysis. Higher temperature may discourage the growth of microorganisms while accelerating the rate of hydrolysis. Unfortunately, the half-life of the enzyme may be reduced at the elevated temperatures necessitating the premature replacement of β-galactosidase.

2.3.2 Acid hydrolysis of whey

Lactose solutions could be hydrolyzed by acidification to pH 1.2 and heating to 150°C until the desired degree of hydrolysis, typically 80%, is achieved (Zadow, 1986). The solution is then neutralized by the addition of alkali. The protein must be removed prior to acid hydrolysis in order to avoid detrimental side reactions, so ultrafiltered (UF) whey permeate would be ideal for this process. Still, the non-protein nitrogen content is a factor which contributes to browning and may be undesirable. Strong acidic ion exchange resins have also been used to hydrolyze UF whey permeate (Boer and Robbertsen, 1981).

Acid hydrolysis is limited to relatively pure solutions of lactose which limits its use and excludes applicability to proteinaceous dairy products such as milk.

2.3.3 Enzymatic hydrolysis of whey

Soluble or immobilized β-galactosidase has also been used to hydrolyze the lactose in whey or ultrafiltered (UF) whey permeate. Guy and Edmondson (1978) suggested that 75% hydrolysis is optimal for the production of a syrup (60-70% solids) in order to minimize the crystallization of galactose and lactose. As with milk, addition of soluble β-galactosidase is costly, especially since the syrup must compete with cheaper, more conventional sweeteners. Processes using fixed enzyme reactors can reduce the cost of the syrup but the whey or UF permeate is typically demineralized and deproteinized to increase the life of the enzyme in addition to improving the sensory properties of the syrup.

In the Corning process (Baret, 1987; Mahoney, 1997; Zadow, 1986), β-galactosidase from the fungus *Aspergillus niger* is covalently bound to a controlled-pore silica carrier of 30/45 US mesh size (0.4-0.8 mm). The whey is typically deproteinized by ultrafiltration to prevent fouling of the reactor and demineralized using ion exchange (Mahoney, 1997). Hydrolysis takes place in a fixed bed reactor operated at the optimum pH of 3.2 - 4.3 (Zadow, 1986) and a temperature of 50°C. The low pH and the relatively high temperature both help to discourage the growth of microorganisms in the reactor. There were two plants in operation in 1987; one in the UK and one in the US (Mahoney, 1997). In the Kentucky facility, UF permeate from cottage cheese whey was hydrolyzed to provide a substrate for the production of baker's yeast (Zadow, 1986). Neither plant is

active at the present time (P. Jelen, 1999, Department of Agricultural Food and Nutritional Science, University of Alberta, personal communication)

The Valio process (Valio Dairy, Finland) utilizes phenol-formaldehyde resin beads to which β-galactosidase from *A. niger* is adsorbed and fixed in place with glutaraldehyde (Mahoney, 1997). The fixed bed reactor operates at pH 3.5 and at sub-optimal temperatures of 20 - 40°C to extend the life of the enzyme. The process has been used for the hydrolysis of lactose in whey and UF whey permeate for several years (Mahoney, 1997). Zadow (1986) also mentions the development of systems designed for the hydrolysis of sweet wheys and milk.

Based on 1980 data, a syrup (65% solids) produced by the Corning process would cost $(US) 0.26-0.46 per kg (Mahoney, 1997). In 1987, a syrup (60% solids) produced by the Valio process was estimated to cost $(US) 0.13 per kg (Mahoney, 1997). For comparison, granular sugar sold for $(US) 0.15-0.50 per kg in 1998 depending on the grade (electronic communication, New York historical data, Coffee, Sugar, Cocoa Exchange, Inc, NY, USA). Use of cheaper supports for enzyme immobilization and continued refinement of the various processes should reduce the cost of hydrolyzing the lactose in milk, whey, or UF whey permeate.

2.4 Hydrolysis using disrupted bacterial cultures

Shah and Jelen (1991) showed that the addition of a sonicated culture of *L. delbrueckii* ssp. *bulgaricus* 11842 to milk was effective in hydrolyzing the lactose. As current methods of hydrolysis used highly purified and expensive β-galactosidase, Jelen (1993) suggested the possibility of using sonicated dairy cultures for lactose hydrolysis.

Further research was needed to evaluate the applicability of using disrupted dairy cultures for the hydrolysis of lactose on a pilot or industrial scale. This requires the growth of a suitable dairy bacterium on, preferably, inexpensive and readily available growth medium. The culture would then have to be disrupted in an industrial facility. An economic evaluation of the process would also be required to ascertain if further research is recommended. The investigations in the following chapters attempt to address these issues.

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Chapter 3

Whey protein concentrate as a nutrient supplement for Lactic Acid Bacteria.*

3.1 Introduction

Fermentation of whey by Lactic Acid Bacteria (LAB) usually focuses on the production of lactic acid (Reddy *et al.*, 1976, Tejayadi and Cheryan, 1995). Alternatively, whey or whey permeate has the potential to serve as a culture medium for the propagation of dairy cultures (Parente and Zottola, 1991). Whey or UF whey permeate are cheap and readily available sources for use as fermentation media, but require supplementation with a complex additive such as yeast extract (Gupta and Gandhi, 1995, Parente and Zottola, 1991) or corn steep liquor (Cox and MacBean, 1977) for LAB to flourish. In the course of our screening experiments with various potential supplements (Appendix, figures A-1 through A-4), it was observed that the addition of a whey protein concentrate (WPC) to whey or UF whey permeate significantly increased both the cell numbers and the lactic acid production of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 and *Streptococcus thermophilus* ST20.

The objective of this communication is to illustrate the effect of the WPC and of the two main whey protein fractions (α-lactalbumin and β-lactoglobulin) as enhancers of microbial growth using the yogurt organisms *L. delbrueckii* ssp. *bulgaricus*. 11842 and *S. thermophilus* ST20.

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* A version of this chapter was published; Bury, D., Jelen, P. and Kimura, K. (1998) *International Dairy Journal*, **8**, 149-151.

3.2 Materials and Methods

3.2.1 Whey or whey UF permeate broths

To prepare the broths, 9 g of industrial sweet whey powder (Kraft, Montreal, QC) or whey UF permeate powder (Protose Separations Inc., Toronto, ON) was added to a 250 mL Erlenmyer flask and dissolved in 150 mL of distilled water.

3.2.2 Supplementation

The broths were supplemented with 1 or 2% (w/v) industrial grade α-lactalbumin or β-lactoglobulin (Protose Separations Inc., Toronto, ON), whey protein concentrate (Alacen 855, New Zealand Milk Products, Inc., Santa Rosa, CA), or Bacto-peptone (Difco, Detroit, MI).

Supplemented and unsupplemented whey or UF permeate broths were sterilized by heating to 121°C for 15 minutes. Alternatively, UF permeate broths supplemented with 1% α-lactalbumin, β-lactoglobulin, WPC, or Bacto-peptone were also pasteurized by heating to 72°C for 20 minutes.

3.2.3 Inoculation and fermentation

L. delbrueckii ssp. *bulgaricus* 11842 and *S. thermophilus* ST20 were propagated in MRS broth (Difco, Detroit, MI) containing 1% lactose (Fisher Scientific Co., Springfield, NJ). To prepare the inoculum culture, 0.5-0.7% of propagation culture was added to 3% whey or UF permeate broth containing 0.8% MRS powder (for inoculation of whey broths) or 1.7% MRS powder (for inoculation of UF permeate broths). The flasks were incubated overnight (14-19 hours) at 42±1°C and ~100 RPM in a controlled environment incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ). Sterilized media were inoculated with 3.2% active *L. delbrueckii* ssp. *bulgaricus* 11842

or *S. thermophilus* ST20 culture and pasteurized media were inoculated with 6.3% culture.

The fermentations were carried out in duplicate flasks at 42±1°C and ~100 RPM in the same incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ). Broths were maintained at pH 5.5±0.4 by the manual addition of 2N NaOH (Fisher Scientific Co., Springfield, NJ). Permeate broths for comparing the growth of *S. thermophilus* ST20 and *L. delbrueckii* ssp. *bulgaricus* 11842 were maintained at pH 6.1±0.1. Growth was monitored by direct microscopic enumeration (using a hemacytometer) and the rate at which NaOH was added.

3.3 Results and discussion

Figure 3-1 shows that unsupplemented whey broth was a better growth medium than unsupplemented whey UF permeate broth for growth and acid production which hints that some growth factors may be removed by ultrafiltration. Not surprisingly, addition of WPC to whey or UF permeate broths resulted in a substantial increase in growth and rate of acidification (Fig. 3-1). Defining productivity as the rate of acid production (or the rate of NaOH addition), productivity increased with the amount of supplementation. For *L. delbrueckii* ssp. *bulgaricus* 11842, supplemented whey broths were more productive than whey UF permeate broths at the same levels of supplementation (Figs. 3-1 and 3-2). *S. thermophilus* ST20 was more productive in supplemented permeate broth than supplemented whey broth, possibly because *S. thermophilus* prefers to grow at the higher pH maintained with the permeate broth in this instance.

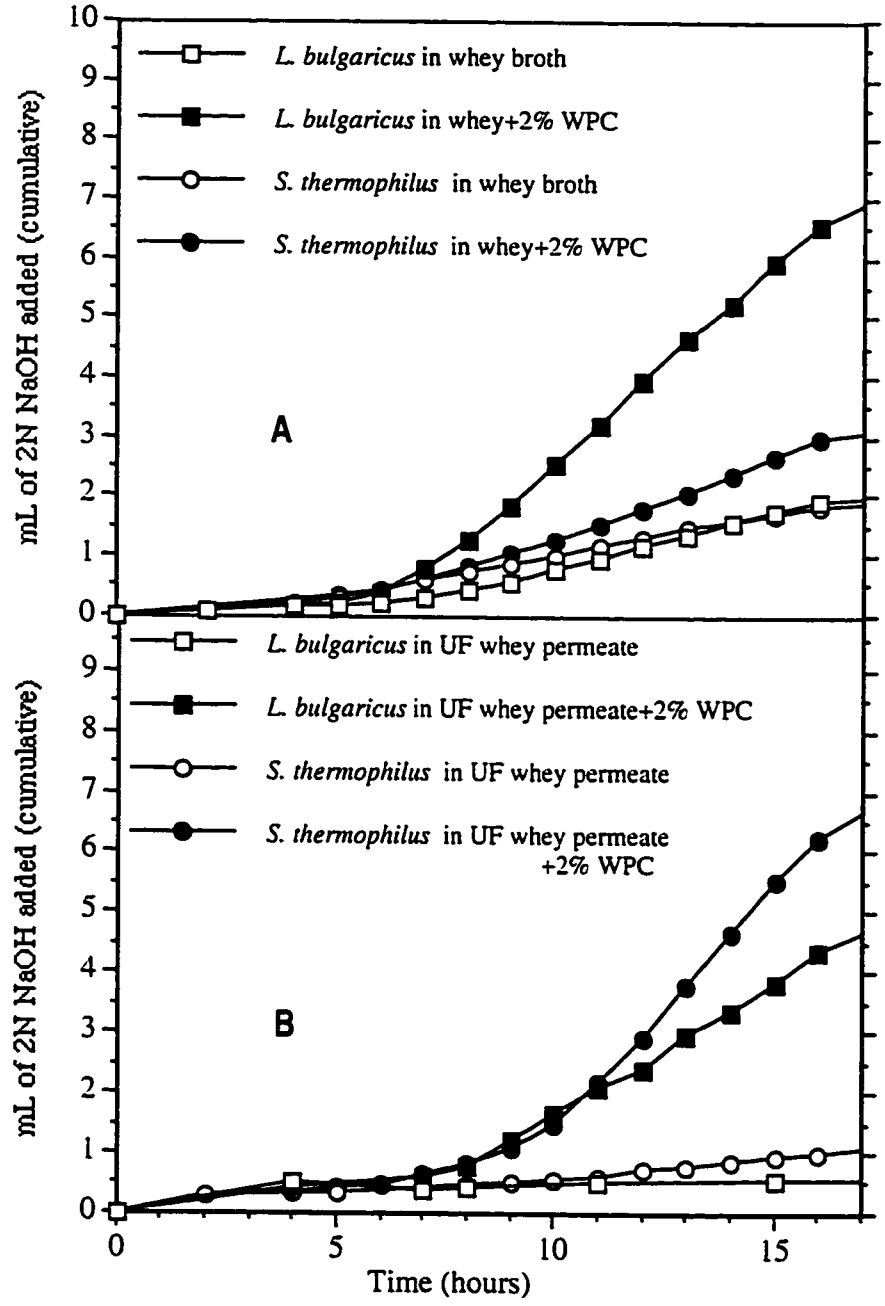

Figure 3-1. Acid production of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 and *Streptococcus thermophilus* ST20 in (A) sterilized (121°C/15 min) whey broth (60 g/L spray dried whey powder) supplemented with 2% WPC; (B) sterilized whey UF permeate broth (60 g/L spray dried whey permeate powder) supplemented with 2% WPC. Average (n=2) for experiment.

The major whey proteins alone may not be responsible for the observed growth as broths with 1% WPC were 1.6 times more productive than broths containing 1% α-

lactalbumin or 1% β-lactoglobulin (Fig. 3-2). *L. delbrueckii* ssp. *bulgaricus* was slightly more productive in whey broth supplemented with β-lactoglobulin than whey broth supplemented with α-lactalbumin, but little difference between the two was observed in UF permeate broth. It is possible that the increased productivity may be due to impurities in the industrial whey protein fractions and not the presence of α-lactalbumin or β-lactoglobulin. Addition of Bacto-peptone to whey UF permeate broth had little or no effect, suggesting that small peptides may not be contributing to the increased growth observed with WPC. Likewise, the extra lactose present within the WPC did not seem to account for the increased growth since preliminary work (Appendix, figures A-3 and A-4) showed little, if any, difference between the rate of acid produced by *L. delbrueckii* ssp. *bulgaricus* with 6% and 8% whey or UF permeate powder. Prior experiments showed that the viability and productivity of *L. delbrueckii* ssp. *bulgaricus* 11842 decreased with solids contents greater than 8% for unsupplemented whey broth and 4% for unsupplemented UF permeate broth. The factor(s) responsible for enhancing the fermentation are obviously heat stable as there was little difference in the growth of *L. delbrueckii* ssp. *bulgaricus* in sterilized or pasteurized UF permeate broths despite the denaturation and flocculation observed in sterilized broths.

3.4 Conclusions

Whey protein concentrates can stimulate the growth of lactic acid bacteria in whey or UF whey permeate broths. Supplementation with the major whey proteins (α-lactalbumin and β-lactoglobulin) was only 63% as effective as WPC. The components responsible for the increased growth are heat stable as the stimulatory effect is not lost after heating to 121°C for 15 minutes. The heat stable components might be α-nucleo-

tides, non-protein nitrogen, or some specific heat stable peptides not present in Bacto-peptone.

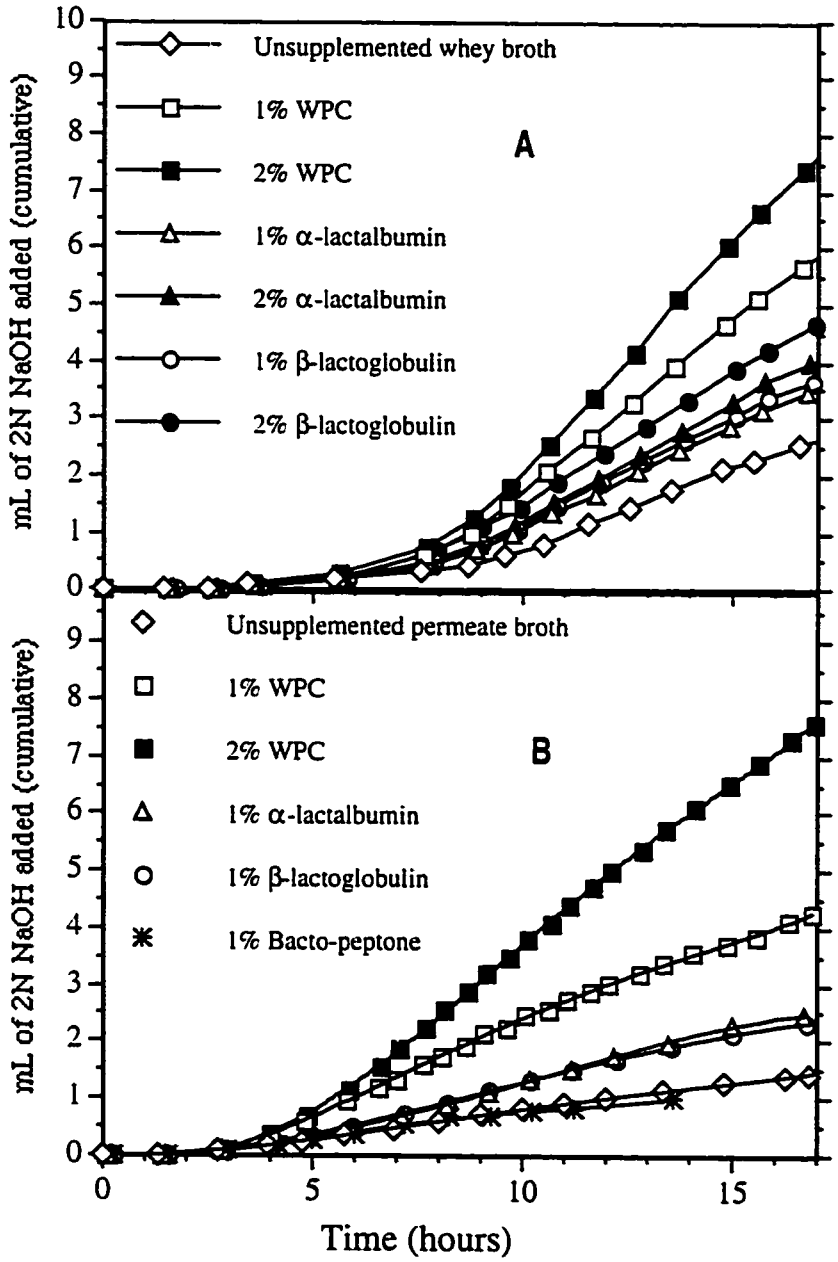

Figure 3-2. A comparison of lactic acid production by *Lactobacillus delbrueckii* spp. *bulgaricus* 11842 grown in (A) sterilized (121°C/15 min) whey broth supplemented with WPC, α-lactalbumin, or β-lactoglobulin; (B) pasteurized (72°C/20 min) whey UF permeate broth (60 g/L spray dried permeate powder) supplemented with WPC, α-lactalbumin, β-lactoglobulin, or Bacto-peptone. Average (n= 2 to 4) for one to three experiments.

3.5 References

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Chapter 4

Growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 in whey supplemented with various whey protein concentrates.*

4.1 Introduction

Whey protein concentrates (WPC) and whey protein isolates (WPI) are gaining popularity and acceptance as functional ingredients in a wide range of nutritional applications and as ingredients in food as well as non-food products (Morr and Foegeding, 1990). For example, whey proteins are recognized as a nutritional supplement for active sports enthusiasts (Durham *et al.*, 1997). In Australia, a whey protein fraction has been produced to replace the fat in sausages (Mann, 1997). WPC's and WPI's may also have therapeutic roles in cancer treatment (Kennedy *et al.*, 1995) or as immunopotentiating agents (Bounous *et al.*, 1988).

