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The use of crude cell extracts of lactic acid bacteria optimized for beta-galactosidase activity to form galactooligosaccharides with lactose, mannose, fucose, and N-acetylglucosamine

by

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Dedicated to my inspiring colleagues and my supportive family who have encouraged my pursuit of higher education

Abstract

Several lactic acid bacteria contain β-galactosidases. Beta galactosidases catalyze lactose hydrolysis and transfer acceptor sugars onto galactose, producing galactooligosaccharides. The aim of this work was to exploit β-galactosidases of lactic acid bacteria as crude cell extracts to produce novel oligosaccharides with mannose, N-acetylglucosamine, and fucose. Of 17 strains of lactic acid bacteria, transferase activity was the strongest in crude cell extracts of *Lactobacillus delbrueckii* subsp. *bulgaricus*, followed by *Streptococcus thermophilus*, *Lactobacillus animalis*, and *Lactobacillus reuteri* in a buffered 19% (w/w) lactose solution. Incorporation of 6 % (w/w) glycerol increased transferase activity and enzyme stability at higher incubation temperatures. Incorporation of 10% (w/w) mannose, N-acetylglucosamine and fucose as acceptor sugars yielded three distinct oligosaccharides with mannose and two with N-acetylglucosamine and fucose, with the composition confirmed using gas chromatography-mass spectrometry. This is the first public report indicating production of oligosaccharides containing N-acetylglucosamine and fucose from β-galactosidases of lactic acid bacteria.

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List of Symbols, Nomenclature, or Abbreviations

β-gal: Beta-galactosidases, β-galactosidases

OS: Oligosaccharides

GOS: Galactooligosaccharides

HOS: Heterooligosaccharides

CCE: Crude cell extracts

LAB: Lactic acid bacteria

NAG: N-acetylglucosamine

MHOS: Heterooligosaccharides containing mannose

NHOS: Heterooligosaccharides containing N-acetylglucosamine

FHOS: Heterooligosaccharides containing fucose

HPAEC-PAD: High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

HPLC: High Performance Liquid Chromatography

SEC: Size Exclusion Chromatography

RID: Refractive Index Detection

ELSD: Electron Scattering Light Detection

GC: Gas Chromatography

MS: Mass Spectrometry

GC-MS: Gas Chromatography – Mass Spectrometry

LC-MS: Liquid Chromatography – Mass Spectrometry

CID: Collision Induced Detection

UF: Ultrafiltration

Introduction

1.1. Oligosaccharides

Carbohydrates are classified as individual mono-, di-, and polysaccharides based on the degree of polymerization of constituent monosaccharides. Oligosaccharides (OS) have between two to 20 constituent monosaccharides. Milk of all placental mammals contains considerable amounts of OS, which play an important physiological role in establishing the growth of intestinal microflora, namely bifidobacteria (Kunz et al., 2000).

OS are known as prebiotics which resist digestion by gastric acids and enzymes until reaching the colon, where they are fermented by select intestinal bifidobacteria. These prebiotic properties are also found in OS of non-milk origin, which are currently added to foods as a source of dietary fibre (Boehme and Stahl, 2003). This gives additional opportunities for use as potential therapeutics in foods and supplements.

1.1.1. Human milk oligosaccharides

Milk OS exist as galactooligosaccharides (GOS) containing galactose, glucose, N-acetyglucosamine (NAG), fucose, and sialic acid on a terminal lactose molecule (Boehm and Stahl, 2003). Synthesis occurs by chemical glycosylation of monosaccharides and disaccharides with glycosidase and glycosyltransferase enzymes (Barreteau et al., 2006). As each constituent monosaccharide has hydroxyl groups to form glycosidic linkages via glycosyltransferase enzymes, the

variety of OS outnumbers peptides and the variety of other biological molecules (Barreteau et al, 2006).

The complex structure and composition of human milk OS contribute "prebiotic" properties by resisting digestion from gastric acid, hydrolysis by mammalian enzymes, and gastrointestinal absorption. Human milk OS are fermented by the intestinal microflora; this selectively stimulates the growth and activity of intestinal bacteria that benefit the health and wellbeing of the host (Gibson, et al., 2004). Human milk OS selectively stimulate the growth of intestinal microbiota (Gibson and Roberfroid, 1995).

Human milk OS additionally protect infants from infection caused by enteropathogenic *Escherichia coli* and other pathogens causing diarrheal diseases. At a concentration of 0.7–1.2 grams per litre, OS are a major component of human milk (Kunz et al., 2000). Human milk has a higher OS concentration than the milk of cows, sheep, goats, and horses, and contains more sugars with fucose, NAG, and sialic acid (Boehme and Stahl, 2003). This implies that the intestinal tract of the human infant is lesser developed than that of animals and requires additional protection from pathogens (Uraschima et al., 2001).

Human milk OS benefit the infantile intestinal microorganisms and provide protection against pathogens. Breast-fed infants also have a greater intestinal population of *B. infantis*, the only microorganism with glycosylhydrolases capable of digesting and fermenting human milk OS (Sela et al., 2008). Glycohydrolase enzymes in *Bifidobacterium infantis* digest human milk OS,

particularly those with NAG, which promote their growth in the infantile intestinal tract (Jenness et al., 1964, Sela, et al., 2008).

In addition to supporting the growth of *B. infantis*, human milk OS serve as passive protection in infants when recognized by pathogens. This is because the composition of human milk OS also mimics OS found on epithelial cells in the intestinal tract. Some intestinal pathogens such as enteropathogenic *E. coli* have multiple receptors that bind to specific OS on the host cell surface (Shoaf et al., 2006). The specific receptor sites of selected pathogens are shown in Table 1.

Table 1. Specific sugars* recognized as surface receptor sites of various pathogens in both intestinal cells and human milk oligosaccharides.

Specificity	Pathogen
Fucosylated oligosaccharides [a]	*Escherichia coli* (heat-stable enterotoxin)
Fucosylated tetra- and pentasaccharides [a]	*E. coli*
GlcNAc(ß1-4)GlcNAc [b]	*E. coli* K1
Gal(α1-4)Gal [b,c]	*E. coli* P
Fuc(α1-2)Gal(ß1-3)[Fuc(α1-4)] Gal [b]	*Helicobacter pylori*
Gal(ß1-4)Glc(NAc) [b]	*Neisseria gonorrhoea*
Gal(ß1-3)Glc(NAc)(ß1-3)Gal(ß1-4)-Glc [b]	*Pseudomonas aeruginosa*
Gal(ß1-4)GlcNAc / Gal(ß1-3)GlcNAc [a,c]	
Gal(ß1-4)GlcNAc / Gal(ß1-3)GlcNAc [c]	*Streptococcus pneumonia*
Neutral oligosaccharides (LNT) [a]	
Gal(α1-4)Gal [c]	*S. suis*
Gal(α1-4)Gal(ß1-4)Glc [c]	
GlcNAc(ß1-3)Gal [c]	

*Gal: galactose, Glc: glucose, Fuc: fucose, GlcNAc: N-acetylglucosamine
a: Kunz et al., 2000; b: Sharon and Ofek, 2000 ; c: Boehm and Stahl, 2003

By recognizing OS on the host cell surface, pathogens adhere and attach onto the cell, and infection may follow. When human milk OS bind to receptor sites

on the pathogen, they prevent their attachment onto intestinal cells, thereby reducing the incidence of diarrheal diseases (Smart, 1991, Newburg, 1996, Martı´n-Sosa et al., 2002, Hopkins and Macfarlane, 2003).

1.1.2. Sources of oligosaccharides from non-milk origin

With their structural and compositional complexity, human milk OS currently cannot be industrially produced. This results in the supplementation of infant formula with simpler GOS structures to mimic the beneficial effects of human milk in stimulating the growth of intestinal bifidobacteria (Boehm et al., 2002, Moro et al., 2002).

In addition to infant formula, OS are supplemented in value-added food products. OS exist in plants as galactooligosaccharides (GOS), soybean OS, fructooligosaccharides (FOS), and xylooligosaccharides. FOS are linear or branched fructose-polymers, which are either β(2-1)-linked inulins in vegetables such as asparagus, onions, and chicory root, or β(2-6)-linked levans in cereals. Xylooligosaccharides are synthesized from xylans in corn cobs, while GOS, lactulose, and lactosucrose are synthesized from lactose (Boehme and Stahl, 2003). FOS are currently added to fermented dairy products such as yoghurt, which also contain lactic acid bacteria (LAB) that benefit the host upon consumption.

Prebiotic properties of FOS, GOS, lactulose, lactosucrose, and even lactose stimulate the growth of intestinal bifidobacteria (Gibson and Roberfroid, 1995, Gibson et al., 2004, Szilagyi, 2004). GOS, however, lack fucose, NAG, and sialic

acid found in human milk OS, and do not provide specific resistance to pathogens (Bode, 2006, Boehme and Moro, 2008, Sela et al., 2008). Production of HOS by transgalactosylation of galactose with NAG and fucose as acceptor sugars with β-gal in CCE of food-grade LAB provides the opportunity to produce OS with composition similar to human milk OS (Mahoney, 1998, Bode, 2006).

1.2. Production of galactooligosaccharides with crude cell extracts of β-galactosidase enzymes of lactic acid bacteria

LAB are comprised of a group of gram-positive, nonrespiring cocci or rods, which produce lactic acid as major end product of glucose fermentation. The term LAB originally described "milk-souring organisms", the first pure culture obtained by J. Lister in 1873 as cited by Axelsson (2004). LAB are associated with nutrient-rich environments such as dairy foods and in the intestinal tract of mammals. LAB associated with food fermentations, such as *Lactococcus lactis* in fermented dairy foods, are considered to be safe (Axelsson, 2004).

Classification of LAB is based on standards set by Orla-Jensen in 1919, which focus on morphology, glucose fermentation method, configuration of lactic acid produced, growth at certain incubation temperatures and in high salt, acid, or alkaline conditions (Axelsson, 2004). A core group of LAB is comprised of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. Glucose metabolism is performed by *Pediococcus* spp., certain *Lactobacillus* spp. and *Streptococcus thermophilus* in a homofermentative Embden-Meyerhof-Parnas pathway, which converts one molecule of glucose into two molecules of lactic

acid (Carr et al., 2002). Glucose is also metabolized in a heterofermentative phosphoketolase pathway by *Leuconostoc* spp. and certain *Lactobacillus* spp., which converts a molecule of glucose into one molecule of each lactic acid, ethanol, and carbon dioxide (Axelsson, 2004, Carr et al., 2002).

Heterofermentative LAB play a role in food fermentation and spoilage, based on the application. *Leuconostoc* spp. are starter cultures in the fermentation of buttermilk and cheese, and cause spoilage in meats (Carr et al., 2002). While *Bifidobacterium* spp. share some of the same features as LAB, they have a unique glucose fermentation method that sets them apart from LAB.

Many LAB are capable of fermenting lactose that is found in food or in the intestinal tract of humans and animals. These LAB harbour β-galactosidase (β-gal) enzymes, which enable lactose utilization shown in Figure 1.