Whey and whey permeate can be difficult to utilize (Jelen, 1991). One means of utilization involves their use as inexpensive natural media for the propagation of lactic acid bacteria (Holsinger *et al.*, 1974). However, to encourage the growth of *Lactobacilli* in whey or whey permeate, complex nutrients such as skim milk powder (Parente and Zottola, 1991), malt sprouts (Abd El-Hafez *et al.*, 1994), corn steep liquor (Cox and Macbean, 1977), yeast extract (Cox and Macbean, 1977; Gupta and Gandhi, 1995), meat extract (Abd El-Hafez *et al.*, 1994), peptides, or regular or hydrolyzed whey proteins (Champagne *et al.*, 1991) must be added. Supplementation of a whey-based medium with a commercial WPC increased both cell counts and the rate of acid production by

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* A version of this chapter was published; Bury, D., Hajsmanova, M. and Jelen, P. (1999). *Milchwissenschaft* **54** (in press).

L. delbrueckii ssp. *bulgaricus* 11842 and *Streptococcus thermophilus* ST20 (Chapter 3). Whey based media have been utilized in the dairy industry for the growth of starter cultures (Jelen, 1991).

The main objective of this research was to compare the effectiveness of several commercial whey protein concentrates (WPC's) and whey protein isolates (WPI's) as promoters of growth and acid production by *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 in reconstituted whey.

4.2 Materials and methods

4.2.1 Microorganism and media

Lactobacillus delbrueckii ssp. *bulgaricus* 11842 was obtained from University of Alberta, Department of Agricultural, Food and Nutritional Science. The culture was propagated every 4 weeks in sterile MRS broth (Difco, Detroit, MI) enriched with 1% w/v of α-lactose (Fisher Scientific Co., Springfield, NJ). The inoculum was grown for 10 hours in 150 mL medium containing reconstituted whey powder (3% w/v, Kraft, Montreal, QC) and MRS (1.3% w/v) just prior to the main fermentation experiments. Whey-based broths contained 6% (w/v) whey powder and 1% (w/v) of one of the alternative whey protein preparations investigated (composition and sources of WPC's and WPI's given in Table 4-1). In one series of experiments, 0.8% (w/v) WPC-B and 0.2% α-lactose was added to the whey broth in order to approximate the protein and lactose content of WPC-A.

Table 4-1: Composition of the investigated whey protein concentrates and whey protein isolates as specified by the suppliers*.

	Composition (% w/w)				
	Protein	Lactose	Fat	Ash	Moisture
WPC's					
A	62	23	5.0	3.0	5.5
B	77	2 - 6	6.0	4.0	4.0
C	78	4	5 - 5.5	4.0	4.0
D	77	4.6	3.4	3.0	5.2
WPI's					
E	88	< 1	< 1	3.0	4.0
F	94	< 1	< 1	1.6	4.0

* The suppliers in random order :

DMV USA, La Crosse, WI, USA.
Land O' Lakes, St. Paul, MN, USA.
Promil, Novy Bydzov, Czech Republic.
Canadian Inovatech Inc., Abbotsford, BC, Canada.
New Zealand Milk Products, Inc., Santa Rosa, CA, USA.

4.2.2 Fermentation

All fermentations were carried out in 300 mL Erlenmeyer flasks containing 150 mL medium sterilized by heating at 121 °C for 15 minutes. The bacteria were grown at 43°C and 160 rpm in a controlled environmental incubator shaker (New Brunswick Co., New Brunswick, NJ). The various growth media were brought to 43°C and inoculated with 3.3 % v/v of the inoculum culture described above. The pH was maintained at 5.8 ± 0.2 by the manual addition of 2N NaOH and measured using a Silver/Silver Chloride Reference probe (Litho, USA) with a digital pH-meter (Orion Research). The cell growth was monitored by the rate of 2N NaOH added, by direct microscopic enumeration, and by counting colonies on plates of MRS agar after incubation (New Brunswick Co., New Brunswick, NJ) at 43°C for 48 hours. All experiments were completely replicated at least twice.

4.3 Results and discussion

In general, the addition of any WPC (but not necessarily WPI) increased the rate of lactic acid production by *L. delbrueckii* ssp. *bulgaricus* 11842. Supplementation with WPC-A had the greatest effect resulting in the highest rate of acid production (Fig. 4-1) while addition of WPI-F did not appear to have any effect. WPC-A had the highest lactose content (23% w/w) and the lowest protein content (62%) while WPI-F had the lowest lactose content (<1%) and the highest protein content (94%).

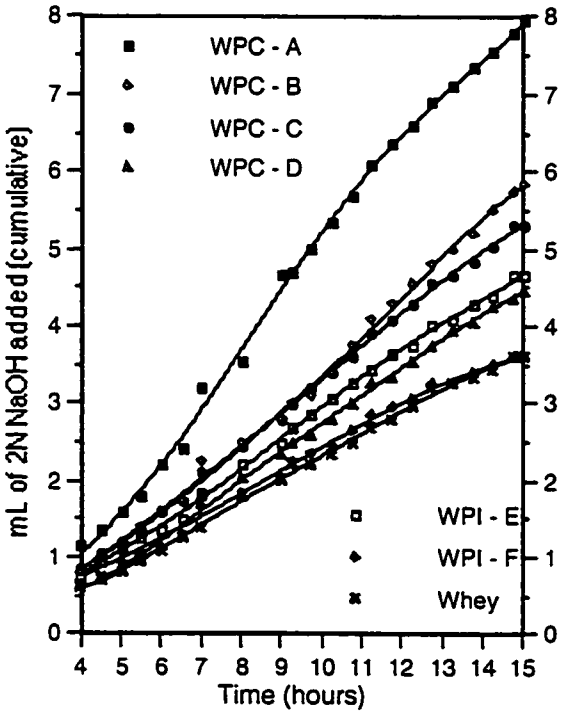

Figure 4-1. Lactic acid production of *L. delbrueckii* ssp. *bulgaricus* 11842 grown on whey broth supplemented with four whey protein concentrates (WPC) and two whey protein isolates (WPI).

In order to see whether the additional lactose had played any role in the increase, α-lactose and WPC-B were blended to match the level of lactose and protein of WPC-A. Figure 4-2 shows that addition of α-lactose to WPC-B had little or no effect on the rate of

acid production suggesting that lactose may not be responsible for the increase shown with WPC-A. Similarly, Bury *et al.* (1998) claimed that increasing the whey broth solids concentration from 6% to 8% did not affect the rate of acid production, but provided extra nutrient resulting in a higher concentration of lactic acid at the end of the fermentation.

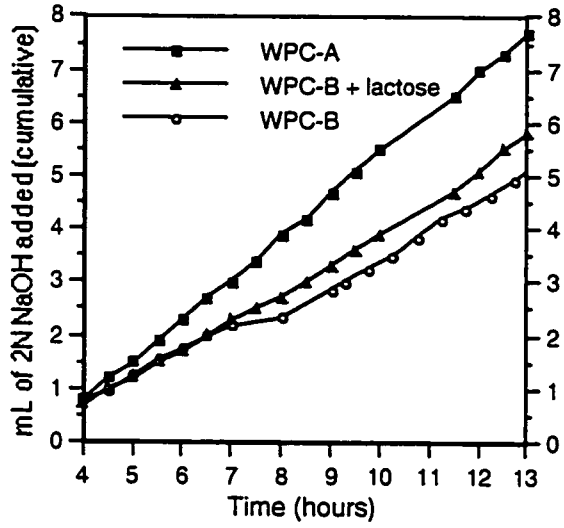

Figure 4-2. Effect of additional lactose on the acid production of *L. delbrueckii* ssp. *bulgaricus* 11842 grown in whey supplemented with two WPC products showing the highest effect.

The rate of acid evolution in whey supplemented with WPI-F was comparable to that produced in whey alone. Similarly, Bury *et al.* (1998) showed that industrial isolates of α-lactalbumin and β-lactoglobulin were not responsible for the increased rate of acid production observed with the addition of a WPC to whey. Since WPI's tend to be rich in α-lactalbumin and β-lactoglobulin, it is not surprising that the addition of WPI-F did not increase the rate of lactic acid production. Supplementation with WPI-E (88% protein) showed a slight increase which was comparable to the rate of acid production with WPC-D (77% protein). In a complementary set of experiments carried out in our laboratory, the

effects of a WPC-35 and a WPI-80 were similar to that of WPC-B and WPC-C in regards to growth and acid production of *L. delbrueckii* ssp. *bulgaricus* 11842 on whey-based media (Appendix, figure A-5). The total protein content does not appear to have a direct influence on the effect observed. It is more probable that the type of protein or the range of proteins in a given WPC would result in increased growth and acid production. The source of whey and its subsequent processing could have a significant influence on the amount and effectiveness of the growth factors present in the various whey protein products. The presence of non-protein nitrogen, carbohydrates, glycoproteins, vitamins, or other components may also have a significant influence on the growth of *L. delbrueckii* ssp. *bulgaricus* 11842. In general, addition of any WPC resulted in higher rates of lactic acid production relative to supplementation with WPI. As WPC's would have more impurities relative to WPI's, it is likely that the impurities may have an important role in the stimulation of growth.

Although addition of most WPC products increased the rate of acidification, a control MRS broth was still approximately 5.6 times more productive than whey-based broth supplemented with WPC-B. Preliminary fermentations with blends of whey, MRS, and WPC-A showed increased growth and acid production in comparison to fermentations in whey or MRS broth alone (Appendix, figure A-6).

In whey broth, a maximum viable cell count of about 2 x 10^8 CFU mL^{-1} was obtained with about 8 hour fermentations (Fig. 4-3). Addition of WPC-C increased the maximum viable cell count to 5 x 10^8 CFU mL^{-1}, while broths containing WPC-A or WPC-B reached maximum viable cell counts of 1 x 10^9 CFU mL^{-1} to 1.6 x 10^9 CFU mL^{-1} 10 to 13 hours after inoculation. It is interesting to note that the production of lactic acid

continued long after the maximum cell count had been reached. The results are comparable to those of Bury *et al.* (1998), and of Beal and Corrieu (1995) who, in fermentations using pure and mixed cultures of *L. bulgaricus* 398 and *Streptococcus thermophilus* 404, observed continued acid production even though the cell concentration was no longer increasing. Goncalves *et al.* (1991) also reported this effect and suggested that lactic acid production by *L. delbrueckii* NRRL B445 on glucose has "growth-associated" and "non-growth-associated" kinetics.

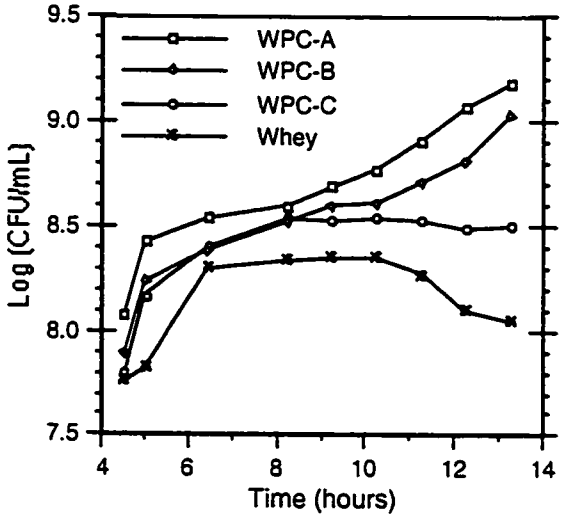

Figure 4-3. Viable cell count (colony forming units per mL) of *L. delbrueckii* ssp. *bulgaricus* 11842 grown on whey broth supplemented with various whey protein concentrates (WPC).

4.4 Conclusions

L. delbrueckii ssp. *bulgaricus* 11842 showed increased growth and acidification in whey broths supplemented with several WPC products, whereas the effect of the two WPI's was lower. The highest lactic acid production and cell growth was supported by Promil, a relatively low protein content WPC. Addition of lactose to WPC-B to a level

comparable to that of the Promil, had little or no influence on the rate of acidification suggesting that the effect of the Promil was not due to the additional lactose.

Further research is needed to elucidate the growth-promoting effects of the WPC for *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 as it appears that the proteins contained in the various preparations may not be the component responsible for these effects. It is possible that other components or impurities such as non-protein nitrogen, glycoproteins, vitamins or other carbohydrates may play a much more important role.

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Chapter 5

Growth and β-galactosidase activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 in whey supplemented with yeast extract.*

5.1 Introduction

It is estimated that 70% of the world's population are lactose maldigesters (Holsinger, 1992) and may experience unpleasant gastrointestinal symptoms such as cramps, bloating, flatulence, or diarrhea after consuming lactose. Hydrolyzing lactose to glucose and galactose would allow lactose maldigesters to consume a wider range of dairy products thereby expanding the market. Current industrially applicable methods of lactose hydrolysis involve the use of β–galactosidase (EC 3.2.1.23) purified from bacteria, yeasts, or fungi. The addition of soluble β–galactosidase to milk or whey is simple but can be costly since the purified enzyme is lost with the product. Other, more cost effective approaches (Mahoney, 1997) may involve immobilization of purified β–galactosidase by entrapping in cellulose acetate fibers, adsorbing to phenol-formaldehyde resin, or covalently binding to porous glass beads; however, in general, these enzyme immobilization technologies may be effective only for high volume operations.

Shah and Jelen (1990, 1991) investigated the hydrolytic activity of β–galactosidase released from sonicated dairy cultures on the substrate o-nitrophenyl-β–D-galactopyranoside (ONPG) with the idea of using disrupted cultures as a potentially economical means to achieve hydrolysis of lactose in milk or whey for certain

* A version of this chapter was submitted for publication. Bury, D. and Jelen, P. (1999) *Food Research International*

applications. The batch addition of a sonicated dairy culture (*L. delbrueckii* ssp. *bulgaricus* 11842) as a source of soluble lactase was shown to be effective under experimental conditions (Jelen, 1993).

Although *L. delbrueckii* ssp. *bulgaricus* 11842 grows very well on MRS or other complex broths, an inexpensive and readily available growth medium, such as whey, would be desirable for fermentation on an industrial scale. *L. delbrueckii* ssp. *bulgaricus* 11842 is able to grow on sweet cheese whey or whey permeate, but the fermentation time can be substantially shortened with complex growth supplements. Addition of corn steep liquor (Cox and MacBean, 1977, Reddy *et al.*, 1976), molasses (Gupta and Gandhi, 1995, Chiarini *et al.*, 1992), whey protein concentrate (Chapters 3 and 4), meat extract (Abd El Hafez *et al.*, 1994), or yeast extract (Gupta and Gandhi, 1995, Abd El Hafez *et al.*, 1994, Parente and Zottola, 1991, Cox and MacBean, 1977, Reddy *et al.*, 1976) increased the rate of growth and acid production by lactic acid bacteria in whey, but the level of supplementation for optimal growth or acid production may not necessarily be the best for the production of β–galactosidase.

The purpose of this study was to examine the production of active β–galactosidase from *L. delbrueckii* ssp. *bulgaricus* 11842 grown in sweet whey supplemented with various amounts of yeast extract, using two different experimental fermentation procedures.

5.2 Materials and Methods

5.2.1 Microorganisms and Media

L. delbrueckii ssp. *bulgaricus* 11842 was obtained from University of Alberta, Department of Agricultural, Food and Nutritional Science. The culture was propagated every two weeks in sterile MRS broth (Difco, Detroit, MI) enriched with 1% w/v alpha-lactose (Fisher Scientific Co., Springfield, NJ). To prepare the culture for fermentation in the whey based broth, about 0.5 mL of refrigerated culture was added to 5 mL of the above MRS / lactose broth. After incubation at 43°C for 8 to 12 hours, 1 mL of active culture was used to inoculate 150 mL of sterile broth containing 3% (w/v) reconstituted whey powder and 1.3% (w/v) MRS powder (WMRS broth). This culture was incubated at 43±1°C and 140±30 RPM in a controlled environment incubator shaker (New Brunswick Co., New Brunswick, NJ) for 10 - 12 hours.

The whey based broths used for the fermentations were made by dissolving 6 g spray dried whey powder (Kraft, Montreal, QC) per 100 mL of distilled water and adding the desired amount of powdered yeast extract (Fermtech, BDH Inc, Toronto). Flasks containing 150 mL of this whey based broth were autoclaved at 121°C for 15 minutes. For larger fermentations, two 2000 mL flask containing 750 mL of whey based broth were autoclaved at 121°C for 20 minutes and aseptically transferred to a sterile 2 L fermenter (New Brunswick Co., New Brunswick, NJ).

5.2.2 Fermentation

Flasks containing 150 mL of sterile whey based broth supplemented with 0, 0.2, 0.4, 0.6, 0.8 or 1% yeast extract (YE) were inoculated with 5 mL of the culture grown in WMRS broth. The culture was grown in the incubator shaker at 40 - 43°C and 140±30

RPM. The pH was monitored using a combination pH probe sterilized with 70% ethanol and maintained at 5.5 ± 0.3 by the manual addition of 2N NaOH (Fisher Scientific Co, Springfield, NJ). Cell growth was followed by direct microscopic enumeration and the rate of acid production was determined as the consumption of 2N NaOH. The culture biomass was determined by centrifuging two 10 mL samples at 10 000 x g for 15 minutes and decanting the supernatant. The pellets were freeze dried and placed in a desiccation chamber at room temperature until constant mass reading was reached. The net biomass content was determined by subtracting the mass of dried pellet obtained from the sterilized broth containing no culture.

The 1.5 L fermentations were inoculated with approximately 50 mL of the culture grown in the WMRS broth. The temperature was controlled by placing the fermenter in a water bath at 43 ± 0.2°C. The pH was maintained at pH 5.0 or 5.5 ± 0.1 by the addition of 2N NaOH via a peristaltic pump (Watson-Marlow, USA) controlled by a pH controller (Omega Engineering Inc., USA). Direct microscopic examination was used to determine the total cell count. The rate of acidification was inferred from the amount of 2N NaOH added during the fermentation. Dry matter was measured in duplicate or triplicate as above using 5 mL samples.

5.2.3 Measuring β–galactosidase activity

A method of measuring β–galactosidase activity using o-nitrophenyl-β–D-galactopyranoside (ONPG) described by Mahoney *et al.* (1975) was adapted as follows.

Approximately 5 mL of culture was sonicated in a 15 mL polypropylene centrifuge tube using the intermediate tip of a Sonic 300 dismembrator (Artek Systems Corporation, Farmingdale, USA) at 60% intensity. The sample was cooled using an ice

water bath to prevent activity loss (Shah and Jelen, 1990) during sonication. The β–galactosidase activity of the culture was measured using the sonicated suspension.

The samples were appropriately diluted using cold (0 - 4°C) 0.1M phosphate buffer (pH 7). The ONPG was hydrolyzed by adding 1.6 mL of cold 0.005 M ONPG in 0.1 M phosphate buffer (pH 7) to 0.1 mL of diluted sample. The dilutions varied to obtain absorbance readings within the maximum sensitivity range. The mixture was incubated at 37 °C for 15 minutes. The hydrolysis was stopped by adding 2 mL of cold 0.5 M sodium carbonate. The concentration of o-nitrophenol was determined from the absorbance at 420 nm (Spectronic 21, Bausch & Lamb, USA). For this assay, the molar absorptivity was 4.8 x 10^3 M^{-1} cm^{-1}. The enzymatic activity was expressed per mL of undiluted culture. All solutions were cooled in ice water. Experiments and all analyses were carried out at least in duplicate. Unless indicated otherwise, results are expressed as average +/- one st. dev. of all available data.Statistical significance of differences was determined by a t-test where appropriate.

5.3 Results and Discussion

5.3.1 Shake flask fermentations

Considerable amounts of precipitated protein were produced by autoclaving the broth, so reliable estimation of cell biomass by optical density was not possible. Instead, the cells were counted by direct microscopic examination (Figure 5-1). Cells grown in whey supplemented with 1.0% yeast extract (YE) were, on average, twice as long and somewhat thicker than cells grown in whey or whey supplemented with 0.2% YE. In

their experiments with *L. bulgaricus* LBR in supplemented whey ultrafiltrate, Cox and MacBean (1977) also observed as much as a twofold variation in the average cell length.

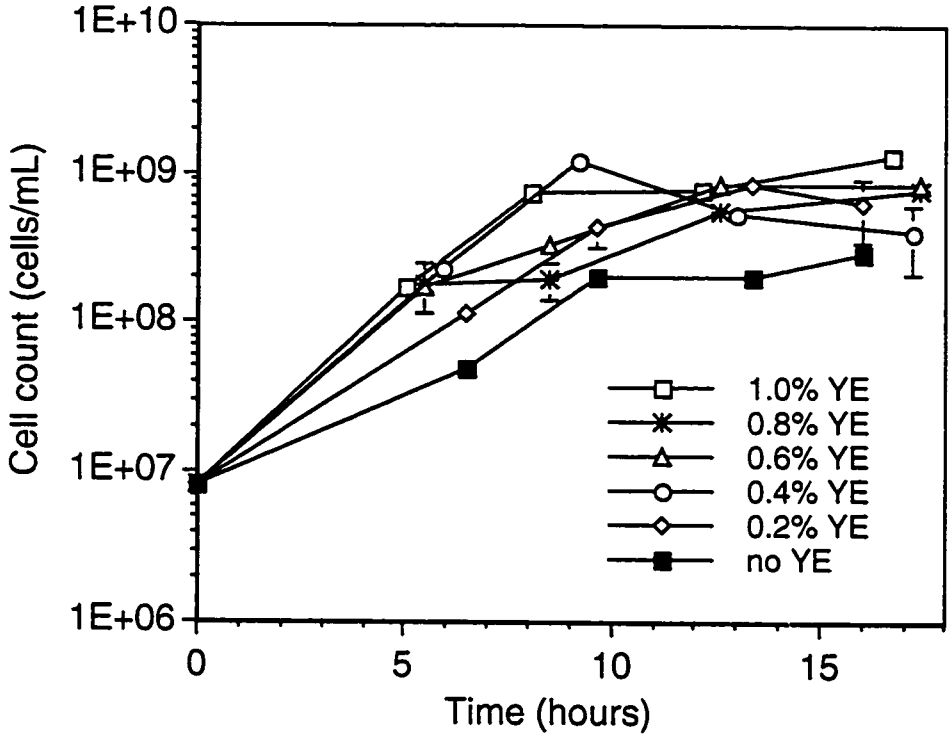

Figure 5-1. Direct microscopic cell count of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 grown in 150 mL of sterilized (121°C/15 min) whey broth (60 g/L spray dried whey powder) supplemented with 0 - 1% yeast extract. The shake flask cultures were incubated at 40 - 43°C and the pH was maintained at 5.5 ± 0.3 using 2N NaOH.

Peak cell counts occurred approximately eight to ten hours after inoculating the shake flasks. The peak cell counts for *L. delbrueckii* ssp. *bulgaricus* 11842 grown in whey ranged from 2 - 3 x 10^8 cells mL^{-1} while whey broths with 0.2 - 1.0% YE had counts of 5 x 10^8 to 1 x 10^9 cells mL^{-1}.

Addition of yeast extract (YE) had a significant effect on the rate of acid production by *L. delbrueckii* ssp. *bulgaricus* 11842 in whey (Figure 5-2). Although increasing levels of supplementation increased the rate of acid production, the cell counts seemed relatively unaffected. The production of acid continued even after the peak cell

count was reached which suggests that the bacteria were directing the available energy towards maintenance rather than growth (Cox and MacBean, 1977, Beal and Corrieu, 1995, Goncalves *et al.*, 1991). Increasing the concentration of YE would increase the amount of nutrients available to the bacteria which could explain the increased rate of acid production.

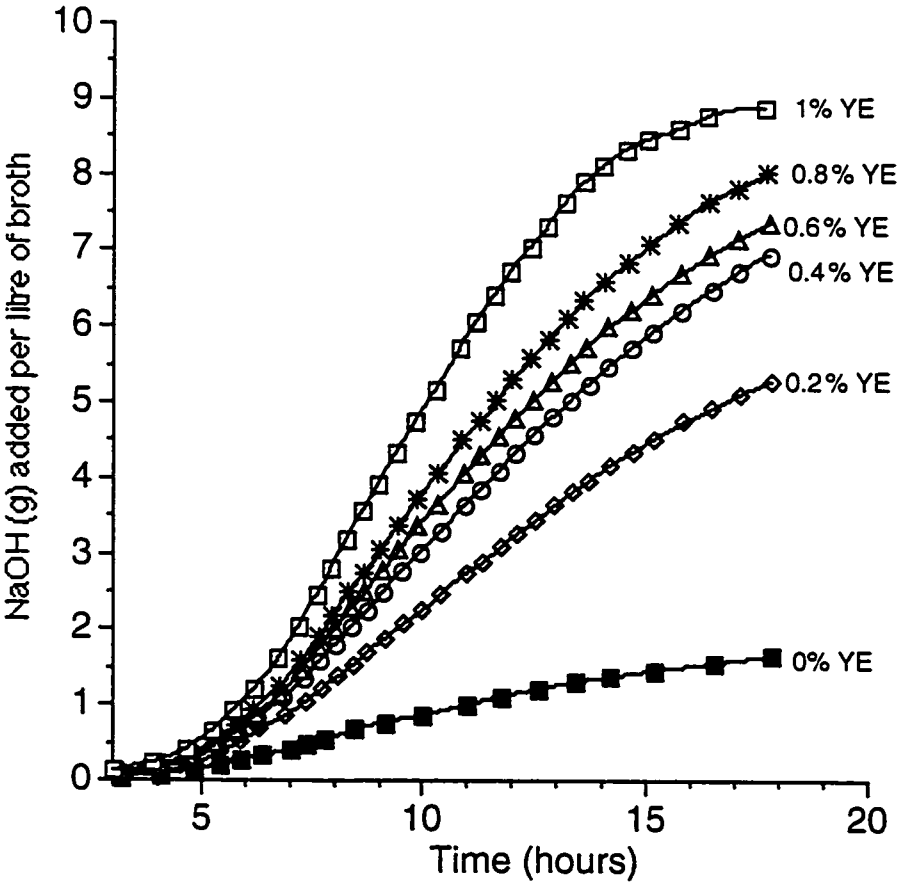

Figure 5-2. Acid production of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 grown in 150 mL of sterilized (121°C/15 min) whey broth (60 g/L spray dried whey powder) supplemented with 0 - 1% yeast extract. The shake flask cultures were incubated at 40 - 43°C and the production of acid was inferred from the amount of NaOH required to maintain the pH at 5.5 ± 0.3.

Figure 5-3 shows the β–galactosidase activity after 15 - 17 hours of growth in whey + YE media. The β–galactosidase activities of cultures grown in flasks

supplemented with 0.2 - 0.8% YE were approximately 2.5 times higher than for cultures grown without YE. Since the β–galactosidase activity did not increase ($P<0.05$) with

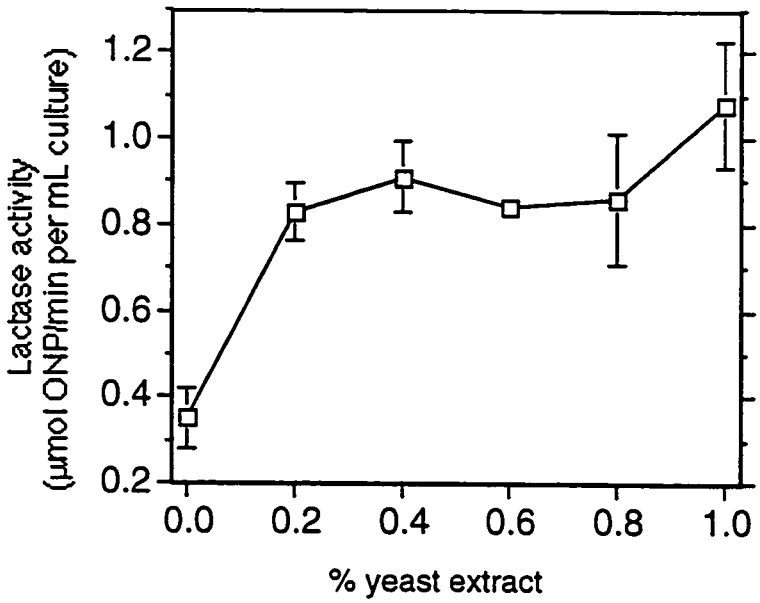

Figure 5-3. The β–galactosidase activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 grown in 150 mL of sterilized (121°C/15 min) whey broth (60 g/L spray dried whey powder) supplemented with 0 - 1% yeast extract. The shake flask cultures were incubated at 40 - 43°C and the pH was maintained at 5.5 ± 0.3 using 2 N NaOH. The β–galactosidase activity at pH 7 was determined by its ability to hydrolyze o-nitrophenyl-β–D-galactopyranoside (ONPG) at 37°C. Bars represent ± 1 standard deviation (n = 4).

supplementation in the range of 0.2 - 0.8% YE, it is possible that the hydrolysis of lactose was not the rate limiting factor for growth or acid production in this range. The amount of β–galactosidase produced with 0.2% YE may have been sufficient to hydrolyze the amount of lactose required for growth with 0.8% YE. Cultures grown in whey supplemented with 1% YE showed a statistically significantly higher ($P<0.1$) β–galactosidase activity of 1.08 ± 0.15 µmol ONP released per min per mL of culture which is three times that obtained from cultures grown in unsupplemented whey. In this

case, the hydrolysis of lactose may have become rate limiting at some point resulting in the increased production of β–galactosidase. Negligible amounts of β–galactosidase were released into the growth medium during fermentation.

5.3.2 1.5 L fermentations

Figure 5-4 (A and B) shows the results from a typical fermentation experiment at pH 5.5 in whey supplemented with 1% YE. The results at pH 5.0 were very similar (Fig. 5-4 C). The cell count was 1.5 x 10^9 cells mL^{-1} after six hours of fermentation and ranged from 2 - 3 x 10^9 cells mL^{-1} for the remainder of the fermentation. The addition of 2N NaOH became linear after six hours which roughly coincided with the maximum cell count. The fermentations were complete in 10 - 11 hours (pH 5.5) or 12 - 13 hours (pH 5.0) at which point the addition of NaOH was no longer required to control the pH.

The β–galactosidase activity and dry matter increased in a manner similar to the addition of 2N NaOH and peaked just before the fermentation was complete. Shortly thereafter, the β–galactosidase activity and dry matter began to decrease (Figure 5-4 a). At the end of the active fermentation, the amount of the β–galactosidase decreased, probably because the cells were not producing the enzyme because of the nutrient depletion. Release of β–galactosidase activity by autolysis was not significant as very little activity was detected in the clarified growth medium.

The cultures grown at pH 5.0 and 5.5 attained an average maximum activity of 7.4 ± 0.6 and 7.5 ± 0.3 μmol ONP released per min per mL of sonicated culture, resp. In comparison, the culture grown in shake flasks containing whey and 1% YE had an

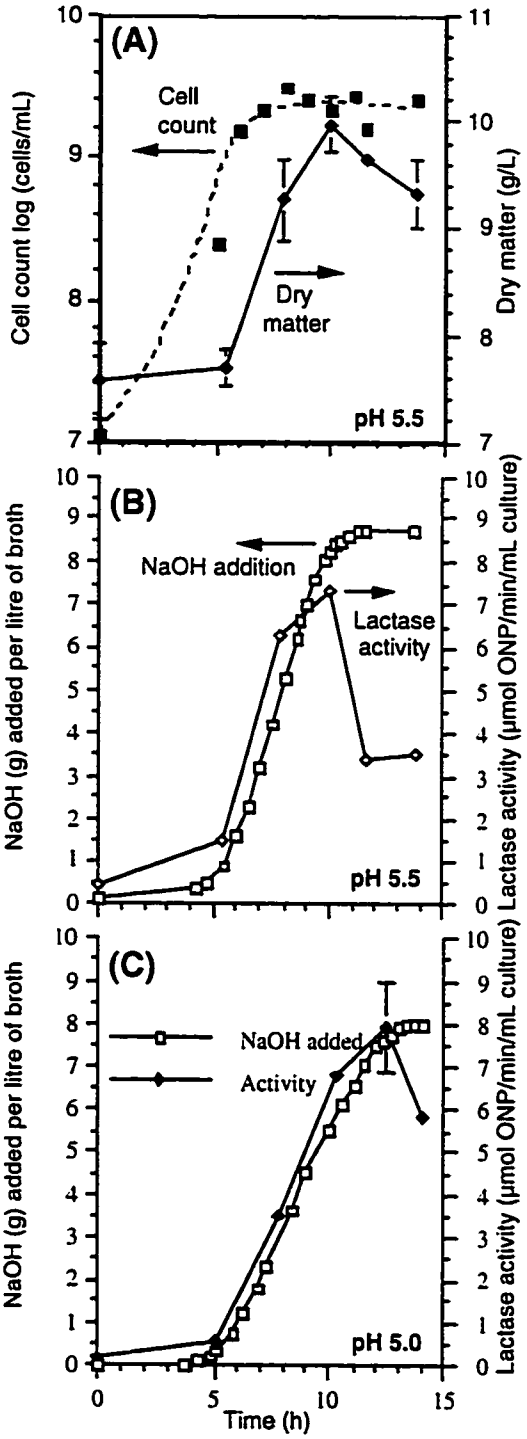

Figure 5-4. Growth, biomass, acid production, and β–galactosidase activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 grown in 1.5 L of whey broth supplemented with 1% yeast extract at 43±0.2°C and pH 5.5± 0.1 (A, B) or 5.0 ± 0.1 (C). The β–galactosidase activity at pH 7 and 37°C was determined using o-nitrophenyl-β–D-galactopyranoside (ONPG). Bars represent ± 1 s.d. (n = 2 or 3) of analyses for a representative run. In most cases the size of the bars was smaller that the datapoint symbols.

activity of 1.08 ± 0.15 μmol ONP released per min per mL of culture. The discrepancy was likely due to the different methods of fermentation. The pH and temperature were tightly controlled for the 1.5 L fermentations. In contrast, the pH in the shake flasks varied from 5.2 - 5.8 and the temperature ranged from 40 to 43°C. Adapting to the environment in the flasks may have placed additional stress on the cells affecting their growth and metabolism. For example, fermentations in flasks with 1% YE required 15 - 17 hours for completion as compared to 10 - 11 hours (pH 5.5) in the 2 L fermentor. For the 1.5 L fermentations at pH 5.5, the maximum rate of NaOH addition was 2.03 ± 0.09 g NaOH per litre of broth per hour as compared to 1.06 ± 0.01 g NaOH per litre of broth per hour attained in shake flask cultures at pH 5.5. The pH and temperature fluctuations in the shake flask cultures may be responsible for the lower acidification rate since the maximum rate of NaOH addition at pH 5.0 (1.27 ± 0.03 g L^{-1} h^{-1}) was higher. Although the rate of addition at pH 5.5 was almost double that of the flask cultures at pH 5.5, both methods required comparable total amounts of NaOH. The 1.5 L fermentations and the shake flask cultures consumed 8.8 ± 0.1 and 8.5 ± 0.6 g NaOH per litre of broth respectively. Preliminary 1.5 L fermentations with 0.6% YE required similar amounts of NaOH (Bury, unpublished) which suggests that a nutrient contained in the whey (probably lactose) is depleted in the course of the fermentation. In shake flask cultures supplemented with 2% YE, increasing the whey content by 33% did not affect the rate of acidification but 33% more NaOH was consumed (Appendix, figure A-3). This also suggests that a nutrient contained in the whey limits the amount of acid produced but other nutrients in YE influence the rate of acidification. The 1.5 L fermentations at pH 5.0 consumed 8.1 ± 0.2 g NaOH per litre of broth. Correcting for the amount of NaOH

required to increase the pH from 5.0 to 5.5, results in NaOH consumption comparable to the fermentations at pH 5.5.

Shah and Jelen(1991) cultured *L. delbrueckii* ssp. *bulgaricus* 11842 in sterile 12% non-fat dry milk at 45°C for 18 hours and reported a β–galactosidase activity of 4.22 μmol ONP per min per g of culture after sonication. The β–galactosidase activity of three strains of *L. bulgaricus* ranged from 0 to 4.45 μmol ONP / min per g of culture at 30°C in a study by Friend *et al.* (1983). Both studies showed much higher β–galactosidase activity than that obtained in the shake flask fermentations with whey supplemented with YE. In contrast, the β–galactosidase activity in the 1.5 L fermentations was almost double that reported by Shah and Jelen (1991). The strict pH control in the 1.5 L fermentations may have been one factor responsible for the greater activity. In addition, the activity measured at 18 hours (Shah and Jelen, 1991) may not have been at the maximum. Unfortunately, it is difficult to compare the activity at 30°C given by Friend *et al.* (1983) to that at 37°C used in this study. However, since the optimum temperature for β–galactosidase from *L. delbrueckii* ssp. *bulgaricus* 11842 is 55°C (Shah and Jelen, 1991), the activity at 37°C should be greater than that at 30°C.

5.4 Conclusions

The addition of yeast extract to whey greatly increased the rate of acidification by *L. delbrueckii* ssp. *bulgaricus* 11842. Cell length increased with the level of supplementation, but the total cell count seemed relatively insensitive to the amount of yeast extract used.

The β–galactosidase activity increased with the addition of yeast extract. Cultures grown in shake flasks with whey containing 0.2 - 0.8% YE produced 2.5 times the β–galactosidase obtained from cultures grown and sonicated in unsupplemented whey. Cultures grown with the addition of 1% YE showed three times the β–galactosidase activity (1.08 μmol ONP per min per mL) after sonication. The additional nutrients supplied by the yeast extract increased the rate of acidification.

The tight pH and temperature control of the 1.5 L fermentation with 1% YE probably resulted in higher β–galactosidase activity and rate of acidification in comparison to shake flask culture. The β–galactosidase activity attained maximums of 7.4 - 7.5 μmol ONP released per min per mL of sonicated culture in fermentations at pH 5.0 - 5.5 . These results illustrated that comparison of data obtained in shake flasks experiments with those generated by using properly controlled fermentors may be misleading.

Environmental factors such as pH and temperature should be carefully controlled in order to increase the production of β–galactosidase by *L. delbrueckii* ssp. *bulgaricus* 11842. Addition of one or more complex growth supplements may result in greater β–galactosidase activity as the hydrolysis of lactose would tend to be rate limiting with an excess of other nutrients.

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Chapter 6

Disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 cells by sonication, high-pressure homogenization, and bead milling.*

6.1 Introduction

Lactose, the most abundant component of milk, is a potentially valuable source of food energy. Unfortunately, most of the world's population is lactose intolerant, a condition limiting the consumption of milk and many dairy products or the use of isolated lactose as an ingredient in other foods. Lactose can be chemically modified to produce more valuable components such as lactulose, lactitol, lactobiose, lactobionic acid, lactobionamides or galactooligosaccharides (Holsinger, 1992; Thelwall, 1992; Timmermans, 1998). A syrup composed of glucose and galactose can be produced by the hydrolysis of lactose. Acid hydrolysis is unattractive for use in food but enzymatic hydrolysis using the enzyme β-galactosidase has been applied on an industrial scale for processing of whey for use in whey beverages as well as for producing lactose-hydrolyzed milk. A recent estimate (Sloan, 1999) indicated that, in the USA alone, the market for lactose-hydrolyzed dairy products may encompass up to 50 million consumers afflicted with lactose intolerance.