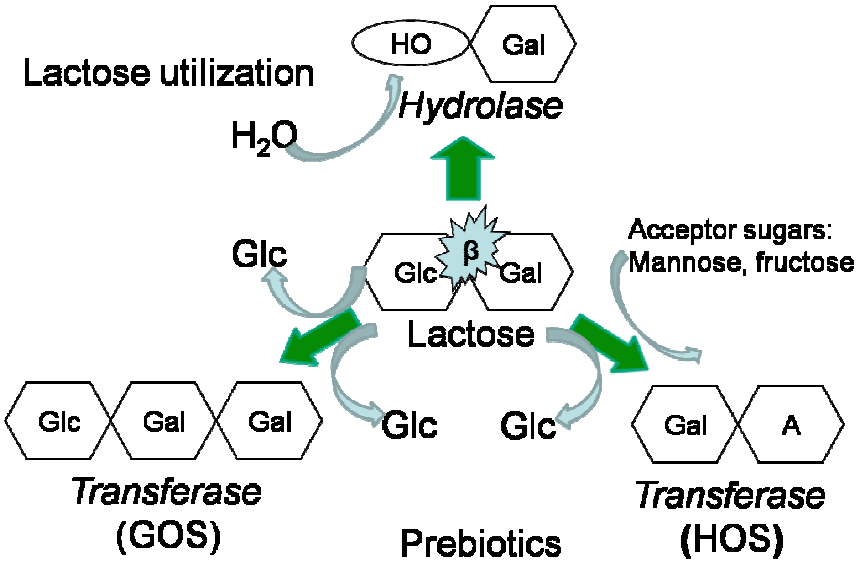

Figure 1. Lactose utilization by hydrolase and transferase activities of β-galactosidases found in many strains of lactic acid bacteria.

Beta-galactosidases are classified in four families of glycosylhydrolase enzymes. Most enzymes of LAB belong to glycosylhydrolase family 2, which require monovalent and divalent metal ions for maximum activity (Nakayama and Amachi, 1999). Beta-galactosidases of *E. coli lacZ* and *Kluyveromyces lactis* also belong to glycosylhydrolase family 2 (Nakayama and Amachi, 1999).

Glycosylhydrolase enzymes break the o-glucosyl group of lactose into glucose and galactose moieties. An acceptor molecule attaches onto the free hydroxyl group of the galactose and glucose moieties. When the acceptor is water, glucose and galactose are formed by hydrolase activity of β-gal. The β-gal enzyme is necessary for LAB in milk and in the infantile intestinal tract to hydrolyze lactose to release glucose for growth.

When the acceptor is another sugar molecule, β-gal enzymes catalyze its transfer to donor molecules by forming glycosidic bonds (Barreteau et al., 2006). GOS are produced by transfer of enzyme-bound galactose to another galactose moiety or a molecule of lactose as an acceptor (Wierzbicki and Kosikowski, 1972, Greenberg and Mahoney, 1983, Prenosil et al., 1987a). A variety of GOS is formed by transgalactosylation of β-gal with the different types of linkages between its constituent monosaccharides.

It is known that *Lactobacillus delbrueckii* subsp. *bulgaricus* (referred to in further text as *Lactobacillus bulgaricus*) produces galactopyranosyl-glucose with β(1-3) and β(1-6) linkages, and galactopyranosyl-D-galactose with a β(1-6) linkage and *Streptococcus thermophilus* produces galactopyranosyl-glucose with

a β(1-2) linkage, trisaccharides and higher OS (Toba, et al, 1981). A spectrum of GOS formed by β-gal of various microbial strains is shown in Table 2.

Mechanical disruption of LAB cells releases intracellular β-gal, producing crude cell extracts (CCE) (Vasiljevic and Jelen, 2002). CCE from food-grade LAB is regarded as a safe, low-cost enzyme source suitable for lactose utilization without purification (Vasiljevic and Jelen, 2002, Gänzle, et al., 2008). CCE have been used industrially for lactose hydrolysis and GOS production from skim milk and lactose-containing effluents such as milk and whey permeates (Kreft and Jelen, 2000, Vasiljevic and Jelen, 2003, Splechtna et al., 2007). Copious quantities of ultrafiltration (UF) permeate are produced by the dairy industry following the manufacture of cheese and processing of milk and whey.

Whey UF permeate is a byproduct of the manufacture of cheese, following removal of whey proteins. Whey permeate contains lactose and mineral salts remaining after ultrafiltration of whey to separate whey proteins, a valuable component of whey, from less valuable components of lactose and minerals (Wong et al., 1978). Whey permeate cannot be easily disposed because of the high biological oxygen demand from its lactose content, resulting in disposal difficulties and environmental issues. The use of UF whey permeate in GOS production could be of interest to the dairy processing industry as a method of byproduct utilization to minimize waste output.

Table 2. Galactooligosaccharides formed from galactose (Gal) and glucose (Glc) by transgalactosylation of β-galactosidases from various microbial strains.

Disaccharides	**Name**
β-D-Gal (1-6)-D-Glc [a,b,c,d]	allolactose
β-D-Gal (1-6)-D-Gal [a,b,c,d]	galactobiose
β-D-Gal (1-3)-D-Glc [a,d]	
β-D-Gal (1-2)-D-Glc [a,d]	
β-D-Gal (1-3)-D-Gal [a,d]	
Trisaccharides	
β-D-Gal (1-6)-β-D-Gal-(1-6)-D-Gal [b,c,d]	6' galactotriose
β-D-Gal (1-6)-β-D-Gal-(1-6)-D-Glc [b,c,d]	6' digalactosyl-glucose
β-D-Gal (1-6)-β-D-Gal-(1-4)-D-Glc [b,c,d]	6' galactosyl-lactose
β-D-Gal (1-3)-β-D-Gal-(1-4)-D-Glc [c,d]	3' galactosyl-lactose
β-D-Gal (1-4)-β-D-Gal-(1-4)-D-Glc [c,d]	4’ galactosyl-lactose
Tetrasaccharides	
β-D-Gal (1-6)-β-D-Gal-(1-6)-β-D-Gal-(1-4)-D-Glc [c,d]	6’ digalactosyl-lactose
β-D-Gal (1-6)-β-D-Gal-(1-3)-β-D-Gal-(1-4)-D-Glc [c,d]	
β-D-Gal (1-3)-β-D-Gal-(1-6)-β-D-Gal-(1-4)-D-Glc [c,d]	
β-D-Gal (1-6)-β-D-Gal-(1-6)-β-D-Gal-(1-6)-D-Gal [b]	
β-D-Gal (1-6)-β-D-Gal-(1-6)-β-D-Gal-(1-4)-D-Glc [b]	
Pentasaccharide	
β-D-Gal (1-6)-β-D-Gal-(1-6)-β-D-Gal-(1-6)-β-D-Gal-(1-4)-D-Glc [c,d]	6’ trigalactosyl-lactose

Gal: galactose , Glc: glucose
a: Toba et al., 1981 ; b: Prenosil et al. 1987; c: Zarate and Lopez-Leiva, 1990; d: Mahoney,1996

UF whey permeate contains naturally-occurring monovalent and some divalent cations, which are known to enhance enzyme activity (Vasiljevic and Jelen, 2002). The composition of UF whey permeate varies in the type of cheeses that were manufactured. Differences in the mineral composition of UF whey permeate are caused by differences in methods used to precipitate the casein and

processing of whey. Sweet UF whey permeate is a byproduct of rennet-coagulated cheeses such as Cheddar, while acidic UF whey permeate is a byproduct of acid-coagulated cheeses such as cottage cheese (Wong et al., 1978). Acidic UF whey permeate has a higher concentration of minerals than sweet UF whey permeate, especially in calcium (Wong et al., 1978). Calcium is retained within the curd of rennet-coagulated cheeses, while ionic calcium are released into the whey upon acid coagulation (Wong et al., 1978). While acidic UF whey permeate has a higher concentration of minerals, its high calcium content inhibits β-gal activity (Garman et al., 1996).

1.3. Optimization of β-galactosidase activity for galactooligosaccharide production

A shift toward the transgalactosylation activity of β-gal can be accomplished by a variety of methods including removing enzyme-inhibiting compounds, adjusting

the substrate concentration, incubation temperature, and by immobilization, using different enzyme sources, and genetic manipulation of the enzyme.

1.3.1. Optimization of reaction conditions

Enzyme activity and stability differs between bacterial strains and by altering the buffering capacity of the substrate. Substrate concentration, pH and addition of mineral cofactors including potassium and magnesium are all known

to affect β-gal-activity (Garman et al., 1996, Rustom et al., 1998, Splechtna et al., 2006). Monovalent and divalent cations such as potassium and magnesium serve as cofactors to enhance β-gal activity of LAB (Garman et al., 1996; Kreft and Jelen, 2000).

1.3.2. Removal of glucose as a competitive inhibitor

Lactose hydrolysis liberates glucose and galactose, which inhibit β-gal activity. Glucose is not desired in GOS manufacture as it is a noncompetitive inhibitor for β-gal that reduces enzyme activity by binding onto the enzyme and altering may be undesirable; its structure to change the conformation of the active site (Flaschel et al., 1982, Prenosil et al., 1987a). Glucose also does not provide the fermentation selectivity for prebiotic carbohydrates. In addition, glucose contributes sweetness which its presence can limit the application of GOS in value-added foods. Therefore, glucose removal is required in GOS and HOS production. Glucose can be oxidized with glucose oxidase or consumed by *Saccharomyces cerevisiae*, where its removal will maintain β-gal activity (Goulas et al., 2007). Purification of sugar solutions with *Saccharomyces cerevisiae* is currently used in the manufacture of FOS (Duvnjak and Koren, 1987).

1.3.3. Effect of incubation temperature and compatible solutes

Enzyme activity is also affected by the water activity. Hydrolase activity dominates over transferase activity in solutions of higher water activity. Thus, transferase activity is enhanced at higher lactose concentrations, corresponding to

reduced water activities. By removing the available water, there is a greater opportunity for sugars to serve as acceptor molecules following lactose hydrolysis. For the theoretical yield of lactose hydrolysis, equimolar amounts of glucose and galactose should be observed at lactose concentrations below 0.050 M (Huber et al., 1976). Increasing the lactose concentration decreases the concentration of available water, where galactose undergoes transgalactosylation to form GOS. At concentrations of 0.5 M lactose or higher, a yield of up to 40% of GOS is produced relative to the initial lactose concentration (Huber et al., 1976). Decreasing the water activity by increasing the substrate concentration can result in sugar crystallization depending on the solubility of the substrate. This can be overcome by increasing the incubation temperature, where the solubility of lactose goes from 18 grams at 25°C to 25 grams at 40°C and 37 grams at 60°C per 100 grams of water (Machado et al., 2000). While increasing the incubation temperature also increases the rate of lactose hydrolysis, enzymes are unstable at high temperatures. The excess heat will thermally denature enzymes by changing their structure, causing them to unfold (Timasheff, 1993, Kilimann et al., 2006). Thermal stability of proteins can be maintained extrinsically by incorporation of compatible solutes such as glycerol. Glycerol or carbohydrates stabilize proteins by preferential hydration, forming a shield around the protein to maintain its water of hydration and its shape (Timasheff, 1993).

1.3.4. The effect of enzyme immobilization on β-galactosidase activity

Enzyme immobilization on industrial surfaces such as glass beads, nylon, chitosan, and graphite has been used in the food industry since the 1970s (Zhou

and Chen, 2001). The purpose of immobilization is to extend the life of the enzyme for continuous processes. Immobilization also increase the thermal stability of the enzyme and shifting the optimal pH to an alkaline value (Zhou and Chen, 2001). Immobilization techniques include entrapment, cross-linking, and adsorption. A combination of these methods is used for ease of regeneration of the immobilized enzyme and low cost operation (Zhou and Chen, 2001). Immobilized enzymes can also be reused in lactose hydrolysis and GOS formation (Vasiljevic and Jelen, 2002). Immobilized β-gal used by Prenosil et al. (1987b) had shown consistent activity in GOS production compared with free β-gal up to a concentration of 10 mmol L^{-1} lactose.

A major disadvantage of immobilization is the reduction in enzyme activity. By increasing the lactose concentration, the enzymatic activity of immobilized enzymes decreased in activity resulting in reduced GOS production compared to free enzymes due to weak mass transfer resistance (Prenosil et al., 1987b). In solutions with greater than 10 mmol L^{-1} lactose, the concentration of lactose is lower inside the industrial surface of the carrier than in substrate solutions. This consequently reduces the degree of β-gal activity in restoring the equilibrium between the substrate solution and immobilization surface.

1.3.5. Use of β-galactosidases from thermophilic microorganisms

Hyperthermophilic microorganisms possessing β-gal can be used for GOS production at higher incubation temperatures and substrate concentrations. The use of β-gal from hyperthermophilic microorganisms such as *Sulfolobus*

solfataricus and *Pyrococcus furiosus* had produced GOS at incubation temperatures up to 85ºC and in a solution of 70% w/v lactose (Hansson and Adlercreutz, 2001).

1.3.6. Genetic manipulation of β-galactosidases by protein engineering

Development of gene-cloning, protein engineering, and recombinant techniques for food-grade LAB and bifidobacteria has demonstrated the potential for the genetic engineering to improve enzyme activity. Modifications of amino acid sequence of the enzyme potentially alter thermal stability and maintain activity at higher incubation temperatures. Hung and Lee (2002) heterologously expressed a mutated β-gal from *B. infantis* in *E. coli*. The recombinant β-gal showed a higher degree of lactose hydrolysis and transgalactosylation, and an increase in β-gal activity at higher incubation temperature and pH.

Jørgensen, Hansen, and Stougaard (2001) used a truncated β-gal enzyme from *Bifidobacterium bifidum*, which converted 90% of the lactose in solution into GOS. These processes, however, require the cloning, expression, and purification of enzymes, which limits their use in food-grade applications.

1.4. Incorporation of acceptor sugars in the transferase activity of β-galactosidases in the formation of heterooligosaccharides

A list of galactosyl acceptors in the transgalactosylation reaction of β-gal from LAB and other microorganisms is shown in Table 3.

Table 3. Galactosyl acceptors for transgalactosylation by β-galactosidases from lactic acid bacteria and other microorganisms.

Galactosyl acceptor	Product	Enzyme source
Sugars, glycosides, and derivatives		
Galactose [a,b,c,d]	β-D-Gal (1-6)-D-Gal (galactobiose)	*S. thermophilus* *E. coli (lacZ)* *Klyuveromyces fragilis* *K. lactis* *Bacillus circulans*
	β-D-Gal (1-3)-D-Gal	*B. circulans* *Aspergillus niger* *K. fragilis*
Glucose [a,b,c,d]	β-D-Gal (1-6)-D-Glc (allolactose)	*S. thermophilus* *E. coli (lacZ)*
	β-D-Gal (1-3)-D-Glc	*K. lactis* *B. circulans*
	β-D-Gal (1-2)-D-Glc	*A. niger* *K. fragilis*
Lactose [a,b,c,d]	β-D-Gal-(1-6)-β-D-Gal-(1-4)-D-Glc	*E. coli (lacZ)*
	β-D-Gal-(1-2)-β-D-Gal-(1-4)-D-Glc	*E. coli (lacZ)*
Mannose [e]	Disaccharide and trisaccharides	*B. circulans*
Fructose [f]	Lactulose	*K. lactis*
Sucrose [g]	Isoraffinose, lactosucrose	*E. coli (lacZ)*
Branched cyclodextrins [h]	Mono- and di-ß-Gal products at their side chains	*B. circulans* *A. oryzae* *Penicillium multicolor*
N-acetylglucosamine and derivatives [i]	ß-Gal-(1-3)-ß-GlcNAc	*E. coli (lacZ)* Bovine
N-acetylgalactosamine [j]	ß-Gal-(1-3)-ß-GalNAc	*E. coli (lacZ)* Bovine
Rubsoside [k]	13-O-[ß-Gal-(1-4)-ß-Glc]-19-O-ß-Glc-steviols	*E. coli (lacZ)* *B. circulans*
Amino acids and peptides		
N-Protected-L-serine methyl esters [l]	3-O-ß-Gal-L-Ser derivatives	*E. coli (lacZ)*
N-Protected dipeptide esters [m]	ß-(1-3)-digalactosyl peptides	*E. coli (lacZ)*

Galactosyl acceptor	Product	Enzyme source
Alcohols		
Alcohols [n,o]	Alkyl ß-Gals	*E. coli (lacZ)*
2-Fluoroethanol [p]	2-Fluoroethyl-ß-Gal and derivatives	*K. lactis*
Allyl alcohol [q]	Allyl ß-Gal	*S. thermophilus*
Vitamins and other bioactive compounds		
Kojic acid [r]	Galactosylkojic acid	*B. circulans*
Oximes [s]	ß-Gal-oxime derivatives	*A. oryzae*

a: Toba et al., 1981; b: Prenosil et al., 1987a; c: Zarate and Lopez-Leiva,1990; d: Mahoney, 1996; e: Miyasatoand Ajikasa, 2004; f: Lee et al., 2004; g: Suyama et al., 1986; h: Kitahata et al., 1992 ; i: Hedbys et al., 1989a; j: Hedbys et al., 1989b ;k: Kitahata et al., 1989 ; l: Fujimoto et al., 1997; m: Cantacuzene and Attal, 1991 ; n: Attal et al., 1992 ; o: Crout et al., 1990; p: Stevenson et al., 1993 ; q: Stevenson et al., 1994 ; r: Hassan et al., 1995; s: Pozo and Gotor, 1993.

Incorporation of fructose and sucrose as acceptor sugars by β-gal produces lactulose and lactosucrose, respectively (reviewed by Gänzle et al., 2008). These carbohydrates have tremendous potential as galactosyl acceptors in the production of OS with a similar composition similar as human milk OS.

1.5. Review of analytical methods for separation and identification of galactooligosaccharides and heterooligosaccharides

Chromatographical methods are a suitable tool to analyze the complex compositions of GOS preparations obtained from bacterial β-gal. High Performance Liquid Chromatography (HPLC) methods can be employed alone or in combination.

1.5.1. High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

The number of products formed by β-gal can be visualized with High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD), a sensitive detection method that separates products based on size and charge (Hardy and Townsend, 1988). HPAEC-PAD is conducted under alkaline conditions to ionize the hydroxyl groups of carbohydrates with pKa values between 12 to 14 (Hardy and Townsend, 1988). Carbohydrates have weak acidic properties by separating and ionizing in alkaline conditions with a water-sodium hydroxide-sodium acetate gradient (Andersen and Sorensen, 2000). Electrochemical detection by PAD detects compounds to the level of picomoles, which is more sensitive than other methods such as refractive index detection (RID) and ultraviolet (UV) signals.

HPAEC-PAD separates neutral OS based on molecular size, sugar composition, and linkage of monosaccharide units. Neutral OS exist as isomers with the same number, type, sequence, and anomeric configurations of monosaccharides, differing in the linkage position of a single sugar. The linkage and positioning of a hydroxyl group affects the retention of analytes on HPAEC-PAD. The acidity of the hydroxyl groups depend on the position of a molecule of glucose. The anomeric hydroxyl group on the reducing carbohydrate is more acidic than hydroxyl groups located on the second, sixth, third, and fourth carbon of a molecule (Hardy and Townsend, 1988).

1.5.2. High Performance Liquid Chromatography

Separating a mixture of glucose, galactose, disaccharides, and neutral GOS formed by bacterial β-gal with HPLC yields a purer sample for further structural analysis. This simplifies the subsequent structural identification of mass fractions by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS).

A detector is coupled to the end of an HPLC column, which indicates the appearance of products eluting from the column. Common detection methods for HPLC include refractive index (RID), ultraviolet (UV) signal, and evaporative light scattering detection (ELSD). Carbohydrates are detectable by UV at low wavelengths of 195 nm, with detection limits in the micrograms (Liu et al., 2005). RI is considered a universal method of detection that is slightly more sensitive than UV, with detection limits in the nanograms (Liu et al., 2005). RI, however, is based on the signal given by the sample compared with the solvent composition and cannot be used with a gradient. ELSD is used in carbohydrate analysis as the response is independent of the spectral properties of the compound and solvent, making it suitable to be used with a gradient. ELSD, however, nebulizes the sample and fractions cannot be collected (Liu et al., 2005). Fraction collection after analysis with ELSD is performed before the compound reaches the detector.

GOS produced by CCE contain galactose and glucose and are classified as neutral OS. Neutral OS can be separated with the use of a polar bonded amine column, which separates based on the size and polarity of the compound. A nonpolar solvent is used in separating based on hydrogen bonding between the

amino groups of the column and the hydroxyl groups of OS (McGinnis et al., 1989, Hayes and Varki, 1995, Blanken et al., 2000). Acetonitrile fosters the hydrogen bonding between amino and hydroxyl groups, while water disrupts the bonding (Hayes and Varki, 1995). The degree of separation can be controlled by starting with a higher concentration of acetonitrile, which would decrease with time. The water-acetonitrile gradient can be adjusted to foster the hydrogen bonding of hydroxyl groups of the OS. GOS and HOS are retained on the column, eluting in the order of increasing size, based on the numbers of hydroxyl groups on the compound (Hayes and Varki, 1995). Increasing the water concentration disrupts hydrogen bonds between the column material and oligosaccharide, resulting in elution from the column.

OS are also separated by molecular weight with size exclusion chromatography (SEC). SEC column materials contain pores which are bypassed by molecules with a higher molecular weight, eluting before smaller molecules. SEC separation results in OS eluting earlier than monosaccharides (McGinnis et al., 1989). While SEC separates based on size classes, it does not separate within a size class. A mixture of lactose and its isomers allolactose with a β(1-3) linkage and galactobiose with two galactose moieties are not resolved from each other with SEC (Molnar-Perl, 2000).

Reverse-phase (RP) HPLC separates compounds based on hydrophobic or nonpolar interactions between the sample and the column material. Monosaccharides are more polar and elute before OS (McGinnis et al., 1989). RP-

HPLC separates compounds based on stereochemical differences, as found in human milk OS containing NAG and fucose (Dua and Bush, 1982).

1.5.3. Gas Chromatography – Mass Spectrometry

Structural analysis of OS can be achieved by GC, which volatilizes compounds in a carrier gas. Carbohydrates contain polar hydroxyl and carbonyl groups that require conversion into a volatile state by derivatization for GC analysis (Biermann, 1989). A method of derivatization is the Hakomori method of permethylation, which replaces all available hydroxyl groups with methyl groups using methyl iodide, shown in Figure 2 (Biermann, 1989).

Coupling GC with MS determines the structure of the compound based on its molecular weight of compounds from the mass to charge ratio and abundance within a compound (Mellon et al., 2000). This is achieved by the collision of electrons and volatile compounds, which are ionized into mass fractions. These mass fractions are assembled to identify the composition and structure of the compound. While GC-MS yields structural information of compounds, it requires an additional step of derivatization in sample preparation, which is time-consuming and has low reproducibility (McGinnis et al., 1989).