Commercial sources of β-galactosidase for the enzymatic hydrolysis of lactose are readily available but these can be costly. The lactic acid bacterium *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842, commonly used in making yogurt, produces relatively high levels of intracellular β-galactosidase (Shah and Jelen, 1990, 1991). It might be feas-

* A version of this chapter was submitted for publication. Bury, D., Jelen, P. and Kaláb, M. (1999) *International Dairy Journal*

ible for a dairy processor to produce "in-house" cultures of *L. delbrueckii* ssp. *bulgaricus* 11842 for the purpose of obtaining a crude, but relatively inexpensive source of β-galactosidase.

Growth of the culture would be the first step in an in-house process. However, in order to achieve acceptable rates of lactose hydrolysis, the enzyme must be released from the cells (Shah and Jelen, 1991). Sonication or other methods of cellular disruption, e.g. forcing the cells through a small orifice at high-pressures (French press) can be used for disrupting the cells in the laboratory. Unfortunately, neither is suited for large scale industrial use. Two methods for large scale cell disruption commonly used in pharmaceutical or biotechnology industries are the high-speed bead mill and the high-pressure homogenizer.

The bead mill is composed of a chamber filled with small glass beads 0.1 - 1 mm in diameter. As the cell suspension is pumped through the chamber, an impeller "activates" the beads creating high shear forces capable of disrupting the cell walls (Kula and Schütte, 1987). The high-pressure homogenizer forces the cell suspension through the small gap between the spring loaded valve and the valve seat. The cells are disrupted by the extremely high shear forces generated in the gap and their subsequent impact against an impact ring perpendicular to the flow from the homogenizing valve (Kula and Schütte, 1987, Siddiqi *et al.*, 1997). The equipment is generally well known in the dairy industry where it is being used for breaking up fat globules for production of homogenized milk at much lower pressures than required for disruption of microorganisms.

As a general rule, Gram-positive bacteria such as *L. delbrueckii* ssp. *bulgaricus* 11842 are more difficult to disrupt in comparison to Gram-negative bacteria and yeasts. The literature tends to focus on the disruption of yeasts with much less information being available on disruption of lactobacilli. The purpose of this study was to compare and evaluate effectiveness of sonication, bead milling and homogenization for disrupting the cells of *L. delbrueckii* ssp. *bulgaricus* 11842.

6.2 Materials and Methods

6.2.1 Preparation of bacterial cultures

Cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 were propagated in MRS (Difco, Detroit, MI) broth containing 1% added α-lactose (Fisher Scientific Co., Springfield, NJ). The culture was prepared for fermentation by adding 1.5 mL of the propagation culture to 150 mL WMRS broth made by dissolving 4.5 g spray dried whey powder (Kraft, Montreal, QC) and 2 g MRS powder in 150 g of distilled water.

For preliminary fermentations, approximately 50 g of the culture grown in WMRS broth was used to inoculate 1560 mL of whey broth supplemented with 1% yeast extract in a 2 L New Brunswick fermentor (New Brunswick Scientific Co. Inc., New Brunswick, NJ). The supplemented whey broth (6% whey powder, 1% powdered yeast extract) was sterilized by heating to 121°C for 20 minutes. The temperature was maintained at 43 ± 0.2 °C by placing the 2 L fermentor in a 10 L circulating water bath. The culture was maintained at pH 5.0 or 5.5 by adding 2N NaOH (Fisher Scientific Co., Springfield, NJ) with a peristaltic pump (Watson-Marlow Bredal Pumps Inc., USA) controlled by an Omega pH controller (Omega Engineering Inc., Stamford, CT). The

biomass was concentrated using a disk stack centrifuge (Alpha-Laval, Tumba, Sweden) and stored at -30°C.

In a larger fermentation trial, a 45 L batch of whey broth (6% whey powder) supplemented with 0.6% powdered yeast extract was pasteurized by heating to 60°C for 20 minutes and cooled to 41 ± 2°C prior to inoculation. The seed culture, about 1.7 L after fermentation, was prepared and grown as in the preliminary fermentations except for the addition of 0.6% instead of 1% yeast extract. Initially, the pH was allowed to decrease from 5.6 to 4.1; for the remainder of the fermentation the pH was maintained at 5.0 ± 0.1 using 50% NaOH. The culture was cooled to 30°C, concentrated to 23% w/w solids using the Alpha-Laval disk stack centrifuge, and stored in 200 mL aliquots at -30°C. The aliquots were thawed and pooled. A portion was centrifuged at 4000 xg for 10 minutes (Beckman model J2-21, Beckman Coulter, Inc., Fullerton, CA, USA). Some of the supernatant was removed and added to an aliquot from the pool while the pellet was re-suspended with the remainder of the supernatant. This produced suspensions containing 12%, 23%, and 46% solids (wet wt.).

6.2.2 Disruption of microbial biomass

Bead mill

The 150 mL chamber of the Dyno-Mill® KDL Special (WAB Maschinenfabrik, Basel, Switzerland; Glen Mills, Inc., Maywood, NJ, USA) was charged with 130 mL of lead-free glass beads (0.2-0.3 mm diameter) and 85 mL of cell suspension. The mill was run with an impeller tip speed of 10 m s^{-1} and the chamber was cooled using glycol/water at 0°C. Samples (0.2 mL) were withdrawn at various time intervals for β–galactosidase analysis.

High-pressure homogenization

A Mini-Lab Rannie high-pressure homogenizer (APV, Wilmington, MA, USA) operating at average pressure of 135 MPa was used for disruption. The suspensions were cooled to 8±2°C using an ice-water bath after each pass. Since the Mini-Lab Rannie high-pressure homogenizer was limited to 150 MPa, a frozen 200 mL sample (23% wet wt) was sent to APV (Wilmington, MA, USA) for disruption in a prototype Rannie Lab 2000 homogenizer at 200 MPa. Upon receipt of the culture (control) and disrupted culture (one passage at 200 MPa) from APV, 5 mL samples were frozen for β-galactosidase analysis and another set of samples was fixed to a final concentration of 3% formaldehyde for electron microscopy.

Sonication

Aliquots of suspensions with cell concentrations of 3.2 - 46% (wet wt) were sonicated using the intermediate probe of an Artek Sonic 300 Dismembrator (Fisher Scientific Co., Springfield, NJ) at 60% intensity. The probe was placed in a 15 mL polypropylene tube containing the cell suspension. Samples (0.2 mL) were withdrawn every minute for 6-8 minutes. Cooling was achieved by placing the polypropylene tube in an ice water bath while sonicating and sampling.

6.2.3 Measurement of β–D-galactosidase

The disrupted samples were diluted with 0.1M sodium phosphate buffer at pH 7 such that the resulting absorbance at 420 nm was in the maximum range of sensitivity. The β–galactosidase activity of the suspension was measured using the method of Mahoney *et al.* (1975) with minor modifications as previously described in chapter 5. To measure the soluble β–galactosidase, 1 mL of the suspension was centrifuged for 3

minutes in a Brinkmann Eppendorf 5414 Centrifuge. To determine total or soluble β–galactosidase activity, 100 µL of the suspension or clarified supernatant, respectively, was added to 1.6 mL of cold 0.005 M ONPG in 0.1 M phosphate buffer (pH 7) and placed in a 37°C water bath for 15 minutes. The hydrolysis of ONPG was stopped by adding 2 mL of cold 0.5 M Na_2CO_3. The concentration of o-nitrophenol was determined from the A_{420} (Spectronic 21, Bausch & Lamb, USA) and a molar absorptivity of 4.8 x 10^3 M^{-1} cm^{-1}. The enzymatic activity was expressed as µmol o-nitrophenol released per minute per gram of biomass (wet wt). All solutions were cooled in ice water. Experiments and all analyses were carried out at least in duplicate. Unless indicated otherwise, results are expressed as average ± 1 st. dev. of all available data. Statistical significance of differences was determined by a t-test where appropriate.

6.2.4 Electron microscopy

The cell slurries were fixed with a formaldehyde solution to 3% final concentration and then prepared for examination by scanning and transmission electron microscopy.

Scanning electron microscopy (SEM)

The slurries were filtered through Nucleopore polycarbonate filters (Costar Scientific Corporation, Toronto, Canada) with pores 0.4 micrometer in diameter. A rather thick (<0.2 mm) bacterial slurry layer was used since the fixed material had a tendency to get washed away from the filters during the subsequent steps even if the filters were treated with polylysine before filtration. The filters with the slurries attached were carefully dehydrated in a graded ethanol series (20, 40, 60, 98, 100%) using a Penetron Swirling Shaker (SPI Supplies, Toronto, ON, Canada) at a low inclination and speed so

as not to disturb the filter cake. The filters with the slurry cakes in 100 ethanol were critical point-dried from carbon dioxide in individual compartments. The dried slurries easily separated as flakes from the filters. The flakes were mounted on aluminum SEM stubs with their tops and also with their bottom facing up. A double-sided sticky tape mounted on the stubs was used for this purpose and contact with the sticky tape was provided by a silver conducting paint (Ladd Research Industries, Inc., Burlington, Vermont).

The mounted samples were sputter-coated (Technics Hummer sputter coater) with approximately 20 nm of gold and examined in a Zeiss DSM 940A scanning electron microscope operated at an accelerating voltage of 15 kV. Micrographs were taken on 35-mm T-Max Kodak 100 ASA film.

Transmission electron microscopy (TEM)

The formaldehyde-fixed bacterial slurries were centrifuged by using a table-top low speed centrifuge (6000 rpm for 10 min). The pellets were soft and not compact. Small (~1 mm^3) lumps on a needle tip were covered with a thin layer of 40°C agar (4%) sol (Kaláb, 1988). The agar gelled rapidly on contact. The resulting beads were washed with 3 changes of 0.05 M veronal-acetate buffer, pH 6.75, within 30 min and then postfixed with a 2% osmium tetroxide solution in the same buffer at 22°C overnight. The postfixed beads were washed with plain buffer and then dehydrated in a graded ethanol series (same as for SEM, see above) within a day. They were passed through 1:2 and 2:1 dilutions of LR White resin with 100% ethanol and then through full-strength resin and polymerized at 60°C overnight. Thin sections (~90 nm thick) on 3 mm copper grids were stained with a 25% methanolic solution of uranyl acetate followed by staining with an

aqueous solution of lead citrate (Reynolds, 1963) and examined in a Zeiss 902 transmission electron micrscope operated at 80 kV. Micrographs were taken on 35-mm film.

6.3 Results and Discussion

6.3.1 Preliminary experiments

The β–galactosidase activity of the disrupted cultures attained a maximum between 2 - 3 minutes of processing in the bead mill. At the maximum, the soluble activity (232 ± 33 μmol o-nitrophenol released per gram of wet biomass) was 71 - 82% of the total activity.

The maximum total β–galactosidase activity was reached after 4 minutes of sonication. In sonicating the cultures grown at pH 5.5, the maximum soluble β–galactosidase activity was also observed after 4 minutes of sonication, but with cultures grown at pH 5.0, the maximum soluble β–galactosidase activity was not reached even after 6 minutes of sonication. The soluble activity was 82% of the total activity for cultures grown at pH 5.5, whereas, the soluble activity was 38 - 55% of the total for cultures grown at pH 5.0. On average, the soluble β–galactosidase activity was 151 ± 82 μmol o-nitrophenol released per gram of wet biomass after 4 - 6 minutes of sonication. It would appear that cultures of *L. delbrueckii* ssp. *bulgaricus* 11842 grown at pH 5.0 are more difficult to disrupt by sonication in comparison to cultures grown at pH 5.5.

Other potentially suitable methods of disruption such as static high-pressure treatment, dynamic or oscillatory high-pressure treatment, and microfluidization (Microfluidics Corp., Newton, MA) were also included in these preliminary

investigations in which the individually grown cultures were used. These methods were not studied in detail because they were not as effective, accessible, or easy to use when compared to the bead mill or the homogenizer, and thus the results are not included in this report. Since the somewhat different conditions of the individually grown cultures could be a source of variability in disruption experiments (Kula and Schütte, 1987), a larger single batch of culture prepared as described above was used in the remainder of the work.

6.3.2 Disruption in a Bead Mill

Figure 6-1 shows that the maximum β–galactosidase activity was released between two and three minutes of milling, after which the activity began to decrease. Although the enzyme release is a first-order process, the enzyme is typically deactivated while milling (Kula and Schütte, 1987) which may explain the maximum shown. Addition of 0.01% mercaptoethanol to prevent oxidation (Feliu and Villaverde, 1994; Kula and Schütte, 1989) and 0.01% polypropyleneglycol P 2000 as antifoam (Kula and Hummel, 1989) may have reduced enzyme deactivation as some foaming was evident. The suspensions remained relatively cool (<18°C) after six minutes of disintegration which should have helped to minimize the loss of activity due to heat.

The release of active β–galactosidase did not appear to be affected by the biomass concentration, ranging from 12 to 46% (wet weight of biomass). Kula and Schütte (1987) reported similar findings with yeasts in literature and suggest an optimal concentration of 40 to 50% cells (wet weight) for disruption by milling.

6.3.3 High-pressure Homogenizer

The soluble β–galactosidase activity increased as a first-order process with the number of passes through the Rannie high-pressure lab homogenizer at 135 MPa

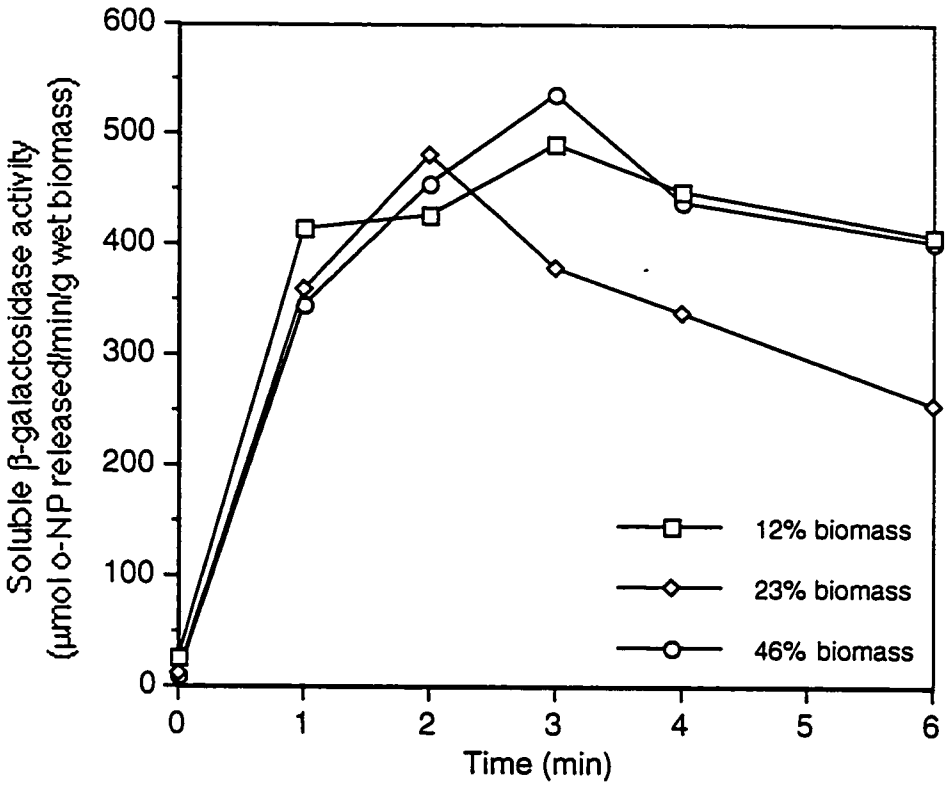

Figure 6-1. The release of soluble β–galactosidase from various concentrations of *L. delbrueckii* ssp. *bulgaricus* 11842 disrupted using a DynoMill KDL Special (WAB Maschinenfabrik, Basel, Switzerland). Tip speed was 10 m/s. Batch disruption using 150 mL grinding chamber and 85% loading with lead-free glass beads 0.2-0.3 mm in diameter.

(Figure 6-2). A temperature increase of 36°C per pass or 0.26°C per MPa was recorded which is consistent with the value of 0.25°C per MPa reported in literature (Kula and Schütte, 1987). The suspension was cooled between each passage to reduce the denaturation of β–galactosidase by heat. After three passes, the soluble lactase activity was comparable to the maximum obtained with the bead mill. There was no increase in activity between the second and third pass when the suspension was not cooled before the

third passage (Figure 6-2). Although additional β–galactosidase may have been released, some heat inactivation may have occurred thereby resulting in no net gain or loss.

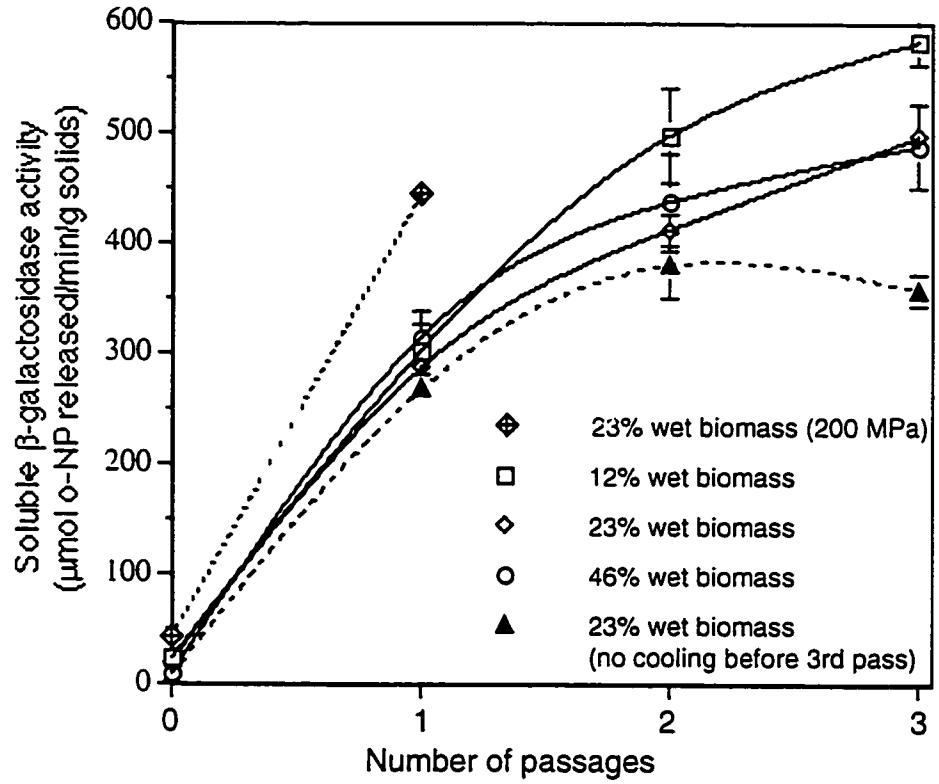

Figure 6-2. The release of soluble β–galactosidase from various concentrations of *L. delbrueckii* ssp. *bulgaricus* 11842 disrupted using a Mini-Lab Rannie high-pressure homogenizer (APV, Wilmington, MA, USA) at 135 MPa unless otherwise indicated. Error bars represent one standard deviation (n = 2 - 4).

Disruptions of suspensions containing 12, 23, and 46% w/w wet biomass did not show any significant differences in the amount of active lactase released per gram of biomass for the first two passages. However, there was slightly, but significantly ($P<0.05$), more activity per gram of wet biomass in the sample containing 12% solids after the third passage. Similarly, Kula and Schütte (1987) reported that the disintegration of yeast with a Gaulin M3 homogenizer was not affected by cell concentrations in the 20 - 60% range.

Evaluation of the suspensions received from the APV laboratory showed that after one pass through the Rannie Lab 2000 homogenizer at 200 MPa, the enzymatic activity (Figure 6-2) was comparable to that after two passes at 135 MPa recorded in our experiments. There was some concern that the cells or the β–galactosidase activity may have been influenced by the conditions of shipping, but the control showed no difference in SEM or activity in comparison to the other controls. By using higher pressures, the desired degree of disintegration can be achieved in significantly fewer passages (Kula and Schütte, 1987), however, the generation of additional heat may denature heat sensitive enzymes. In this case, it appears that because the β–galactosidase from *L. delbrueckii* ssp. *bulgaricus* 11842 is relatively heat stable it may be able to withstand the heat generated by a passage at 200 MPa.

6.3.4 Sonication

The amount of active soluble β–galactosidase released after six minutes of sonication (Figure 6-3) was less than half that liberated by the bead mill (3 min) or by three passages through the high-pressure homogenizer. Shah and Jelen (1991) claimed that it took approximately four minutes of sonication to release the maximum lactase activity from *L. delbrueckii* ssp. *bulgaricus* 11842. In the present study, preliminary disruptions using sonication also showed that four minutes of sonication was sufficient to release the maximum amount of active β–galactosidase from cultures before or after freezing. The culture was well into stationary phase and experienced two freeze/thaw cycles which might have accounted for the increased difficulty of disruption by sonication in this instance. However, the same suspensions were used in the homogenizer and bead mill experiments so the results should be comparable. To avoid the variability

introduced by culture conditions, aliquots from the same fermentation were used to compare the various methods of disruption. Therefore, the differences in cell disruption and subsequent release of β–galactosidase cannot be due to the variations in fermentation conditions or procedures.

In the small volumes used (six to seven milliliters), the concentration of cells (12 to 46% wet biomass) did not appear to affect the release of active β-galactosidase under the sonication conditions employed.

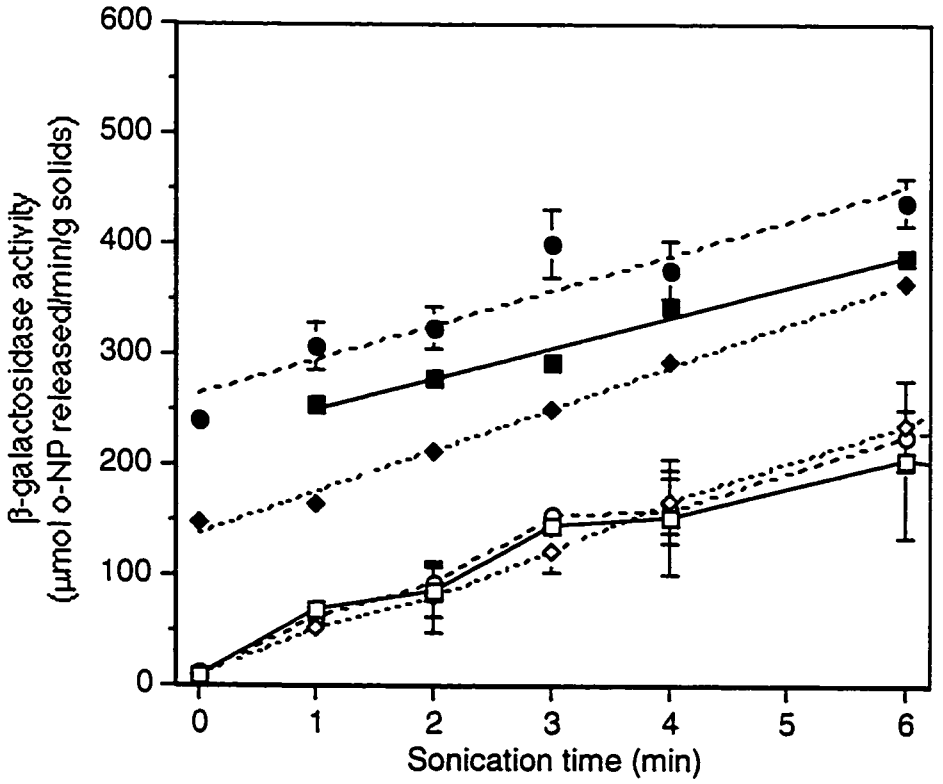

Figure 6-3. The release of β–galactosidase from various concentrations of *L. delbrueckii* ssp. *bulgaricus* 11842 disrupted by sonication. The Artek Sonic 300 Dismembrator (Fisher Scientific Co., NJ, USA) was operated at 65% intensity with the intermediate tip. Soluble activity from 12% biomass —□—, 23% biomass ----◇----, and 46% biomass ---○---. Total activity, which includes cell debris, is indicated by filled symbols. Error bars represent one standard deviation (n = 2 - 4).

6.3.5 Scanning Electron Microscopy

Figures 6-4 and 6-5 show the results of the scanning electron microscopy (SEM) investigations of the effectiveness of disruption of *L. delbrueckii* ssp. *bulgaricus* 11842 cells. The culture appeared to be intact after freezing as evidenced by Figure 6-4a and by the relatively low levels of enzymatic activity prior to disintegration. There is extensive

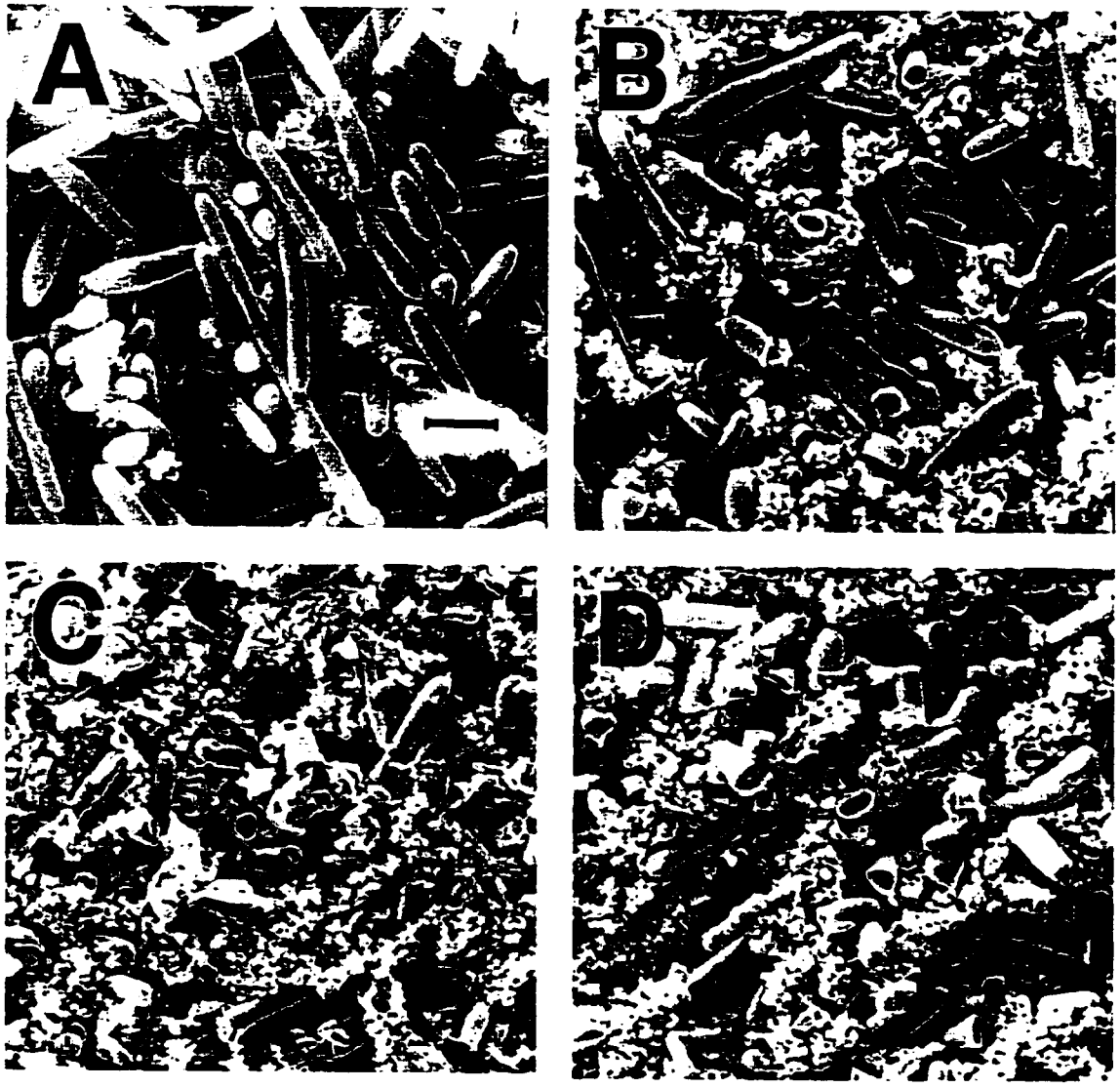

Figure 6-4. Scanning electron micrographs of disrupted cultures of *L. delbrueckii* ssp. *bulgaricus* 11842. (a) Culture prior to disruption. (b) Culture after sonicating for 6 minutes, (c) processing in a bead mill for 6 minutes, or (d) after one passage through a high-pressure homogenizer at 200 MPa. Bar: 2 µm.

cellular destruction after 6 min of processing with the bead mill (Figure 6-4c), as well as after one pass at 200 MPa (Figure 6-4d) or after two passes at 135 MPa (Figure 6-5c) through a homogenizer. The β–galactosidase activity after 6 min of sonication was substantially lower than that released by the other methods. Figure 6-4b shows that after six minutes of sonication there were considerably more intact cells in comparison to the other methods which provided a visual confirmation of the results of the lactase assays.

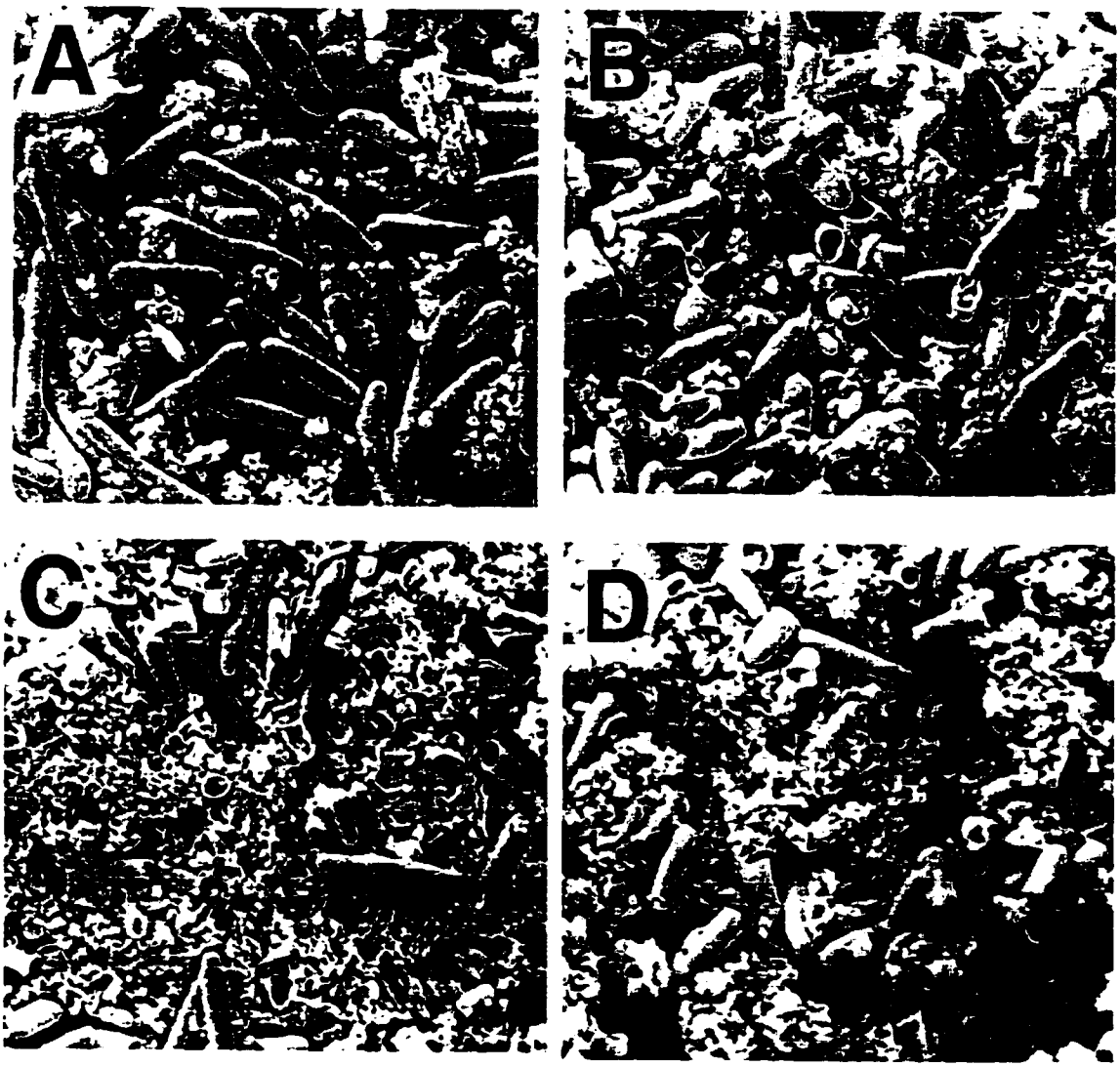

Figure 6-5. Scanning electron microscopy of disrupted cultures of *L. delbrueckii* ssp. *bulgaricus* 11842. (a) Culture prior to disruption. (b,c,d) Culture after one, two, or three passages through a Mini-Lab Rannie high-pressure homogenizer operated at 135 MPa.

Caution must be exercised in interpreting these images as the degree of destruction observed in the SEM does not necessarily reflect the amount of active enzyme released from the cells. For example, the enzymatic activity decreased with the time of milling once the maximum had been reached. Similarly, the excessive heat generated by high-pressure homogenization may cause denaturation even though the degree of disruption and release of protein continues to increase.

6.3.6 Discussion

A decrease in β–galactosidase activity was observed when the undisrupted cells (Figure 6-3, 0 min) or the cell debris (Figure 6-3, 1-6 min) were removed by centrifugation. The activity of the undisrupted controls (166 ± 43 μmol o-nitrophenol released per minute per gram of biomass) was quite variable and much higher in comparison to the minimal activity obtained from cell-free supernatants (14± 6 μmol o-nitrophenol released per minute per gram of biomass). This suggests that negligible amounts of soluble and active β–galactosidase are released to the growth medium by autolysis or other means such as freezing. Since there is considerable activity associated with the undisrupted cells, it is possible that the ONPG may diffuse into the cells and be hydrolyzed by the β–galactosidase entrapped within. For suspensions disrupted in the bead mill or high-pressure homogenizers, the maximum soluble β–galactosidase activity was 80-100% of the maximum activity obtained prior to centrifugation, whereas the soluble activity from sonicated cultures was only 50-65% of the total activity in the samples (Figure 6-6). Similarly, Feliu and Villaverde (1994) reported a maximum recovery of 70% for a recombinant β–galactosidase from *E. coli* after a single 15 min sonication treatment. It is likely that some of the active β–galactosidase might be

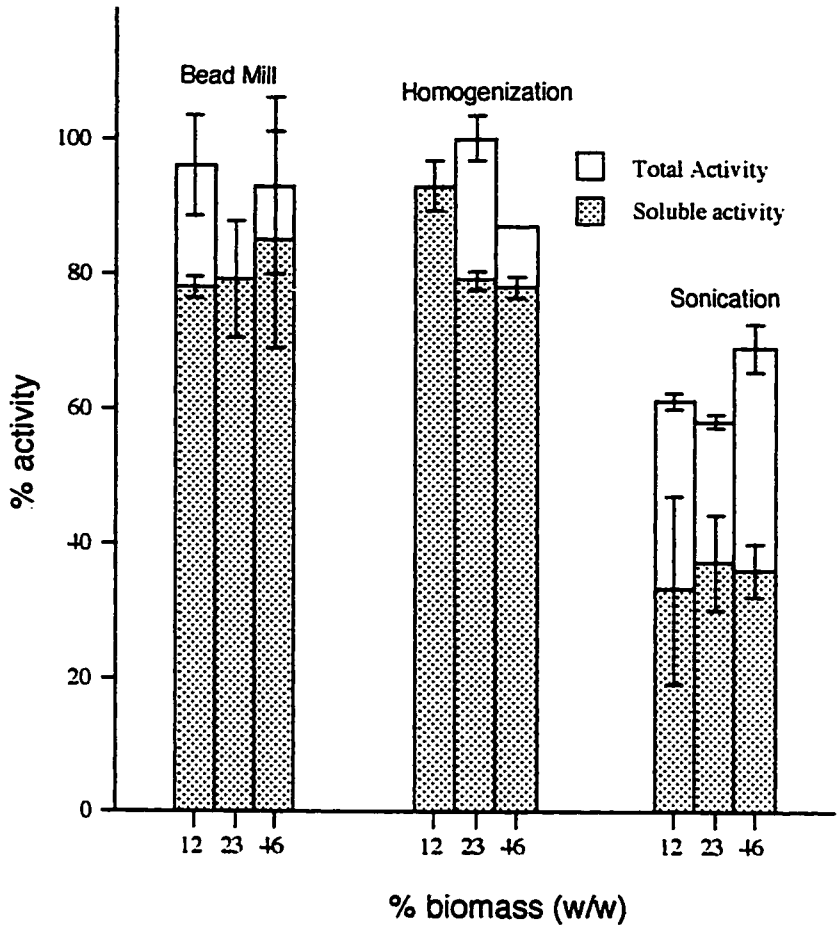

Figure 6-6. A comparison of the of β–galactosidase activity from mechanically disrupted cultures of *L. delbrueckii* ssp. *bulgaricus* 11842 at various concentrations of biomass. Total activity refers to the maximum activity measured from the disrupted suspension which includes the cells and cell debris. Soluble activity refers to the maximum activity in the supernatant of the sample. Error bars represent one standard deviation (n = 2 - 4).

associated with cell fragments in addition to that within intact cells and would therefore be removed by centrifugation. The soluble β–galactosidase activity increased slightly if the disrupted culture was allowed to rest for a period of time before centrifugation (Appendix, figure A-7). The possible explanation might be the escape or solubilization of β–galactosidase from cell fragments or damaged cells. Transmission electron microscopy (TEM) of the bacterium also helps to explain the decrease in activity after centrifugation.

Figure 6-7a, the control, shows intact cell walls and a membrane containing cytoplasm. The sonicated cultures (Figure 6-7b) show some cells with holes in their cell walls but, more importantly, intact membranes containing cytoplasm. Removal of the cells by centrifugation would remove the β–galactosidase associated with them. In contrast, the TEM of the culture disrupted using the bead mill (Figure 6-7c) shows fragments of cell walls with no intact membranes.

The pH, available nutrients, temperature, and other factors affecting microbial growth can influence the strength of the cell wall and its disruptibility. Generally, it is easier to disrupt rapidly growing cells. Typically, the cell wall is strengthened upon entering the stationary growth phase (Kula and Schütte, 1987) which tends to increase the difficulty of disruption.

There may have been some effects of freezing of the cells related to the ease of disruption but this was not considered important in the conditions of this study. The cell suspension was frozen after concentration with the disk stack centrifuge in order to minimize the variability of the cells used for disruption. By using the suspensions from a single fermentation, the results regarding the effectiveness of the various means of disruption can be considered directly comparable.