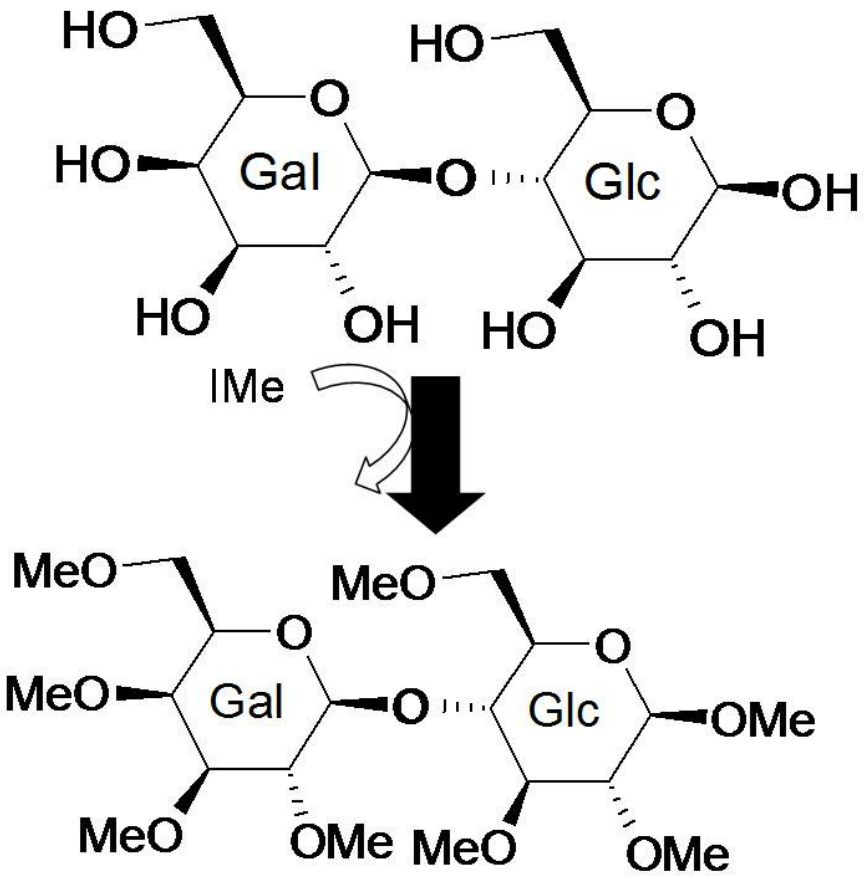

Figure 2. Derivatization of lactose with the Hakomori method of permethylation of free hydroxyl groups with methyl iodide (IMe) into methylated groups.

1.5.4. Liquid Chromatography – Mass Spectrometry

Structural identification of OS with LC-MS is becoming increasingly available as a method for analyzing OS. LC-MS is a sensitive method that can detect as little as 50 picograms with ESI-MS (Liu et al., 2005). Underivatized OS are separated and characterized using columns that have a graphitized carbon, amino-bonded, or cyclodextrin stationary phase and ionization methods such as electrospray ionization (ESI) MS. Separation of isomers with the same molecular weight and numbers of hydroxyl groups is achieved if each compound has

different configuration of hydroxyl groups that interact with the stationary phase (Liu et al., 2005).

The main advantage of LC-MS is that the samples are readily identified following HPLC separation (McGinnis et al., 1989). GC-MS requires the additional sample preparation step of derivatization, which is time-consuming. While the structure of OS is readily analyzed upon HPLC separation with a mass detector, LC-MS is still a new field in carbohydrate analysis, where the ionization and detection need to be configured for carbohydrates.

1.6. Goals of present research

The goals of this research were the following:

1. Investigate the use of CCE from disrupted LAB cells as source of β-gal
2. Optimize CCE for β-gal activity in pure and industrial sources of lactose
3. Develop novel HOS with mannose, NAG, and fucose and analyze the composition of HOS formed by CCE of β-gal from LAB

This study was based on the hypothesis that β-gal of disrupted cells of food-grade LAB can utilize lactose in a pure solution or in UF whey permeate to form GOS and novel HOS with mannose, NAG and fucose as acceptor carbohydrates. To test this hypothesis, buffer optimization and transgalactosylation activity based on glucose removal and effect of incubation temperature and addition of glycerol as a compatible solute were also examined. Structural analysis of HOS produced by β-gal of *L. bulgaricus* ATCC 11842 with NAG and fucose was performed using GC-MS and LC-MS.

2. Experimental

2.1. Strains and enzymes used in this study

All microorganisms used in this study were obtained from the strain collection in the Food Microbiology laboratory in the Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada as listed in Table 4.

Table 4. Microorganisms obtained from the Food Microbiology Laboratory stock culture collection in the Department of Agriculture, Food, and Nutritional Sciences at the University of Alberta, Edmonton, AB.

Name	Strain
Lactobacillus reuteri [a]	100-23
Lactobacillus animalis [a]	FUA 3045
Lactobacillus reuteri [a]	FUA 3054
Lactobacillus acidophilus [b]	FUA 3066
Streptococcus thermophilus [b]	FUA 3068
Pediococcus acidilacti [b]	FUA 3072
Enterococcus spp. [a]	FUA 3074
Enterococcus faecalis [a]	FUA 3095
Carnobacterium maltaromaticum [c]	FUA 3103
Lactobacillus delbrueckii subsp. bulgaricus [b]	ATCC 11842
Lactobacillus fermentum [d]	ATCC 14931
Enterococcus faecalis [a]	ATCC 19433
Enterococcus faecium [a]	ATCC 19434
Carnobacterium maltaromaticum [c]	ATCC 43225
Bifidobacterium infantis [a]	ATCC 2012
Bifidobacterium breve [a]	ATCC 2013
Bifidobacterium longum [a]	ATCC 2014

Obtained from a: animal; b: dairy; c: meat; d: cereal sources

All strains were thawed from storage at -80°C before streaking onto an agar plates. Plates were incubated under microaerophilic conditions (1% O_2, balance N_2) at 37ºC for 24 hours, except for *P. acidilacti* FUA 3072 and *C. maltaromaticum* FUA 3103, which were incubated at 30ºC. All bifidobacteria strains were incubated under anaerobic conditions (10% H_2, 5% CO_2, 85% N_2) at 37ºC for 24 to 48 hours.

One colony from each agar plate was transferred to 1 mL of modified DeMan-Rogosa-Sharpe (MRS) lactose broth containing beef extract 5.0 g L^{-1}, peptone 10 g L^{-1}, yeast extract 5.0 g L^{-1}, NH_4Cl 3.0 g L^{-1}, K_2HPO_4 4.0 g L^{-1}, KH_2PO_4 2.6 g L^{-1}, cysteine hydrochloride 0.6 g L^{-1}, Tween-80 1.0 g L^{-1}, $MgSO_4*7H_2O$ 0.1 g L^{-1}, $MnSO_4*4H_2O$ 0.05 g L^{-1} with 50 g L^{-1} lactose, 1 mL vitamin blend with 8 mg each thiamin, riboflavin, pyridoxine, cobalamine, folic acid, and pantothenic acid with a pH of 6.2. The working bacterial culture was incubated with the above conditions.

The working bacterial culture was transferred to 50 mL modified MRS lactose broth and incubated overnight with the above conditions. Cells were harvested, washed twice, and resuspended in 1 mL of 50 mmol L^{-1} sodium phosphate buffer (pH 6.8) with 20% glycerol and 1 mmol L^{-1}dithiolthreitol. Cells were transferred to screw cap tubes with 0.5 mL of 0.1 mm Zirconia/Silica beads and disrupted in a Mini Beadbeater-8 Fisher Scientific model 693 (BioSpec) for 1.5 minutes, followed by chilling in ice and repeating for a total of 3 minutes. Disrupted cells were centrifuged (Micromax RF ThermoIEC) at a relative centrifugal force of

15300 x g for 20 minutes at 4ºC. The supernatant was collected as CCE and stored at -20ºC until use.

Purified β-galactosidase of *Kluyveromyces lactis* was obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Determination of β-galactosidase activity in crude cell extracts

Activity of β-gal was measured with O-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma Aldrich (St. Louis, MO, USA) as the substrate. An aliquot of 10 μL of CCE was added to 240 μL of 50 mmol L^{-1} ONPG prepared in 50 mmol L^{-1} sodium phosphate buffer (pH 6.8). Purified β-gal from *Kluyveromyces lactis* was used as a positive control. Enzyme activity was determined by absorbance over a period of 10 minutes at 30ºC at 420 nm taken at 1-minute intervals in a Varioskan Flash spectrophotometer (Thermo Fisher Scientific, Asheville, NC, USA). One unit of β-gal activity was defined as the liberation of 1 μmol of ONP from hydrolysis of ONPG per minute per mL at 30ºC, pH 6.8. All CCE and β-gal from *K. lactis* were standardized to 30 units of β-gal activity for use in enzymatic reactions. All assays were performed with CCE and β-gal from *K. lactis* with 30 units of β-gal activity.

2.3. Screening of β-galactosidase activity in crude cell extracts

CCE from 17 strains of LAB and bifidobacteria and a positive control of β-gal from *K. lactis* were screened for hydrolase and transferase activity in lactose based on chromatograms from HPAEC-PAD. Hydrolase activity was indicated

by the presence of glucose and galactose on chromatograms from HPAEC-PAD. Transferase activity was indicated by production of GOS as peaks eluting after lactose on chromatograms from HPAEC-PAD.

An aliquot of 0.2 mL CCE or β-gal from *K. lactis* was applied to 1 mL of sugar solutions with a starting concentration of 300 grams of lactose in 1 L of 50 mmol L^{-1} sodium phosphate buffer (pH 6.8). This was equivalent to 230 grams lactose per kilogram of buffer. All sugar solutions were sterilized by filtration before use. The final concentration after addition of CCE or β-gal from *K. lactis* was 19% (w/w) lactose. Solutions were incubated at 37ºC for 24 hours and stopped by heating at 95ºC for 15 minutes to inactivate the enzyme. Assays were performed in duplicates using CCE from two independently-grown cultures.

To determine the transferase activity of β-gal in CCE and from *K. lactis,* samples were analyzed with HPAEC-PAD on a Dionex ICS-3000 system consisting of an AS50 autosampler, an electrochemical cell detector with a gold working electrode and an Ag/AgCl reference electrode (Dionex Corporation, Sunnyvale, CA, USA). Separations were performed at 25ºC on a CarboPac PA-20 Carbohydrates column (4x 250 mm). Samples were diluted in water (1:999 ratio) before injecting an aliquot of 10 μL into the AS50 autosampler of the HPAEC-PAD unit. Samples were analyzed at a flow rate of 0.25 mL min^{-1} for 42 minutes at 25ºC. OS were separated with eluents A (water), B (0.2 M sodium hydroxide), C (1M sodium acetate) in the following gradient: 0 minutes 30.4% B, 1.3% C; 25 minutes 30.4% B, 15% C; 28 minutes 0%B, 50% C; 31 minutes 73% B,17% C; 31.1 minutes 30.4% B, 36.3% C; 34 minutes 73% B,17% C; 37 minutes

73% B,17% C; 40 minutes 30.4% B, 1.3% C. HPAEC-PAD detector signals were reported in nanocoulombs (nC).

2.4. Effect of the source of lactose on β-galactosidase activity

Dried UF whey permeate powder (Saputo, Montreal, QC) with a composition of 83% lactose and a pH of 6.0 was dissolved in deionized water at 60ºC for 30 minutes in a waterbath shaker (AquaTherm, New Brunswick Scientific Co. Inc., Edison, NJ, USA) to attain the equivalent of 23% (w/w) lactose. Whey permeate solution was centrifuged for 5 minutes at 5300 x g in an Allegra 25R centrifuge (Beckman Coulter, Miami, FL, USA) to remove insoluble materials before sterilizing by filtration. Aliquots of 1 mL CCE or purified β-gal of *K. lactis* and CCE were added to 5 mL of UF whey permeate for a final concentration of 19% (w/w) lactose. After incubating at 37ºC for 24 hours, samples were analyzed for transferase activity as per section 2.3. Assays were performed in duplicates using CCE from two independently-grown cultures.