6.4 Conclusions

The β–galactosidase activity after three passages through the homogenizer at 135 MPa was comparable to the maximum attained after two to three minutes in the bead mill. The activity released after one passage through the homogenizer at 200 MPa was similar to that obtained between two and three passes at 135 MPa. A simultaneous release

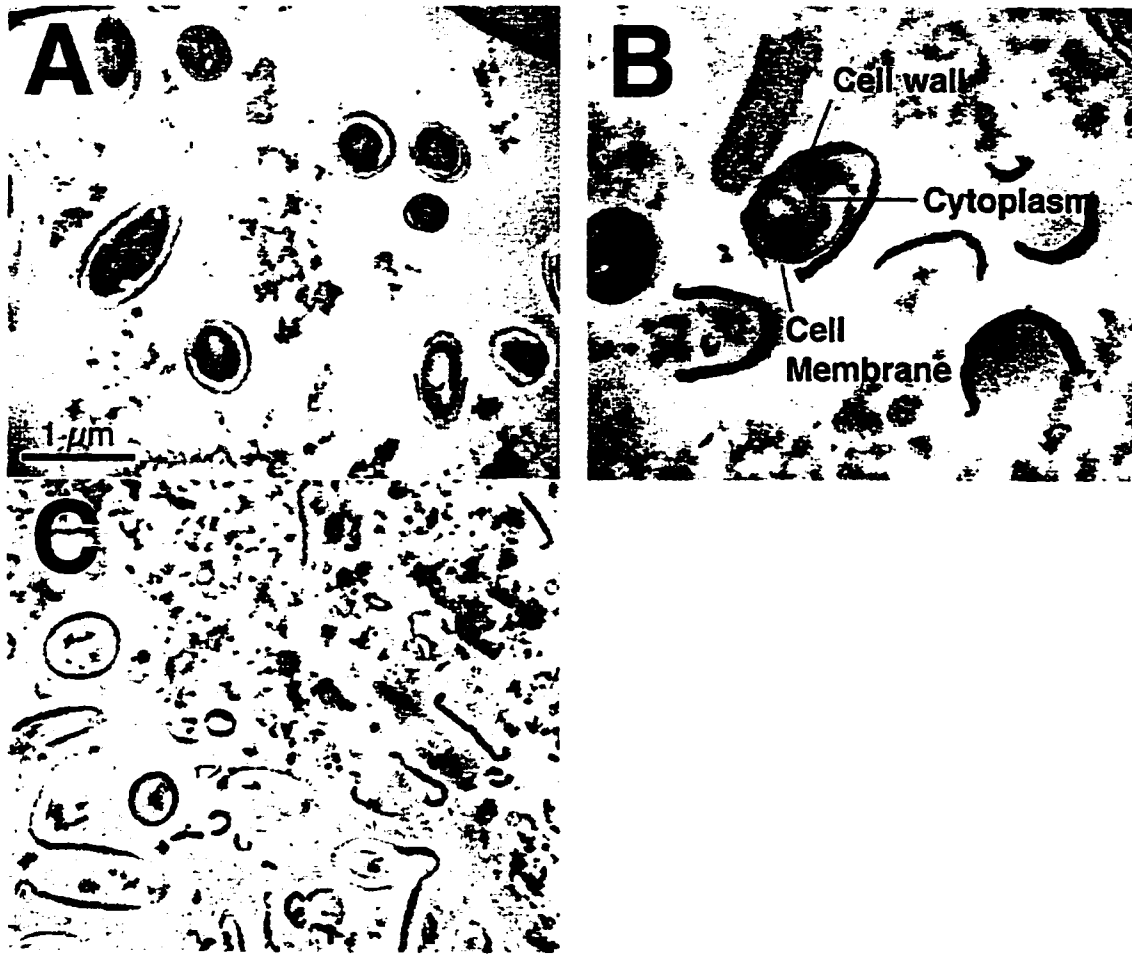

Figure 6-7. Transmission electron microscopy of disrupted cultures of *L. delbrueckii* ssp. *bulgaricus* 11842. (a) Culture prior to disruption. (b) Culture after sonicating for 6 minutes or (c) disrupting in a bead mill for 6 minutes. Bar: 1 µm.

and deactivation of β–galactosidase can occur under certain process conditions as evidenced by the maximum observed with the bead mill. Three to four discrete passes through the high-pressure homogenizer should be sufficient for the maximum release of β–galactosidase, however, efficient cooling needs to be provided between the individual passes.

In general, the release of β–galactosidase was not dependent on the cell concentration (12 to 46% wet biomass). Only after three passages through the high-

pressure homogenizer, there appeared to be slightly but significantly ($P<0.05$) more activity released from suspensions containing 12% cells (wet weight) in comparison to those with 23% and 46% cells, expressed per g of the wet biomass. In all other cases, the differences in the cell concentrations had no statistically significant effect on the activity released.

The bead milling and the high-pressure homogenization appear equally suitable for the large scale disruption and subsequent release of β–galactosidase from *L. delbrueckii* ssp. *bulgaricus* 11842. Sonication is limited to small bench-top volumes and its effectiveness seemed to be lower in comparison to the other two methods as shown by enzymatic assays, SEM, and TEM.

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Chapter 7

Lactose hydrolysis using a disrupted dairy culture: Evaluation of technical and economical feasibility.*

7.1 Introduction

Traditionally, cheese whey has been considered a waste and the dairy industry tended to focus on its disposal. Current trends aim at the recovery of valuable components from whey or seek to utilize whey by other means. For example, the protein fraction can be removed and concentrated by ultrafiltration (UF) to produce whey protein concentrates and a lactose rich UF permeate. Addition of β-galactosidase would hydrolyze the lactose in the whey or permeate to a mixture of the monosaccharides galactose and glucose which are sweeter and more soluble. Use of lactose hydrolyzed whey in ice cream would help to minimize the potential problems of sandy texture caused by the formation of lactose crystals while contributing to the sweetness. The low solubility of lactose limits the concentration of whey or permeate for transport. Lactose hydrolysis prior to the concentration process would allow for a higher degree of water removal thus lowering the cost of transportation. The resulting syrup could then be used to supplement or replace conventional sweeteners such as sucrose. Finally, the hydrolysis of lactose in many dairy products could make them available to consumers suffering from lactose intolerance. A potential market of some 50 million persons was estimated to exist in the USA alone (Sloan, 1999). Since the cost of purified β-galactosidase is prohibitive in most instances, Jelen (1993) proposed the use of sonicated dairy cultures to produce a

* A version of this chapter was submitted for publication. Bury, D. and Jelen, P. (1999) *Canadian Agricultural Engineering*

relatively impure source of β-galactosidase for a potentially more economical process of lactose hydrolysis.

Growth media based on whey or whey permeate have been used for the production of lactic acid, ethanol, biomass, and the propagation of dairy starter cultures. *L. delbrueckii* ssp. *bulgaricus* 11842, a bacterium used in the production of yogurt, would be suitable for the "in-house" production of β-galactosidase as the microorganism is capable of producing relatively high levels of intracellular β-galactosidase in comparison to other dairy cultures (Friend *et al.*, 1983; Shah and Jelen, 1990, 1991). Sweet cheese whey supplemented with 0.6% yeast extract (Bury and Jelen, 1999a) would be used for the growth medium. Once the fermentation is complete, the cells would be concentrated by centrifugation and disrupted. The suspension could then be clarified for use in products such as milk or used as is in the case of ice cream, yogurt, pudding, or other products. The objective of this investigation was to verify the effectiveness of the proposed approach and to examine its general economic feasibility.

7.2 Materials and Methods

7.2.1 The proposed process

L. *delbrueckii* subsp. *bulgaricus* 11842 would be grown on sweet whey containing 0.4–0.6 % (w/v) yeast extract. The pH would be controlled by addition of 12N NaOH, KOH, or 10N NH_4OH. Seed cultures would be grown in sterile broth or direct-to-vat inoculation could be used. The fermentation broth would be pasteurized by heating to 70°C for 10 min and cooled to 43°C for fermentation. The fermentation would take approximately 14 – 18 hours at which point the culture would be concentrated using a

disk stack centrifuge (Figure 7-1). The concentrated cell slurry would be cooled in a jacketed holding vessel and adjusted to 50% solids (wet weight) for disruption. Two passes through a high pressure homogenizer or a pass through a bead mill would be required for disruption and subsequent release of β-galactosidase. The suspension could then be added directly to milk, whey, permeate, concentrated permeate, or clarified by centrifugation or filtration prior to use. This procedure was followed in a small scale laboratory verification experiment providing a basis for the economic evaluation.

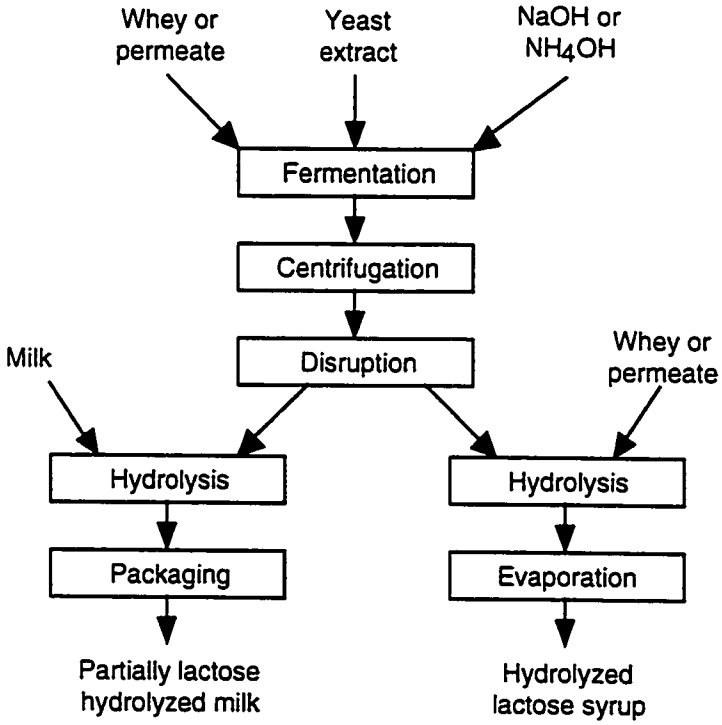

Figure 7-1: An overview of lactose hydrolysis processes using a disrupted dairy culture as considered in this study.

7.2.2 Experimental Procedures

The basic experimental setup was as described by Bury and Jelen (1999a,b). *L. delbrueckii* subsp. *bulgaricus* 11842 was maintained in MRS broth (Difco, Detroit, MI) containing 1% α-lactose (Fisher Scientific Co., Springfield, NJ). The culture was grown

in 150 mL of broth containing 3% w/v sweet whey powder (Kraft, Montreal, QC) and 0.8% MRS powder. The culture was incubated for 12-14 h in an incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ) maintained at 40 - 43°C and 100 RPM. About 50 mL of this culture was used to inoculate a two liter fermentor (New Brunswick Scientific Co., New Brunswick, NJ) filled with 1560 mL of broth containing 5.8% whey power and 0.6% yeast extract (Fermtech, BDH Inc., Toronto, ON). Temperature was maintained at 43 ± 0.2°C by placing the two liter fermentor in a circulating water bath. When the culture reached pH 5.0 ± 0.1, it was maintained at this level for the remainder of the fermentation by addition of 2 N NaOH (Fisher Scientific Co., Springfield, NJ) using a peristaltic pump (Watson-Marlow Bredal Pumps Inc., USA) controlled by a pH controller (Omega Engineering Inc., Stamford, CT). Growth was monitored by recording the amount of NaOH added and by direct microscopic enumeration of the cells.

The fermentation broth was cooled to approximately 10°C by placing the fermentor in an ice water bath once the consumption of NaOH began to slow down (about 16h). The biomass was recovered by centrifuging 200 mL aliquots of culture at 4000 x g for 10 minutes. Two pellets, representing 400 mL of culture, were pooled and resuspended in 70 mL of cold 2% milk in preparation for the disruption in a bead mill (Dyno-Mill KDL Special, WAB Maschinenfabrik, Basel, Switzerland; Glen Mills, Inc., Maywood, NJ, USA). The cell suspension was introduced to the 150 mL grinding chamber followed by 130 mL of lead free glass beads (0.2 - 0.3 mm diameter). Approximately 15 mL of cold 2% milk was added to fill the chamber. The cells were milled for two minutes using an agitator tip speed of 10 m/s. The suspension of cells and glass beads was drained from the chamber. Cold 2% milk was used to rinse the chamber

and dilute the suspension to 400 mL of 2% milk. The milk was decanted to remove the glass beads and placed at 4°C for hydrolysis. The amount of lactose hydrolyzed was determined by measuring the freezing point depression as a function of time (Zarb and Hourigan, 1979).

7.2.3 Economic analysis

Whey was assumed to be readily available for the fermentation. The cost of processing the spent fermentation broth was not taken into consideration. Capital and operating costs were based on estimates from manufacturers, suppliers, Kalk and Langlykke (1986), and Ulrich (1984). Costs were adjusted to reflect their current value using the Chemical Engineering Plant Cost Index (Anonymous, 1999). Estimates are shown in terms of Canadian dollars ($CDN). A discounted cash flow analysis (Ulrich, 1984) based on a project lifetime of 10 years with straight line deprecitation was used to estimate the discounted cash flow rate of return (DCFRR). The DCFRR can be used to compare alternative investments. Since the DCFRR is not a true interest rate, a high DCFRR does not necessarily result in high returns. Errors and variability in the estimation of capital and operating expenses result in uncertainty, so a DCFRR of 30% was used in this instance to determine if the project might be economically feasible and therefore warrant a more detailed (pilot scale) study.

The commercial price of the product, partially lactose hydrolyzed milk (PLHM) with 60% of the lactose hydrolyzed, was estimated by adding the cost of production to the local price of a 1 L container of 2% milk. The income was estimated by subtracting the price of the current commercial lactose hydrolyzed milk product (99% hydrolyzed lactose) found in the marketplace from the estimated price of PLHM (60% hydrolyzed

lactose). For example, if a competitor's product sells for $1.79 per L and the price of PLHM is $1.64 per L, the income would be $0.15 per L assuming a similar markup.

7.3 Results and discussion

7.3.1 Laboratory scale verification of the process concept

A dilution of 1:1 represents the amount of culture obtained from the original volume of the batch fermentation and used to hydrolyze the lactose in an equal volume of milk. For example, at a 1:1 dilution, the biomass recovered from 400 mL of the fermented broth was disrupted and resuspended in 400 mL of 2% milk. After 24 hours at 4°C, 37% of the lactose in the milk was hydrolyzed (Figure 7-2). Increasing the dilution, thus reducing the amount of β-galactosidase, resulted in a corresponding increase in the time required for hydrolysis.

The disrupted culture contains viable microorganisms capable of growth in milk or whey, therefore, the rate of lactose hydrolysis must be greater than the rate of bacterial growth during hydrolysis. For a target of 60% hydrolysis, the process would take approximately 80 hours at 4°C. The time could be reduced substantially by increasing the temperature but the growth of microorganisms could become significant. In their studies with sonicated dairy cultures, Shah and Jelen (1991) reported that the optimal temperature for β-galactosidase activity from *L. delbrueckii* subsp. *bulgaricus* 11842 was 55°C. This temperature is considerably higher than the optimal growth temperature so the effects from continued fermentation by *L. delbrueckii* subsp. *bulgaricus* 11842 should be minimal with hydrolysis at higher temperatures. Selection of a suitable temperature (e.g.

50°C) and other technological adjustments should lead to at least 60% lactose hydrolysis in a given solution within 24 hrs.

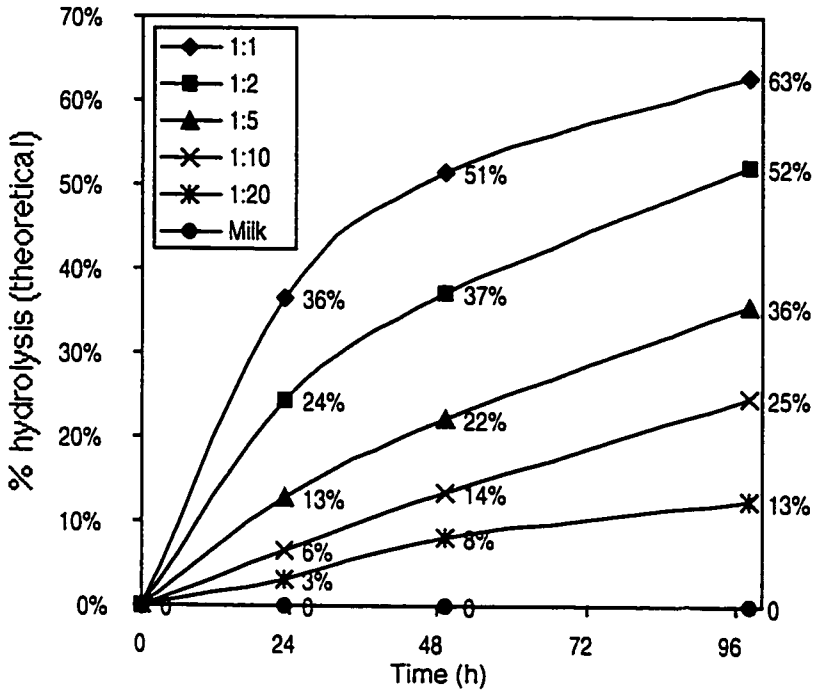

Figure 7-2. Hydrolysis of lactose in 2% milk at 4°C by direct addition of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 disrupted using a high speed bead mill.

The efficiency of the process could likely be improved by selecting a dairy organism that is capable of producing more enzyme (β-galactosidase) with an optimum activity at the desired temperature; genetic manipulation of a specific dairy organism to increase the amount of enzyme produced several fold; or by genetic modification of the enzyme to improve its stability and activity at the desired temperature of hydrolysis. Although an industrial process based on the results obtained here would not be realistic, the data provided a useful background for the economic evaluation of the proposed approach.

7.4 Economic evaluation

7.4.1 1000 - 2000 L batch fermentation

It was assumed that the facility already has a 1000 L or 2000 L vessel that can be utilized for the fermentation. In addition, the pumps, heat exchangers, and holding vessels required for pasteurization, fermentation and cooling would also be available for use. The capital cost estimation was based on the assumption that the facility already has the capability to maintain and culture the dairy organism for fermentation. The centrifuge, required for concentration of the biomass, is the largest single capital expense (Table 7-1) having an estimated installed cost of $450k. Although the bactofuges in some facilities would be capable of the concentration, it is likely they would be too large to efficiently handle the small volume used for this process.

Table 7-1. Fixed capital investment for the hydrolysis of lactose in milk using a disrupted culture of *L. delbrueckii* ssp. *bulgaricus* 11842. Basis: 1 000 -2 000 L of milk per batch.

Item	Cost ($CDN)	Ref
Direct costs		
1. Installed equipment		
Bactofuge	450 000	a
Bead mill (1.4 L chamber)	42 000	b
Cooling unit	20 000	c
2. Total direct costs	512 000	
Indirect costs		
Contingency and fee (30% of 2)	154 000	
Fixed capital investment	666 000	

a) Personal communication (1998) Alfa Laval Inc., Scarborough, ON, Canada
b) Personal communication (1998) Glen Mills Inc., Clifton, NJ, USA
c) Personal communication (1999) Neslab Instruments, Inc., Portsmouth, NH, USA

The high pressure homogenizer and the high speed bead mill are comparable in cost and disruptive ability. To maximize the disruption, at least two passes through the high pressure homogenizer would be required with the option of placing two homogenizers in series. The bead mill is jacketed and therefore able to maintain cooler product temperatures. The high pressure homogenizer causes a significant temperature increase of 0.26°C per MPa (Bury and Jelen, 1999b) which requires a downstream heat exchanger or jacketed vessel for cooling. However, the capital cost of a small and simple heat exchanger required for the small volume and low flow rate would be insignificant in comparison to the total capital cost. Similarly, the cost of small jacketed holding vessels (~20 L) and pumps were also neglected. The total capital expenditure is estimated to be $666k for a 1000 - 2000 L fermentation.