2.5. Optimization of transferase activity of β-galactosidase

2.5.1. Buffer optimization with pH, cations, incubation temperature and time

All buffer optimization reactions were performed in duplicates using 0.2 mL CCE from two independently-grown cultures in 1mL of sugar solutions with 23 % (w/v) lactose in 1.5 mL plastic Eppendorf tubes. All sugar solutions were sterilized by filtration. Reactions were stopped by heating at 95ºC for 15 minutes

to inactivate the enzyme. Enzyme activity was estimated by determining GOS formation by CCE in all conditions.

Buffer optimization was based on pH, addition of cationic salts, and incubation temperature and time. The optimum pH was determined by incubating 0.2 mL of CCE with 30 units of β-gal activity in 23% (w/v) lactose with 50 mmol L^{-1} of sodium acetate (pH 5.4) and sodium phosphate (pH 6.8) buffers with 2 mmol L^{-1} $MgCl_2$ at 37ºC for 24 hours. Temperature optimization was determined by incubating CCE at 37, 45, 55, 65ºC in 23% (w/v) lactose with 50 mmol L^{-1} of sodium phosphate buffer (pH 6.8) with 2 mmol L^{-1} $MgCl_2$ for 24 hours. Effect of cationic salts was determined by incubating CCE in a control solution of 23% (w/v) lactose in 50 mmol L^{-1} of sodium phosphate buffer (pH 6.8) and solutions of 19% (w/w) lactose in 50 mmol L^{-1} of sodium phosphate buffer (pH 6.8) with 2 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} $MgCl_2$ and 100 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$ and 100 mmol L^{-1} KCl, and a combination of 2 mmol $L^{-1}$$MgCl_2$ and 100 mmol L^{-1} each NaCl and KCl for 24 hours in at 37ºC for 24 hours. Incubation times for the progression of enzymatic reactions were examined at 1, 6, 12, 18, and 24 hours in solutions of 30% w/v lactose in 50 mmol L^{-1} sodium phosphate buffer (pH 6.8) with 2 mmol L^{-1} $MgCl_2$ at 37ºC.

Samples were diluted in water (1:999 ratio), then an aliquot of 10 µL was analyzed with HPAEC-PAD at a flow rate of 0.25 mL min^{-1} for a total run time of 60 minutes at 25ºC with the gradient used in section 2.3. that was modified to: 0 minutes: 30.4% B, 1.3% C; 30 minutes 30.4% B, 15% C; 35 minutes 0% B, 50% C; 40 minutes: 73% B, 17% C; 40.1 minutes: 30.4% B, 36.3% C; 45 minutes

73% B, 17% C; 50 minutes: 73% B, 17% C; 55 minutes: 30.4% B, 1.3% C.

2.5.2. Kinetics of transferase activity of β-galactosidases

All experiments using *L. reuteri* 100-23 and *L. animalis* FUA 3045 were conducted with 1 mL of CCE in 5 mL of solutions of 23% (w/v) lactose dissolved in 50 mmol L^{-1} sodium acetate buffer (pH 5.4), 100 mmol L^{-1} KCl and 2 mmol L^{-1} $MgCl_2$. All experiments with *L. bulgaricus* ATCC 11842 were conducted in solutions of 23% (w/v) lactose dissolved in 50 mmol L^{-1} sodium phosphate buffer (pH 6.8), 100 mmol L^{-1} KCl and 2 mmol L^{-1} $MgCl_2$. CCE of *S. thermophilus* FUA 3068 were not used in these experiments. The final concentration of lactose was 19% (w/w). All assays were performed in duplicates using CCE from two independently-grown cultures. Solutions were incubated at 37ºC for 24 hours, where aliquots were collected after 1, 2, 4, 8, and 16 hours and stopped by heating at 95ºC for 15 minutes to inactivate the enzyme.

Samples were diluted in water (1:999 ratio) before injecting an aliquot of 10 µL into the AS50 autosampler of the HPAEC-PAD unit. Samples were analyzed at 25ºC for a total run time of 40 minutes at a flow rate of 0.25 mL min^{-1}. The concentration of glucose and galactose were quantified with eluents A (water) and B (0.2 M sodium hydroxide) in the following gradient: 0 minutes: 6% B; 20 minutes 0A, 100B; 32 minutes 100% B; 32.1 minutes: 6% B, 40 minutes: 6% B. The total activity of β-gal was calculated as the concentration of free glucose in solution. Hydrolase activity was calculated as the concentration of free galactose

in solution. Transferase activity was calculated as the difference in the concentration of glucose and galactose, expressed in mmol L^{-1}.

2.5.3. Effect of *Saccharomyces cerevisiae* and glucose oxidase on the transferase activity of β-galactosidases

Glucose, a noncompetitive inhibitor of β-gal activity, was removed using *S. cerevisiae* or glucose oxidase in solutions of 19% (w/w) lactose to enhance transferase activity of CCE and β-gal of *K. lactis*. An overnight culture of *S. cerevisiae* FUA 4002 was grown in 5 mL of modified MRS broth with 10 g L^{-1} of each malt extract and maltose and 5 g L^{-1} of each fructose and glucose in place of lactose and vitamins at 30ºC for 24 hours. The cell suspension was washed twice in 10% (w/w) lactose before resuspending in 5 mL of 19% (w/w) lactose.

An aliquot of 1mL CCE with 30 units of β-gal activity was applied to 5 mL of cell suspension of *S. cerevisiae* in 23% (w/v) lactose. Alternatively, an aliquot of 1 mL CCE with 30 units of β-gal activity was applied to 5 mL of 0.1 mg mL^{-1} glucose oxidase (Sigma Aldrich, St. Louis, MO, USA) in 23% (w/v) lactose. All samples, along with a control solution of 1 mL CCE with 30 units of β-gal activity in 5 mL of 23% (w/v) lactose, were incubated in shaking test tubes at 37ºC. The reaction was stopped by heating samples were heated to 95 ºC for 5 minutes. Samples were centrifuged for 10 seconds after enzyme inactivation at 10600 x g to remove *S. cerevisiae* from solution. Assays were performed as per section 2.5.2.

2.5.4. Effect of incubation temperature and addition of glycerol as a compatible solute

An aliquot of 1 mL of CCE or β-gal from *K. lactis* with 30 units of enzyme activity were applied to 5 mL of solutions of 23% (w/v) lactose with 10 % (w/v) glycerol and a control 23% (w/v) lactose solution for a final concentration of 19% (w/w) lactose and 6% (w/w) glycerol. Solutions were incubated in water baths at 40, 50, 60, and 70ºC. Assays were performed as per section 2.5.2.

2.6. Application of acceptor carbohydrates

Solutions of 23% (w/v) lactose were prepared with 12% (w/v) of each mannose, NAG, and fucose as acceptor sugars. All sugars were dissolved in 50 mmol L^{-1} sodium acetate buffer (pH 5.4) with 100 mmol L^{-1} KCl and 2 mmol L^{-1} $MgCl_2$ for *L. reuteri* 100-23 and *L. animalis* FUA 3045. Sugars were dissolved in 50 mmol L^{-1} sodium phosphate buffer (pH 6.8) with 100 mmol L^{-1} KCl and 2 mmol L^{-1} $MgCl_2$ for *L. bulgaricus* ATCC 11842 and *S. thermophilus* FUA 3068. All sugar solutions were sterilized by filtration.

Aliquots of 0.2 mL CCE or β-gal from *K. lactis* were applied to 1 mL of the above sugar solutions for a final concentration of 19% (w/w) lactose. Solutions were incubated at 37ºC for 24 hours and stopped by heating to 95ºC for 15 minutes. Samples were prepared and analyzed for transferase activity by HPAEC -PAD as per section 2.3. HOS were identified as additional peaks present in chromatograms of enzymatic reactions in solutions of lactose with acceptor sugars that were absent in lactose. NAG was also applied as an acceptor sugar in UF

whey permeate, with 23% (w/v) NAG in UF whey permeate with 23% (w/v) lactose. All reactions were performed with CCE from two independently-grown cultures as per section 2.4., with analysis performed as per section 2.5.

2.7. Separation of crude mixtures with High Performance Liquid Chromatography

Further structural analysis of HOS was performed with NAG and fucose only, as HOS containing NAG and fucose have different molecular weights than GOS. Mannose is an isomer of glucose and galactose, having the same molecular weight. HOS with mannose would not be resolved from GOS with MS.

Solutions of CCE in solutions of lactose with NAG and fucose were analyzed with HPLC on a Varian 9000 system consisting of an autosampler and ELSD (Varian, Sunnyvale, CA, USA). Separations were performed at 25ºC on a Supelcosil LC-amino column (4x 250 mm). Aliquots of 50 µL of undiluted samples were injected and analyzed at a flow rate of 1.8 mL min^{-1} for 40 minutes. Eluents A (acetone), B (water) were used in the following gradient: 0 minutes 11% B, 1 minute 13% B, 35 minutes 23%B, 37 minutes 11% B. Samples were run once with this program to indicate the appearance of peaks before collecting fractions in 15mL plastic screw-cap tubes. Fractions were collected based on peaks appearing in addition to glucose, galactose, lactose, and GOS.

Fractions of each peak that was collected were dried under nitrogen gas to remove acetone, followed by overnight lyophilization. Dried samples were stored at room temperature until further analysis.

2.8. Structural analysis of oligosaccharides with Gas Chromatography – Mass Spectrometry

Dried OS containing HOS with NAG and fucose were derivatized with permethylation using methyl iodide. Samples were prepared according to Hansson and Karlsson (1993). Samples were mixed with 1mL DMSO with addition of 50 µg of powdered NaOH. Permenthylation was performed by adding methyl iodide was added to samples and mixed for 10 minutes at room temperature and stopped by adding 2 mL of 0.1N HCl, followed by addition of 2 mL of chloroform. Samples were washed with water for three times to separate the aqueous and organic phases, where the organic phase was retained with each subsequent wash. The organic phase was transferred to a clean glass tube and dried under N_2 gas at 40ºC. Samples were resuspended in 500 µL of ethyl acetate, where 1 µL was injected into the Agilent 6890 GC-MS system consisting of an autosampler and mass spectrometer (Agilent, Santa Clara, CA, USA). Separations were achieved with a 30-metre HP-5 column using a temperature gradient of 70ºC with a 1-minute delay, a linear temperature increase of 10ºC per minute was used until 320ºC followed by holding for 5 minutes. Mass spectra of peaks on the gas chromatogram were analyzed with GC/MSD ChemStation Software (Agilent, Santa Clara, CA, USA). Structural analysis was performed with ISIS Draw (MDL Information Systems Inc., Hayward, CA, USA).

2.9. Structural analysis of oligosaccharides with Liquid Chromatography – Mass Spectrometry

LC-MS analysis of HOS with NAG and fucose was carried out by Brenna Black as part of her graduate research project. Lyophilized samples from section 2.7. and standards of glucose, galactose, NAG, and fucose were dissolved in acetonitrile/water 1:1 (v/v) to a concentration of 1 mg mL^{-1} and filtered with a 0.2 μm nylon membrane (VWR International, Mississauga, ON, Canada). Samples were analyzed in positive and negative ion ESI-MS and Collision Induced Detection tandem MS with a QStar® Elite Hybrid orthogonal Q-TOF mass spectrometer equipped with a TurboIon Spray® Source (Applied Biosystems /MDS Sciex, Concord, ON, Canada). Ammonium acetate was used as an additive for the formation of negative ions and sodium chloride was used as an additive for positive ions. Each working solution contained a final concentration of 10 mmol L^{-1} additive and 0.01 mg mL^{-1} OS. Standard solutions were directly infused at a rate of 20 μL min^{-1} for ionization and analysis.