If we assume that 1000L of fermentation broth could be used to produce 1000 L of milk in which 60% of the lactose is hydrolyzed, there would be an additional cost of $0.53 per liter of milk. Similarly, a 2000 L fermentation would produce 2000 L of product with an additional cost of $0.31 per liter of PLHM.

The cost of labour for a 1000 L fermentation was estimated to be half (50%) of the total operating expenses (Table 7-2). When the batch size is doubled from 1000 L to 2000 L, the labour requirements remain unchanged and the total operating expenses would only increase by 16%. Fermentations larger than 2000 L would likely require additional capital with the possibility of a utilities upgrade.

The fermentation cycle (start to end of CIP) should be about 24 hours. It would, therefore, be convenient to carry out the hydrolysis at an appropriate temperature, pasteurize, and package the product such that the cycle time for the holding tank is about

24 hours as well. Based on this assumption, a facility using a 1000 L fermentation should be able to produce 1000 L of PLHM per day.

Table 7-2. Operating expenses for the hydrolysis of lactose in milk using a disrupted culture of *L. delbrueckii* ssp. *bulgaricus* 11842. Basis: 1 000 -2 000 L of milk per batch with an operating factor of 0.85.

Basis		1 000 L batch		2 000 L batch	
	Unit cost	Quantity per batch	Cost ($/batch)	Quantity per batch	Cost ($/batch)
Raw Materials					
Whey [a]	$ 0.40/kg	57.0 kg	22.80	114.0 kg	45.61
Yeast Extract [b]	$ 4.00/kg	5.7 kg	22.80	11.4 kg	45.61
12 N NaOH (~50%) [c]	$ 0.42/L	11.8 L	5.02	23.6 L	10.04
Direct Labour					
Process Operators	$ 20/h	10 h	200.00	10 h	200.00
Supervision (15% Operators)	15%		30.00		30.00
Benefits (15% of Labour)	15%		34.50		34.50
Utilities					
Steam [d]	$ 0.015/kg	433 kg	6.49	865 kg	12.98
Cooling [d]	$ 0.929/m^3	29 m^3	27.09	58 m^3	54.18
Electricity [e]	$ 0.019/kWh	21 kWh	0.39	40 kWh	0.74
Repairs					
Assume 4% of fixed Capital			72.94		72.94
Overhead (~40% of Direct Labour)			105.80		105.80
Total Operating Expense			$ 527.84		$ 612.40
Volume produced (L)			1 000 L		2 000 L
Cost per Volume ($/L)			$ 0.53		$ 0.31
Cost of 2% milk per L (retail, 1999)			$ 1.05		$ 1.05
Total cost per L			$ 1.58		$ 1.36

[a] Personal communication (1999) P. Jelen, University of Alberta, Edmonton, AB, Canada
[b] Personal communication (1999) Alberta Research Council, Edmonton, AB, Canada
[c] Personal communication (1996) Prairie Chem Inc., Edmonton, AB, Canada
[d] Kalk and Langlykke (1986)
[e] Personal communication (1996) Edmonton Power, Edmonton, AB, Canada

A cash flow analysis (Figure 7-3) shows that the discounted cash flow rate of return (DCFRR) is about 9% for an annual production of 312 000 L PLH milk (1000 L

per day with an 85% operating factor). A 2000 L fermentation has a DCFRR of 9% at an operating factor of 20% and a DCFRR of 36% at an operating factor of 85%.

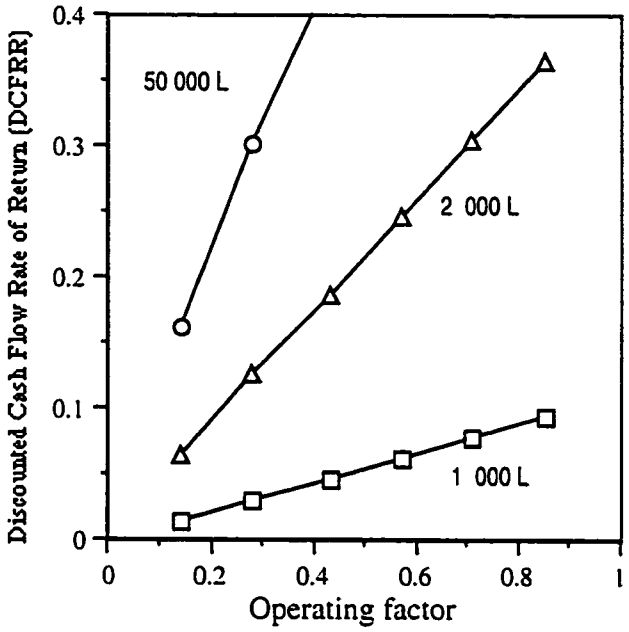

Figure 7-3. Discounted cash flow rate of return in regards to the hydrolysis of lactose in milk using a disrupted culture of *L. delbrueckii* ssp. *bulgaricus* 11842. Basis: 1 000 -50 000 L of milk per batch. Operating factor = days of production per year / number of days per year.

Based on the criterion of 30% DCFRR, a process based on a 1000 L fermentation would not be advisable, while a 2000 L fermentation appears more viable as long as the operating factor is greater than 70%. In response to competition, the price would probably be reduced thus lowering income. Consumer demand must also be considered. For instance, although the process might be capable of an annual production of 625 000 L, there is no guarantee the marketplace will absorb all the product.

If the facility would have access to a bactofuge, the fixed capital investment would be substantially lower. A cash flow analysis in this case reveals a DCFRR of 30% for a facility using a 1000 L fermentation at an operating factor around 27% . The

DCFRR for a process based on a 2000 L fermentation is about 30% for an operating factor of 8% warranting a pilot scale fermentation and hydrolysis in order to confirm the favorable economic analysis.

7.4.2 50 000 L batch fermentation for hydrolysis of lactose in milk

A fermentation of this size would require the installation of a 50 000 L fermentor, seed tanks, heat exchangers, centrifuges, holding tanks, pumps, piping, etc. The fixed capital investment in this scenario is estimated to be $7.5 M (Table 7-3).

Table 7-3. Fixed capital investment for the hydrolysis of lactose in milk using a disrupted culture of *L. delbrueckii* ssp. *bulgaricus* 11842. Basis: 50 000 L of milk per batch.

Item	Cost ($CDN)	Ref
Direct costs		
1. Installed equipment		
50 000 L fermentor	900 000	a
2 000 L seed fermenter	230 000	a
100 L seed fermenter	142 000	a
20 L seed fermentors 2 x 35 000	70 000	a
Heat exchanger for broth	360 000	b
Disk centrifuges 4 x 800 000	320 000	a
Bead mill (215 L chamber)	271 000	c
50 000 L Holding vessel for hydrolysis 2 x 243 000	486 000	a
Pumps and Misc	90 000	a
2. Total direct costs	5 749 000	
Indirect costs		
Contingency and fee (30% of 2)	1 751 000	
Fixed capital investment	7 500 000	

a) Kalk and Langlykke (1986)
b) Ulrich (1984)
c) Sharma (1994)

The utilities required by the process may be more than the existing facility is able to provide, in which case, additional capital would be necessary for upgrading or installing

equipment to meet the demand. The cost of equipment for packaging was not included in the list of fixed capital expenses. Table 7-4 shows the operating expenses.

Table 7-4. Operating expenses for the hydrolysis of lactose in milk using a disrupted culture of *L. delbrueckii* ssp. *bulgaricus* 11842. Basis: 50 000 L of milk or 500 000 L of whey (6.5% solids) per day with an operating factor of 0.85.

Basis		50 000 L milk		500 000 L whey	
	Unit cost	Quantity per day	Cost ($/day)	Quantity per day	Cost ($/day)
Raw Materials		Milk		Whey	
Whey	$ 0.40/kg	2 965 kg	1 186	25 911 kg	Assumed $0
Yeast Extract [a]	$ 4.00/kg	296 kg	1 186	2 591 kg	10 364
12 N NaOH (~50%) [b]	$ 0.42/L	615 L	261	5 373 L	2 283
Direct Labour					
Process Operators	$ 20/h	22 h	440	66 h	1 320
Supervision (15% Operators)	15%		66		198
Benefits (15% of Labour)	15%		76		228
Utilities					
Steam [c]	$ 0.015/kg	5 623 kg	84	44 468 kg	667
Cooling [c,d]	$ 0.929/m^3	1 459 m^3	1 354	13 129 m^3	782
Electricity [e]	$ 0.019/kWh	2 395 kWh	1 484	28 137 kWh	400
Repairs					
Assume 4% of fixed Capital		4%	822	3%	1 644
Overhead (~40% of Direct Labour)			233		698
Total Operating Expense			$ 5 753		$ 18 584
Volume produced (L)			50 000 L		500 000 L
Cost per Volume ($/L)			$ 0.12		$ 0.04
Cost of 2% milk per L (retail, 1999)			$ 1.05		
Final mass of syrup (65% solids)					50 000 kg
Cost of syrup (65% solids) neglecting cost of evaporation [f]					$ 0.40 /kg
Estimated cost of evaporation ($/kg of syrup) [g]					$ 0.20 /kg
Total cost			$ 1.17 /L		$ 0.60 /kg

[a] Personal communication (1999) Alberta Research Council, Edmonton, AB, Canada
[b] Personal communication (1996) Prairie Chem Inc., Edmonton, AB, Canada
[c] Kalk and Langlykke (1986)
[d] Ulrich (1984)
[e] Personal communication (1996) Edmonton Power, Edmonton, AB, Canada
[f] Total operating expense / final mass of syrup
[g] Mahoney (1997)

The cash flow analysis (Figure 7-3) shows that the DCFRR is larger than 30% for operating factors greater than 28%. If an additional $7.5 M is required for a utilities upgrade or packaging system, an operating factor of 57% would be required for a DCFRR of 30%. Reducing the price of PLHM from $1.79 to $1.37 results in a DCFRR of 30% for an operating factor of 85%. Thus, the process should remain profitable even if the anticipated selling price should drop by 23%. As mentioned previously, the DCFRR, in this instance, is based upon selling 100% of what is produced. In this case, there would have to be a demand for 5 000 000 L of PLHM per year (30% operating factor) at a selling price of $1.79. A detailed cost estimate would be highly recommended.

7.4.3 500 000 L fermentation for the production of UF permeate syrup

It was assumed that the process was built on to an existing whey processing facility capable of handling 1 million liters of whey or UF permeate per day. The fermentation would use 500 000 L while the other 500 000 L would be used for hydrolysis. The additional facility would cost approximately $20 million (Table 7-5) to build and equip (excluding multiple effect evaporator as the existing facility would already have one). The permeate (6.5% solids) was assumed to have zero value.

The hydrolyzed permeate (60% hydrolysis) would cost about $0.40 per kg syrup (prior to concentration) with an additional $0.20 per kg syrup for evaporation (Table 7-4). Total cost of production would be $0.60 per kg of syrup which is higher than the cost of competing sweeteners. For example, in 1998 raw sugar sold for $0.23-0.74 per kg depending on the grade (electronic communication, New York historical data, Coffee, Sugar, Cocoa Exchange, Inc, NY, USA). In order to "break even" in 10 years, the

hydrolyzed whey syrup (60% hydrolysis, 65% solids) would have to be sold at $(CDN) 1.01 per kg syrup, a very unfavorable price.

Table 7-5. Fixed capital investment for the hydrolysis of lactose in whey using a disrupted culture of *L. delbrueckii* ssp. *bulgaricus* 11842. Basis: 500 000 L of whey per batch.

Item			Cost ($CDN)	Ref
Direct costs				
1. Installed equipment				
250 000 L fermentor	2 x	2 612 000	5 224 000	a
4 000 L seed fermentor	2 x	250 000	500 000	a
100 L seed fermentor	2 x	138 000	277 000	a
20 L seed fermentors	4 x	37 000	148 000	a
Disk centrifuge	10 x	800 000	8 000 000	a
Bead mill (275 L chamber)	4 x	300 000	1 200 000	b
2. Total direct costs			15 348 000	
Indirect costs				
Contingency and fee (30% of 2)			4 652 000	
Fixed capital investment			20 000 000	

a) Kalk and Langlykke (1986)
b) Sharma (1994)

7.5 Conclusions

Experiments with *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 have shown that the disrupted biomass from 1 L of broth could be capable of hydrolyzing 60% of the lactose in 1L of milk within 24 hrs given an appropriate process optimization. The production of partially lactose hydrolyzed (PLH) milk using the disrupted culture from 1000 to 2000 L of fermented whey-based broth does not appear to be economically viable unless the production facility already owns a bactofuge. If the facility only has to purchase the equipment for cell disruption, one 2000 L fermentation per week would result in a discounted cash flow rate of return (DCFRR) greater than 30%.

The production of 50 000 L of PLH milk appears feasible even though the facility would have to spend a minimum of $ 7.5 million in fixed capital. The process should remain profitable even if the selling price would fall by 23%. Demand is the biggest uncertainty in this case. If there is little demand for the product or the market is already saturated, the process may not be economically viable.

The production of whey or permeate syrup (65% solids, 60% hydrolysis) does not appear feasible based upon a 500 000 L fermentation. Whereas PLHM is a high value added product, the syrup must compete against inexpensive sweetener commodities. In this case, the hydrolyzed whey syrup costs considerably more than other sweeteners such as refined beet sugar.

7.6 References

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Chapter 8

Conclusions and recommendations for future research.

8.1 Conclusions

The lactose in milk, whey, or UF whey permeate was hydrolyzed by the direct addition of disrupted cultures of *L. delbrueckii* ssp. *bulgaricus* 11842. The cells were grown on whey-based media, harvested, mechanically disrupted, and used for hydrolysis. The process was shown to be technically possible in the laboratory and should be applicable on an industrial scale given further research and financial motivation.

L. delbrueckii ssp. *bulgaricus* 11842 grows poorly on whey and especially UF whey permeate. The addition of whey protein concentrates (WPC's) increased the rate of growth and acidification to a small extent in most cases, but the advantages of using WPC's to encourage the growth of lactic acid bacteria were minimal in comparison to supplementation with yeast extract. Increasing the amount of yeast extract increased the growth rate and acid production of *L. delbrueckii* ssp. *bulgaricus* 11842 but the production of β-galactosidase appeared relatively insensitive to the addition of 0.2-0.8% yeast extract. Supplementation with 1% yeast extract resulted in the highest levels of β-galactosidase in the range studied, however, the cost of using higher concentrations of yeast extract was considered to be prohibitive. Therefore, 0.5-0.6% yeast extract can be recommended for the growth of *L. delbrueckii* ssp. *bulgaricus* 11842 in sweet whey.

The bead mill and the high-pressure homogenizer were equally effective in disrupting cultures of *L. delbrueckii* ssp. *bulgaricus* 11842 for the release of β-galactosidase and appear to be suitable for use on an industrial scale. In comparison,

sonication did not appear to be as effective even with the small laboratory volumes and has little potential for scale-up.

The production of 1000-2000 L of partially lactose hydrolyzed milk (PLHM) would require minimal capital investment other than the purchase of disrupting equipment and a bactufuge. The feasibility of the process is questionable unless the facility is able to utilize existing equipment to concentrate the biomass in which case the process appears feasible. An economic evaluation for the production of 50 000 L of PLHM per batch suggests that the process would be quite feasible provided there is sufficient demand for PLHM.

The cost of producing sweetening syrups by hydrolyzing lactose in whey or UF whey permeates using the direct addition of disrupted dairy cultures is estimated to be much higher than competing products. If the disposal of the spent fermentation broth was taken into account, the cost of producing the syrup would be even higher.

8.2 Recommendations for future research

The economic parameters of the lactose hydrolysis in dairy products or other food or non-food materials may be improved with further research into technical and biological aspects. For instance, although *L. delbrueckii* ssp. *bulgaricus* 11842 was used because it showed the most promise at the time when this project was initiated, continued screening may result in the discovery of a more productive dairy microorganism. The optimum temperature for the β-galactosidase used in this study was 55°C (Shah and Jelen, 1991). Hydrolysis at higher, near optimum temperatures should significantly increase the rate of hydrolysis and discourage the growth of microorganisms including

the cells of *L. delbrueckii* ssp. *bulgaricus* 11842 that survived disruption. Lower temperatures would slow or prevent the growth of the microorganisms but also decrease the rate of hydrolysis. Selection of a more appropriate dairy microorganism would help to optimize the hydrolysis at the desired processing temperature.

Genetic modification may enhance the production of β-galactosidase by *L. delbrueckii* ssp. *bulgaricus* 11842 or other microorganisms such as *E. coli*. It may also be possible to use genetic engineering to increase the activity and stability of β-galactosidase at a specified temperature. However, at the present time, the public perception of genetically modified foods is somewhat negative and could have a detrimental impact on the use of genetically modified microorganisms for the hydrolysis of lactose in foodstuffs in the foreseeable future.

Although the addition of disrupted cultures of *L. delbrueckii* ssp. *bulgaricus* 11842 reduced the lactose content in milk and whey, further research is required in evaluating the sensory aspects of the products. For example, the hydrolysis of lactose in milk at higher temperatures (50°C) seemed to promote the production of "off-flavors" when compared to hydrolysis at lower temperatures (4°C). As the "off-flavors" were not unpleasant, the process of using disrupted dairy cultures has an unlimited potential for the creation of novel dairy-based foods, beverages, or other products. For instance, the fermentation broth could be formulated for consumption by humans or animals. There is the possibility of probiotic applications or the production of beneficial oligo-saccharides. The process of using disrupted dairy cultures for lactose hydrolysis applications is technically possible and may even be economically viable for the reduction of lactose in

milk for fluid markets, but there is still a need for further research in developing commercially realistic applications and elucidating the various parameters of the process.

8.3 References

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Appendix

Preliminary and supplemental data.

This appendix provides the preliminary and supplemental data referred to in the previous chapters.

Figures A-1 through A-4 show the results of the preliminary screening experiments that were referred to in chapter 3. The figures demonstrate the growth and lactic acid production by *L. delbrueckii* ssp. *bulgaricus* 11842 grown in whey or ultrafiltered (UF) whey permeate supplemented with yeast extract, whey protein concentrate, or casein. Reference to figure A-3 was also mentioned in Chapter 5 regarding the production of lactic acid using 6 and 8% whey solids.

The cultures in figures A-1 through A-4 were grown in 150 mL of broth made by dissolving spray dried whey or UF whey permeate powder (60 g/L or 80 g/L) in distilled water and adding up to 2% (w/v) dried supplement. The 250 mL flasks were sterilized by heating to 121°C for 15 minutes and the cultures were grown at 43°C. Total cell counts were estimated by direct microscopic enumeration (hemaocytometer). Lactic acid production was estimated by the amount of 2N NaOH required to maintain the pH at 5.5±0.4. The averages of duplicated experiments are shown.

Chapter 4 compared the growth and lactic acid production of *L. delbrueckii* ssp. *bulgaricus* 11842 grown on whey broth supplemented with various whey protein concentrates (WPC's) and isolates (WPI's). Figure A-5 shows the acidification resulting from supplementation with WPC-35 or WPI-80. Although the addition of most WPC's increased the rate of acidification, whey-based broths containing Lactobacilli MRS powder (Difco, Detroit, MI) were substantially more productive (figure A-6).

Chapter 7 discussed the release of β-galactosidase from cells of *L. delbrueckii* ssp. *bulgaricus* 11842 disrupted using sonication, high-pressure homogenization, and bead milling. The soluble (free) β-galactosidase activity was defined as the activity remaining in the supernatant after the cell debris was removed by centrifugation. Delays before centrifugation tended to show a slight increase in soluble activity. Figure A-7 shows the solubilization of β-galactosidase at 0 and 20°C after processing the cells in a bead mill for 2 minutes.