Optimization of all parameters and further data acquisition was performed with Analyst® QS 2.0 software. Negative-mode ionization was set with nebulizer gas 50 (arbitrary units), ionspray voltage -4500V, curtain gas 25, declustering potential (DP) -45V, focusing potential -170V, and DP2 = -20V with a mass range of 50-1300 *m/z* for the mass spectrometer. Fragmentation was achieved from Collision Induced Detection with nitrogen gas. Optimal collision energy varied between -10 to -25V based on standards. Positive ion mode was set with nebulizer gas 45 (arbitrary units), ionspray voltage 5000V, curtain gas 25,

declustering potential 45V, focusing potential 170V and DP2 20V with a scan range of 50-900 *m/z*. Collision energy for positive ions was adequate between 25 to 40V based on standards.

HPLC separation was performed at 25ºC with an isocratic gradient of acetonitrile/water 80:20 (v/v) at a flow rate of 1mL min^{-1} on a Supelcosil LC-NH_2 column (250mm x 4.6mm I.D., 5 µm) (Sigma Aldrich, Oakville, ON, Canada) on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA, USA). An aliquot of 25 µL of sample at a concentration of 0.1 mg mL^{-1} was injected. Post-column addition of ammonium acetate (100mM) or sodium chloride (100mM) was added in 10% of the 0.5 mL min^{-1} flow rate entering the ion source via Harvard Pump 11 Plus syringe pump (Harvard Apparatus, Holliston, MA, USA). Auxillary gas flow was optimized at 60 arbitrary units and the source temperature at 400 ºC for both negative-ion mode and positive-ion mode.

OS samples were analyzed analogously to the standards. In the case of low intensity signals from the previous chromatographic system, an Ascentis™ RPAmide column (150mm x 2.1mm I.D., 3 µm) (Supelco, Bellefonte, PA, USA) was used. An isocratic gradient of either water/methanol 50:50 (v/v) was employed for positive analysis or acetonitrile/water 70:30 (v/v) for negative analysis. Flow rates were maintained at 0.2 mL min^{-1} with the same 10% post-column addition of ion-mode specific additive.

3. Results

3.1. Screening of β-galactosidase activity for the use of crude cell extracts of lactic acid bacteria

Screening of LAB using CCE with 30 units of β-gal activity in lactse had shown that five of 17 strains (*L. reuteri* 100-23, *L. animalis* FUA 3045, *L. bulgaricus* ATCC 11842, *S. thermophilus* FUA 3068, and *B. breve* ATCC 2013) had both hydrolase and transferase activity. The *B. breve* ATCC 2013 strain was not used in further experiments. The remaining strains had either exhibited no β-gal activity or did not produce GOS. The most GOS was produced by *L. bulgaricus* ATCC 11842 and *S. thermophilus* FUA 3068, followed by *L. animalis* FUA 3045 and *L. reuteri* 100-23, as shown in Figure 3.

The HPAEC-PAD gradient used in this screening process had separated GOS, but did not resolve glucose from galactose, which appeared as one peak.

3.2. Optimization of transferase activity of the β-galactosidase enzymes in crude cell extracts of lactic acid bacteria

3.2.1. Buffer optimization with pH, cations, incubation temperature and time

Solutions of lactose were prepared in various buffers to optimize for transferase activity by β-gal of CCE. Addition of cations, pH, incubation temperature and time were considered in the optimization. Results are shown in Figure 4. Optimum buffer conditions were pH 6.8 for *S. thermophilus* FUA 3068

and *L. bulgaricus* ATCC 11842, and pH 5.4 for *L. reuteri* 100-23 and *L. animalis* FUA 3045. The optimum incubation time, temperature and mineral addition were the same for all four organisms, which were all incubated at 37ºC for 24 hours with addition of 100 mmol L^{-1} KCl and 2 mmol L^{-1} $MgCl_2$.

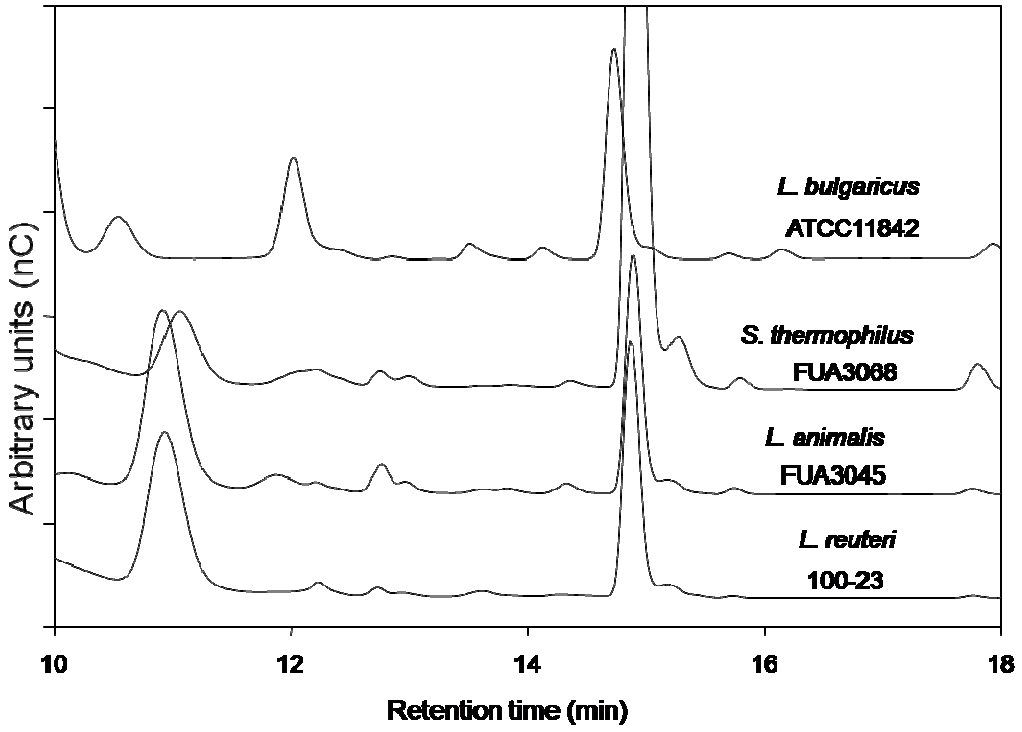

Figure 3. Separation of galactooligosaccharides produced by β-galactosidases in crude cell extracts of *L. reuteri* 100-23, *L. animalis* FUA 3045, *S. thermophilus* FUA 3068, and *L. bulgaricus* ATCC 11842 in 19% (w/w) lactose incubated at 37ºC for 24 hours. Galactooligosaccharides are shown as peaks with retention times of more than 10 minutes, analyzed with High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection in nanocoulombs (nC). Chromatograms were overlaid to show oligosaccharide peaks. Results are representative for two independent experiments.

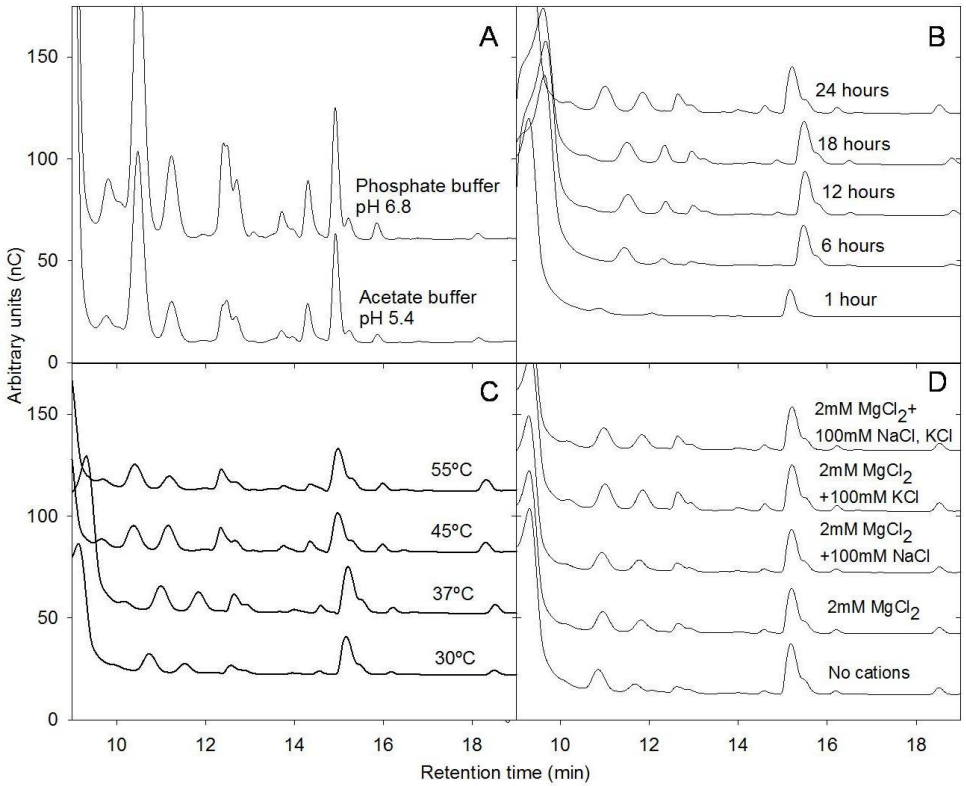

Figure 4. Buffer optimization based on galactooligosaccharide production with 0.2 mL of crude cell extracts of *S. thermophilus* FUA 3068 in 1mL of 23% (w/v) lactose with A) 50 mmol L^{-1} of sodium acetate (pH 5.4) and sodium phosphate (pH 6.8) buffers with 2 mmol L^{-1} $MgCl_2$ at 37ºC for 24 hours, B) incubation time (1, 6, 12, 18, and 24 hours) in 50 mmol L^{-1} sodium phosphate buffer (pH 6.8) with 2 mmol L^{-1} $MgCl_2$ at 37ºC, C) incubation temperatures of 37, 45, 55, 65ºC with 50 mmol L^{-1} of sodium phosphate buffer (pH 6.8) with 2 mmol L^{-1} $MgCl_2$ for 24 hours, and D) effect of having no cationic salts, 2 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} $MgCl_2$ and 100 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$ and 100 mmol L^{-1} KCl, as well as 2 mmol L^{-1} $MgCl_2$ and 100 mmol L^{-1} each NaCl and KCl for 24 hours in at 37ºC for 24 hours. Results are representative for two independent experiments.

3.2.2. Effect of *Saccharomyces cerevisiae* and glucose oxidase

Glucose generated by lactose hydrolysis by β-gal in CCE was removed by incorporation of *S. cerevisiae* or glucose oxidase into the buffered lactose solutions prior to enzymatic reactions. The effect of *S. cerevisiae* FUA 4002 and glucose oxidase in the enzymatic reaction of CCE are shown in Figure 5. Comparable results were attained for CCE of *L. reuteri* 100-23, *L. animalis* FUA 3045, *L. bulgaricus* ATCC 11842, and β-gal of *K. lactis* (data not shown).

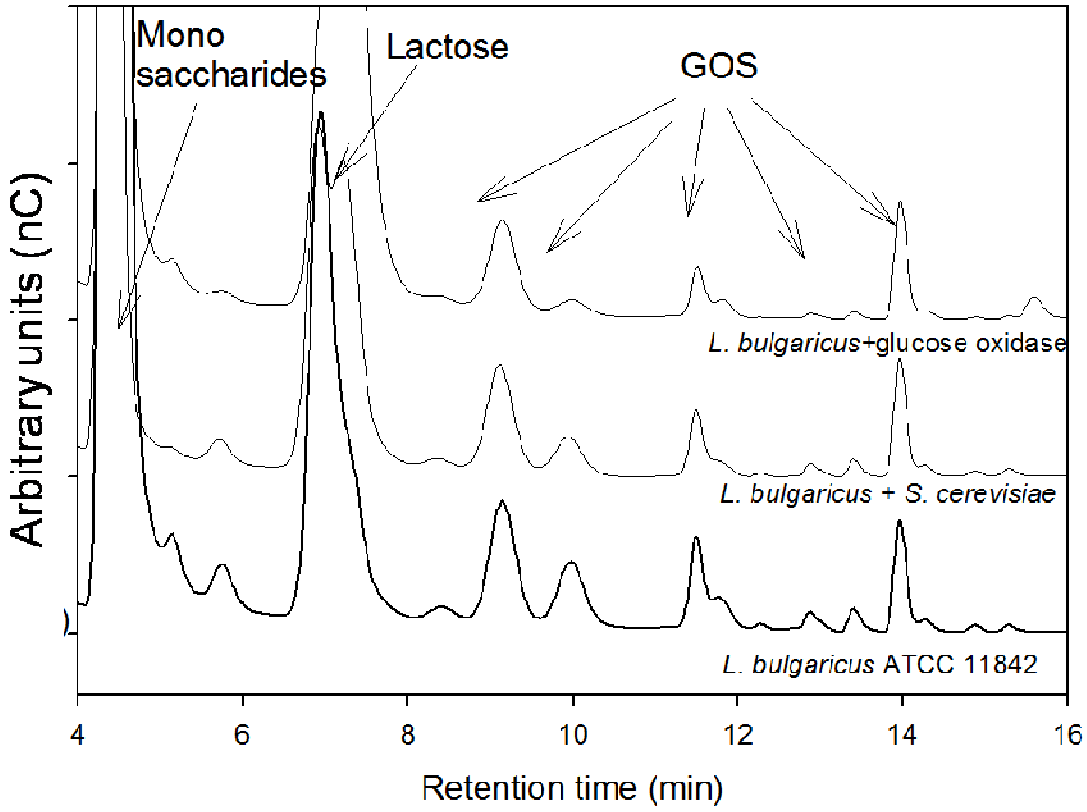

Figure 5. Separation of galactooligosaccharides produced by crude cell extracts of *L. bulgaricus* ATCC 11842 in 19% (w/w) lactose with addition of *S. cerevisiae* FUA 4002 and glucose oxidase using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection in nanocoulombs (nC). Results are representative for two independent experiments.

There were no major differences in the effect of incorporating *S. cerevisiae* FUA 4002 and glucose oxidase on transferase activity in GOS production by CCE and β-gal of *K. lactis*. Thus, glucose removal did not enhance transferase activity in GOS production. This was discontinued from further experimentation.

3.2.3. Effect of temperature and addition of glycerol as a compatible solute

To determine the effect of temperature and glycerol addition on the βgal activity, the activity of pure β-gal of *K. lactis* and CCE of *L. bulgaricus* ATCC 11842 were tested in solutions of lactose with and without incorporation of 10% glycerol (w/v) at incubation temperatures of 40, 50, 60, and 70ºC. Results are shown Table 5 and Figures 6 A and 6B.

Figure 6A shows that more GOS were formed when β-gal of *K. lactis* was incubated in lactose solutions with added glycerol at 40 and 50ºC. While the enzyme activity was lower at 70ºC, it was higher in the presence of glycerol. Figure 6B shows that there was a shift in transferase activity in the CCE of *L. bulgaricus* ATCC 11842 at 40, 50 and 70ºC. In addition, the CCE of *L. bulgaricus* ATCC 11842 remained active in the presence or absence of glycerol, even at 70ºC. Table 5 shows the effect of glycerol on β-gal activity of *K. lactis* and CCE of *L. bulgaricus* ATCC11842 when compared without the use of compatible solutes at each incubation temperature.

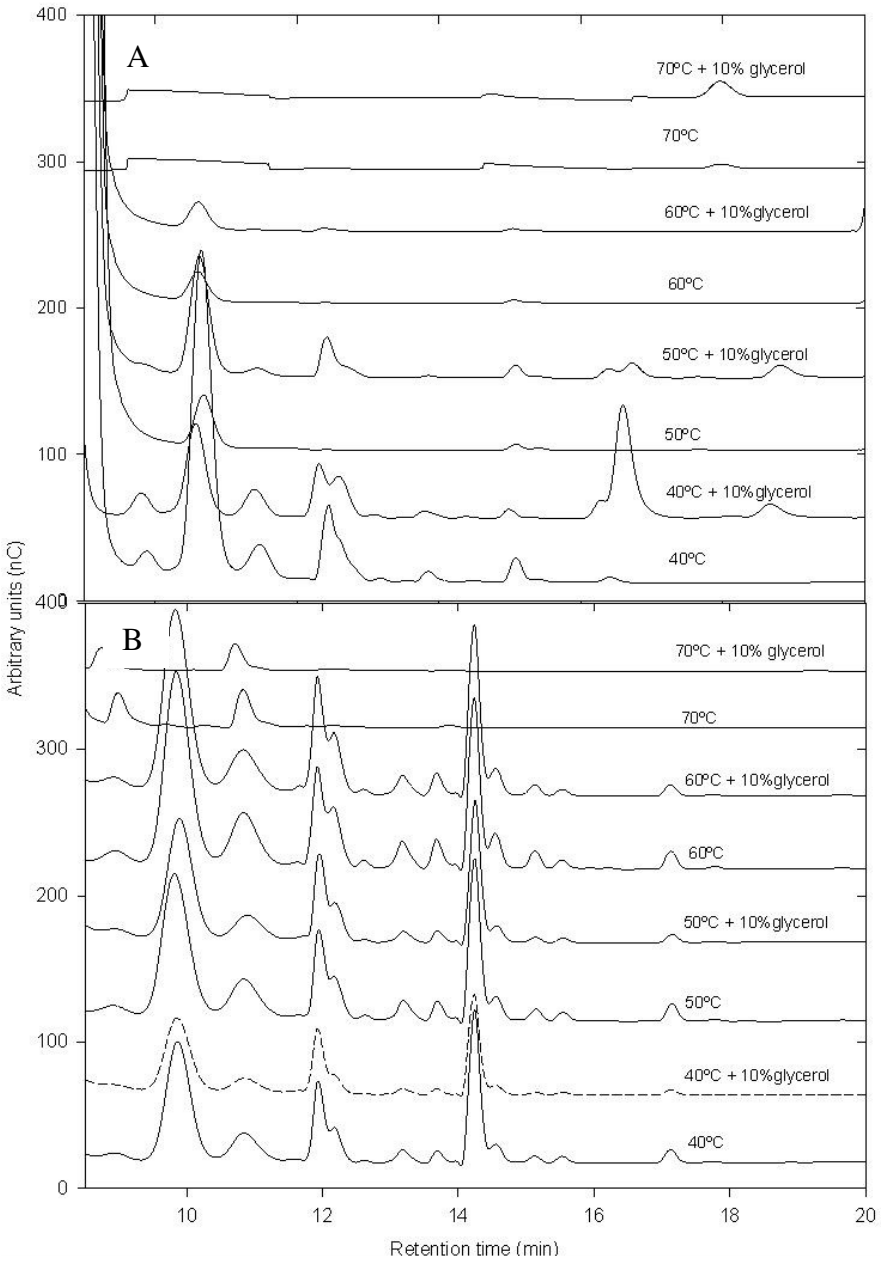

Figure 6. Separation of oligosaccharides produced by β-galactosidases of A) *K. lactis* and crude cell extracts of B) *L. bulgaricus* ATCC 11842 in solutions of 19% (w/w) lactose incubated in water baths at 40, 50, 60 and 70ºC for 16 hours with addition of 6% (w/w) glycerol analyzed with High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection in nanocoulombs (nC). Results are representative for two independent experiments.

Table 5. Lactose utilization by crude cell extracts of *L. bulgaricus* ATCC 11842 with β-galactosidases optimized for transferase activity by water activity in solutions of 19% (w/w) lactose with 0 and 6 % (w/w) glycerol incubated in water baths at 40, 50, 60, and 70ºC for 16 hours analyzed with High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection. Results are shown as means ± standard deviation of two independent experiments.

	Total activity [Glucose] (mmol L^{-1})		**Hydrolase [Galactose] (mmol L^{-1})**		**Transferase [Glucose] - [Galactose] (mmol L^{-1})**	
	K. lactis	***L. bulgaricus***	***K. lactis***	***L. bulgaricus***	***K. lactis***	***L. bulgaricus***
40ºC	503 ± 4	525 ± 3	253 ± 13	118 ± 1	250 ± 9	407 ± 2
+10% glycerol	534 ± 2	537 ± 4	182 ± 12	81 ± 0	352 ± 10	456 ± 4
50ºC	151 ± 0	527 ± 28	45 ± 0	146 ± 11	106 ± 0	381 ± 17
+10% glycerol	374 ± 2	557 ± 5	70 ± 1	106 ± 4	303 ± 0	451 ± 1
60ºC	173 ± 123	506 ± 13	39 ± 13	122 ± 17	134 ± 110	385 ± 4
+10% glycerol	169 ± 118	477 ± 76	36 ± 16	84 ± 25	133 ± 102	393 ± 51
70ºC	86 ± 13	439 ± 6	15 ± 1	104 ± 0	71 ± 12	335 ± 6
+10% glycerol	198 ± 4	440 ± 15	23 ± 3	69 ± 2	176 ± 0	372 ± 12

The protective effect of glycerol was apparent for β-gal of *K. lactis* at 70ºC, with a higher glucose concentration in solutions of lactose with glycerol. While enzymes had less activity at 70ºC than at 40 and 50ºC, glycerol had stabilized the β-gal of *K. lactis* to continue lactose hydrolysis at higher incubation temperatures.

Reaction kinetics of CCE of *L. bulgaricus* ATCC 11842 in Figure 7 and Table 5 affirms that glycerol did not influence the total activity of CCE from *L. bulgaricus* ATCC 11842 at any temperature. However, at 40, 50, and 70ºC, glycerol induced a substantial shift from hydrolase to transferase activity, which is also shown in Table 5. A higher concentration of glucose and lower concentrations of galactose was seen in the presence of glycerol, with more galactose being incorporated into GOS by the β-gal of *L. bulgaricus* ATCC 11842.