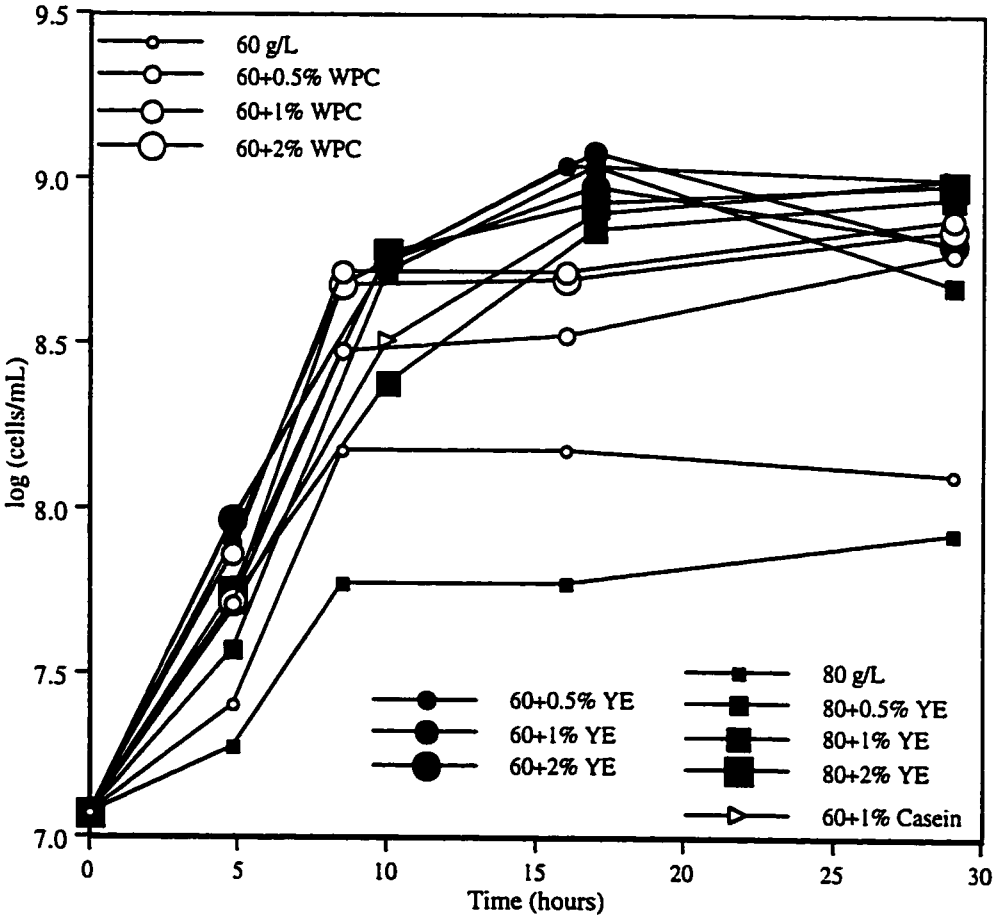

Figure A-1. Total cell count of *L. delbrueckii* ssp. *bulgaricus* 11842 grown in sterilized whey broth (60 g/L or 80 g/L spray dried whey powder) supplemented with whey protein concentrate (WPC), yeast extract (YE), or casein.

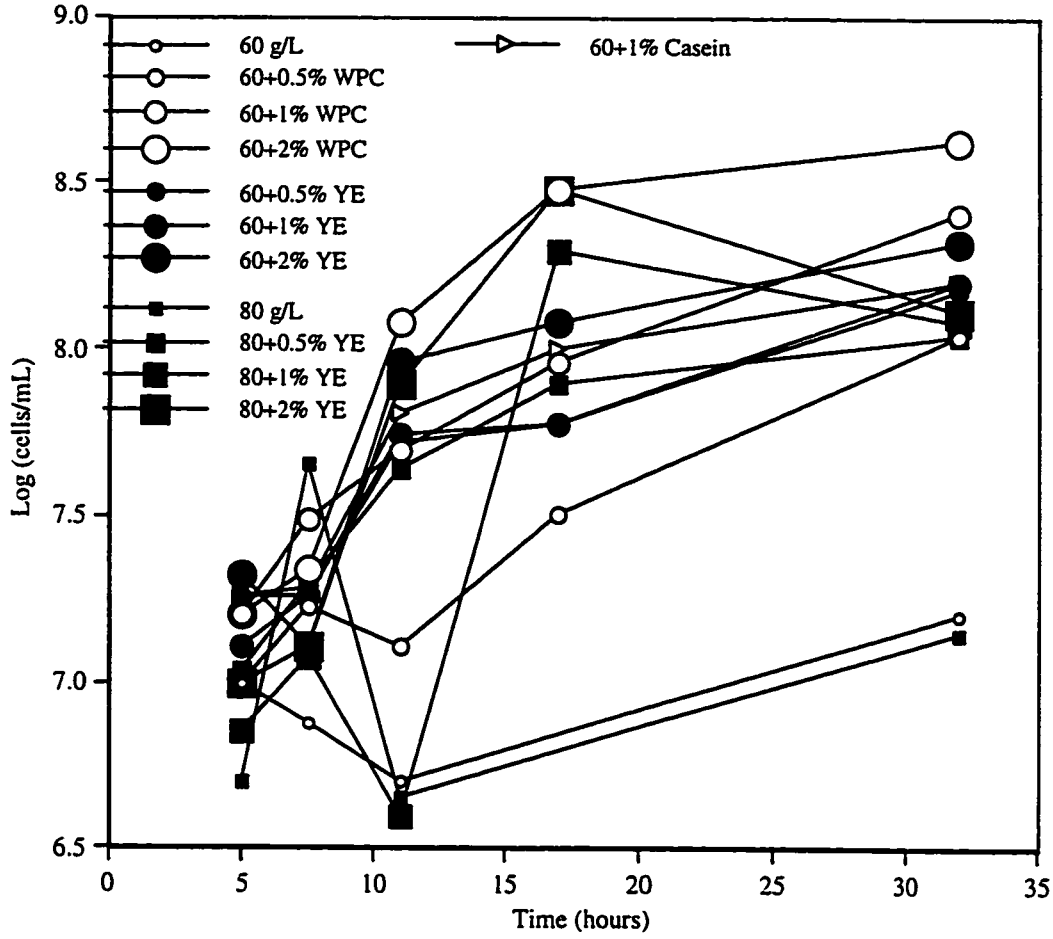

Figure A-2. Total cell count of *L. delbrueckii* ssp. *bulgaricus* 11842 grown in sterilized ultrafiltered (UF) whey permeate broth (60 g/L or 80 g/L spray dried UF whey permeate powder) supplemented with whey protein concentrate (WPC), yeast extract (YE), or casein.

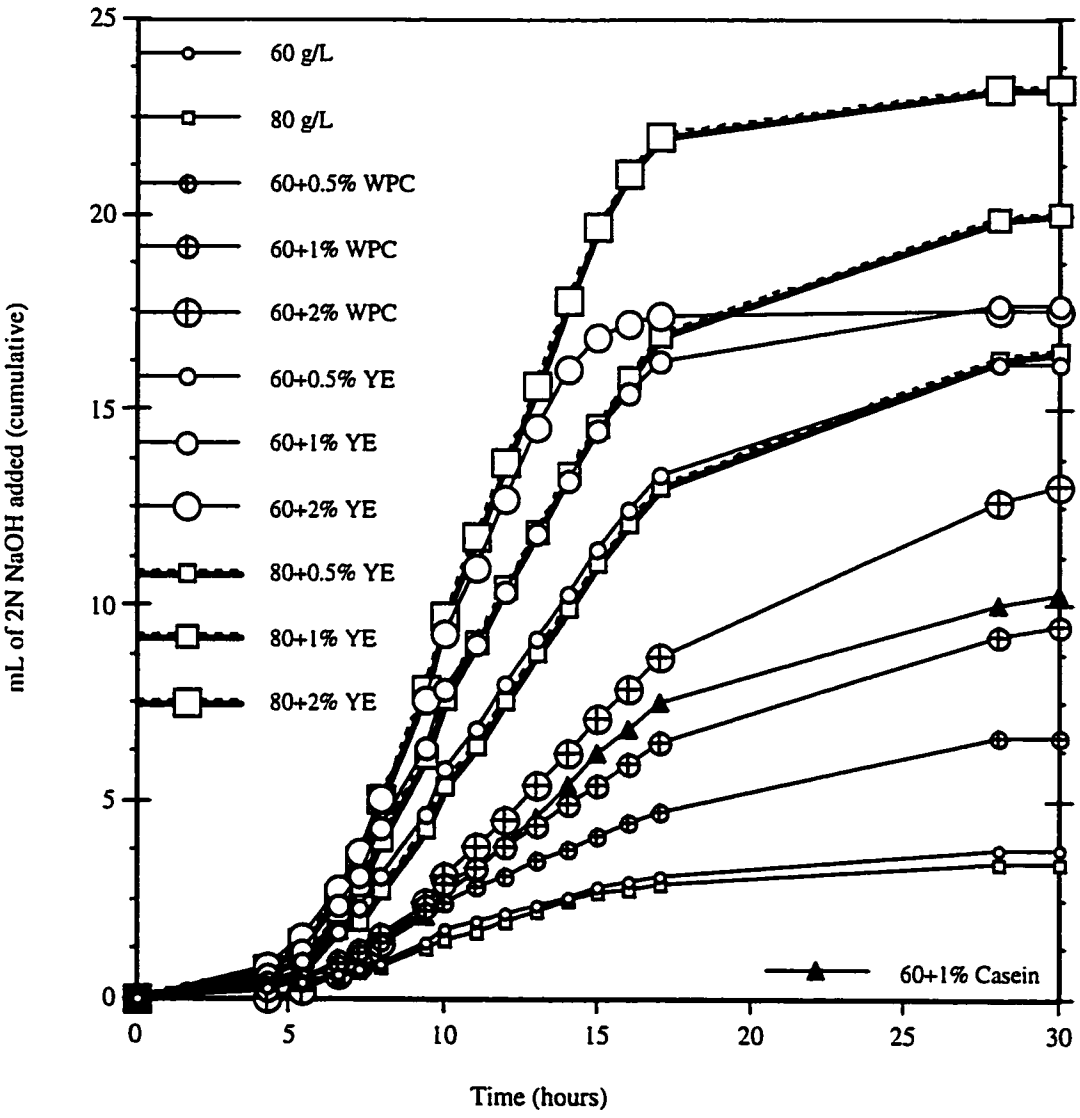

Figure A-3. Lactic acid production by *L. delbrueckii* ssp. *bulgaricus* 11842 grown in sterilized whey broth (60 g/L or 80 g/L spray dried whey powder) supplemented with whey protein concentrate (WPC), yeast extract (YE), or casein.

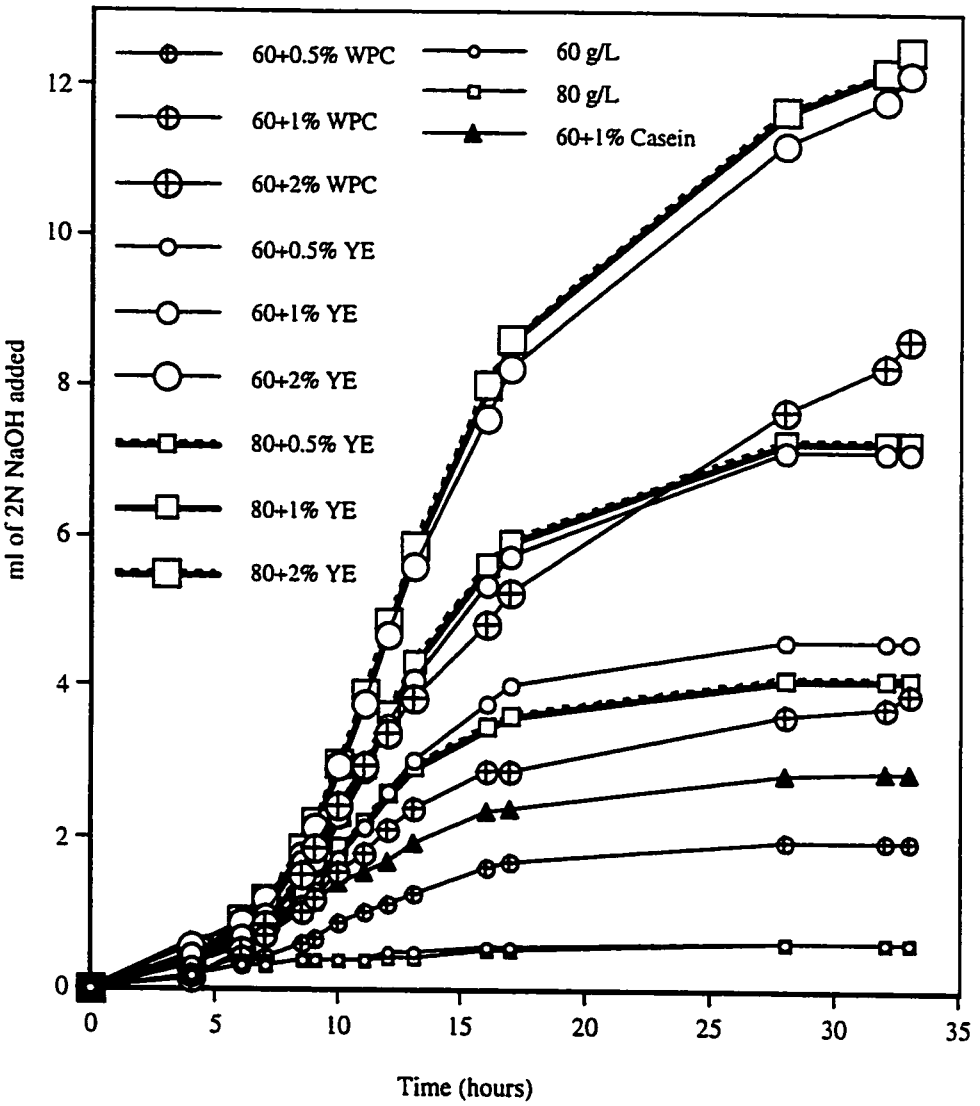

Figure A-4. Lactic acid production by *L. delbrueckii* ssp. *bulgaricus* 11842 grown in sterilized ultrafiltered (UF) whey permeate broth (60 g/L or 80 g/L spray dried UF whey permeate powder) supplemented with whey protein concentrate (WPC), yeast extract (YE), or casein.

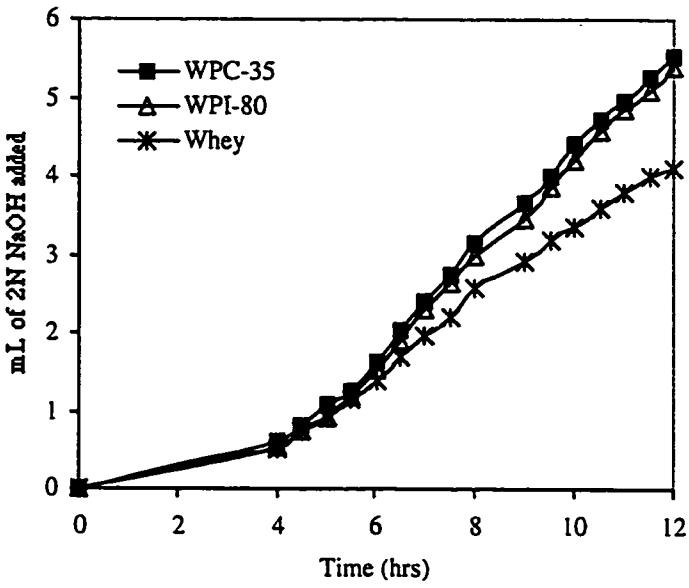

Figure A-5. Lactic acid production by *L. delbrueckii* ssp. *bulgaricus* 11842 grown in whey broth (60 g/L) and whey broth supplemented with 1% (w/v) whey protein concentrate (WPC-35) or whey protein isolate (WPI-80).

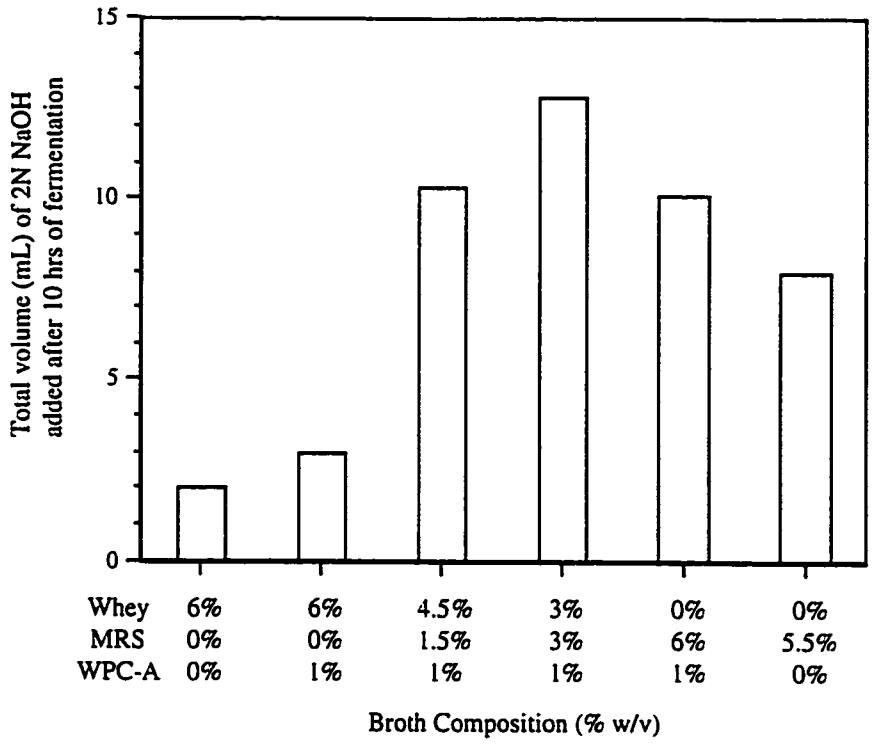

Figure A-6. Lactic acid production by *L. delbrueckii* ssp. *bulgaricus* 11842 grown in broths composed of various amounts of whey, MRS, and WPC-A (Promil).

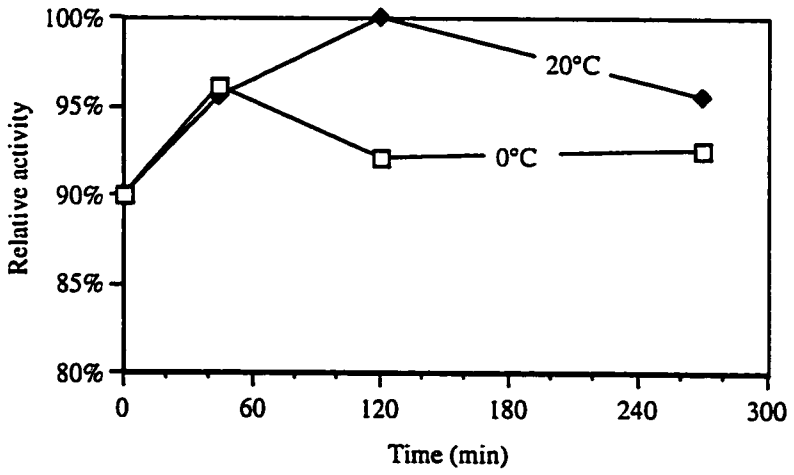

Figure A-7. Release of soluble (free) β-galactosidase from cells of *L. delbrueckii* ssp. *bulgaricus* 11842 at 0 and 20°C. The culture was disrupted by processing in a bead mill for 2 minutes. Samples of the disrupted culture at 0 and 20°C were centrifuged to remove cell debris. The β-galactosidase activity in the supernatant was measured using ONPG as the substrate.