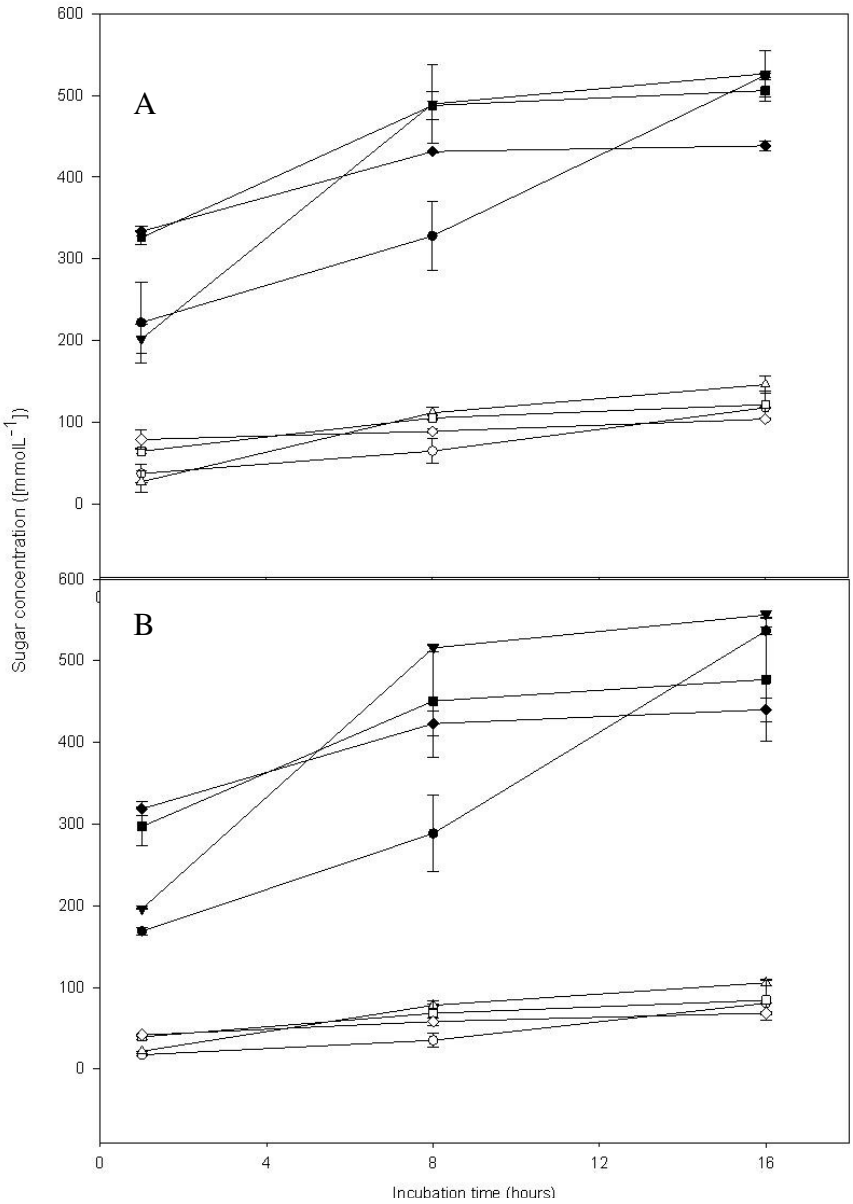

Figure 7. Reaction kinetics of β-galactosidases of crude cell extracts of *L. bulgaricus* ATCC 11842 in A) 19% (w/w) lactose and B) 19% (w/w) lactose with 6 % (w/w) glycerol in terms of hydrolase and total enzyme activity from the concentration of galactose (open symbols) and glucose (closed symbols) formed, respectively in mmol L^{-1}. Samples were inducated ad 40°C (circles), 50°C (triangles), 60°C (squares), or 70°C (diamonds). Results are shown as means ± standard deviation of two independent experiments.

3.3. Application of ultrafiltration whey permeate for production of galactooligosaccharides by β-galactosidases of lactic acid bacteria

UF whey permeate is a by-product of cheese-making, which is composed mainly of lactose. GOS formation in whey permeate was compared to GOS formation from lactose. CCE from the above four LAB strains had shown equivalent activity in solutions of a buffered lactose solution and UF whey permeate in terms of lactose conversion into GOS. A chromatogram of CCE from *L. bulgaricus* ATCC 11842 in both lactose and whey permeate in 19% (w/w) lactose is shown in Figure 8.

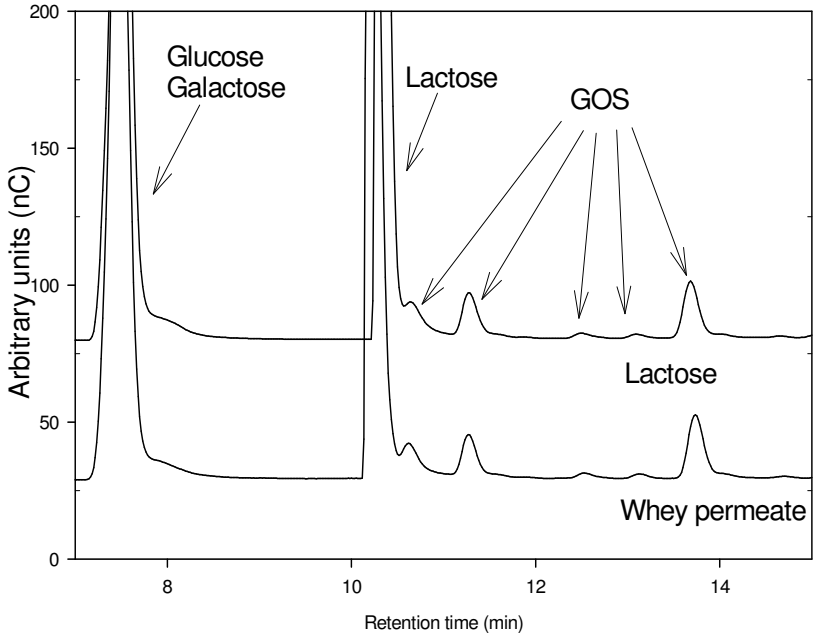

Figure 8. Separation of lactose and galactooligosaccharides produced by β-galactosidases of *L. bulgaricus* ATCC 11842 in 19% (w/w) lactose per kilogram of a buffered solution and an equivalent of 19% (w/w) lactose per kilogram of ultrafiltration whey permeate via High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection in nanocoulombs (nC). Results are representative for two independent experiments.

Adjusting the pH of UF whey permeate to a pH of 6.5 had shown equivalent activity of CCE compared to a buffered lactose solution in terms of lactose conversion into GOS in Table 6. By quantifying the concentration of glucose and galactose formed, the total activity of CCE was similar when incubated in buffered lactose solution and UF whey permeate.

Table 6. Lactose conversion by crude cell extracts of *L. bulgaricus* ATCC 11842 incubated at 37ºC for 24 hours in solutions of buffered lactose at pH 6.8, UF whey permeate at pH 6.5, and UF whey permeate at pH 6.0 with 19% (w/w) lactose. Total activity was expressed as the concentration of glucose, hydrolase activity as the concentration of galactose, and transferase activity as the difference in the concentration of glucose and galactose following lactose hydrolysis. Results are shown as the mean ± standard deviation of two independent experiments.

	Total activity [Glucose] (mmol L^{-1})	Hydrolase [Galactose] (mmol L^{-1})	Transferase [Glucose]-[Galactose] (mmol L^{-1})
Lactose	463 ± 12	141 ± 1	322 ± 11
Permeate, pH 6.0	383 ± 90	190 ± 29	193 ± 62
Permeate, pH 6.5	428 ± 59	178 ± 7	250 ± 52

3.4. Application of acceptor carbohydrates

Mannose, NAG, and fucose were added to solutions of lactose to determine if it is incorporated as acceptor sugars in the formation of HOS (Figure 9). Mannose, NAG and fucose were incorporated into HOS by *L. bulgaricus* ATCC 11842, *S. thermophilus* FUA 3068, *L. reuteri* 100-23, and *L. animalis* FUA 3045,

producing three types of HOS with mannose, two types of HOS each with NAG and fucose that were shown on the HPAEC-PAD chromatogram.

Figure 9 shows that HOS were made at the expense of GOS. Less GOS were formed in the presence of acceptor sugars compared to solutions of lactose as the sole galactosyl acceptor. While all strains had produced two HOS from NAG and fucose, β-gal had demonstrated varying degrees of GOS and HOS. Figure 9 shows that *L. bulgaricus* ATCC 11842 had produced the most GOS and HOS, followed by *S. thermophilus* FUA 3068, *L. animalis* FUA 3045, and *L. reuteri* 100-23. Data on HOS structures could not be obtained via HPAEC-PAD. As HOS have different molecular weights, linkages, and compositions, only assumptions on the type of HOS could be made. It is possible that NHOS and FHOS could have one type of disaccharide and trisaccharide.

The effect of acceptor sugar NAG was investigated in solutions of reconstituted UF whey permeate. Results with β-gal from *L. bulgaricus* ATCC 11842 in solutions of lactose and UF whey permeate with addition of NAG are shown in Figure 10. The degree of lactose hydrolysis appeared to be the same in solutions of lactose and UF whey permeate. Transferase activity, however, was higher in solutions of UF whey permeate with addition of NAG as an acceptor sugar. Another type of HOS in addition to GOS and HOS was produced in solutions of UF whey permeate with NAG. While glucose and galactose were separated with this gradient, the glucose concentration could not be determined when NAG was used as an acceptor carbohydrate. NAG had the same retention time as glucose, which elutes as one large peak.

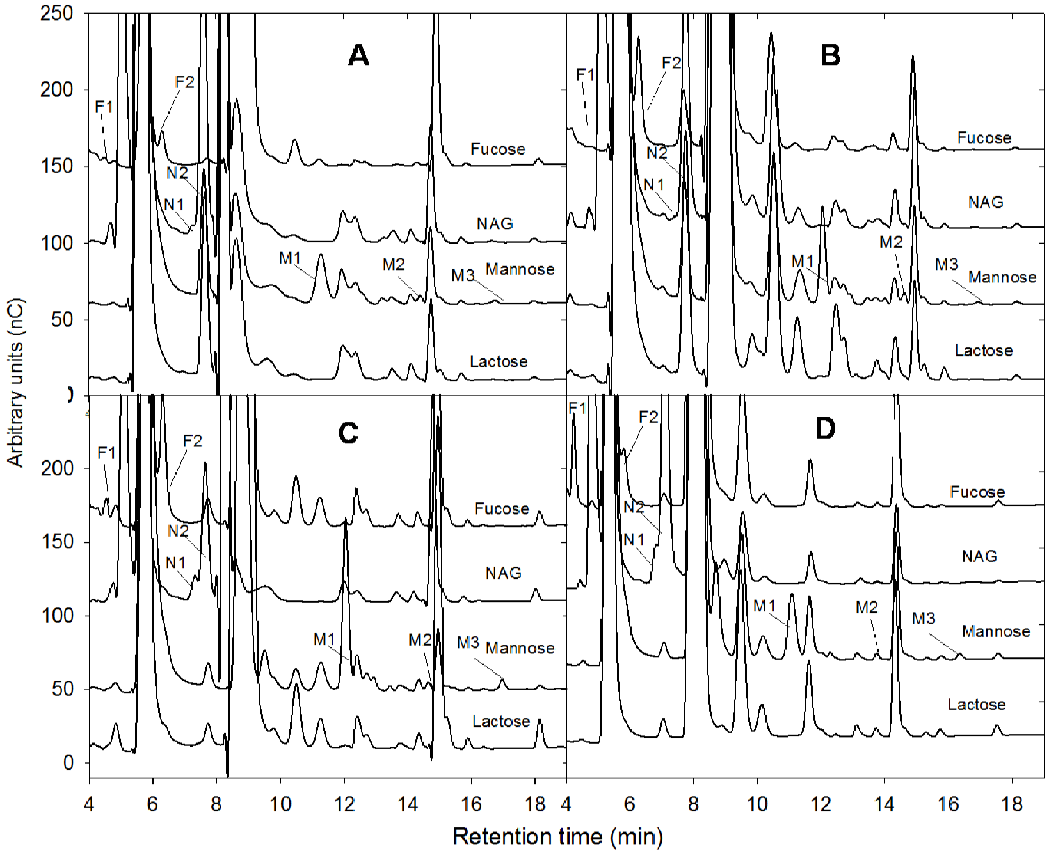

Figure 9. Separation of galactooligosaccharides and heterooligosaccharides profile of crude cell extracts of A) *L. reuteri* 100-23, B) *L. animalis* FUA 3045, C) *S. thermophilus,* and D) *L. bulgaricus* ATCC 11842 in 19% (w/w) lactose with 10% (w/w) each mannose, N-acetylglucosamine, and fucose per kilogram of solution, incubated at 37ºC for 24 hours. Lactose, heterooligosaccharides (M1, M2, M3, N1, N2, F1, F2), and galactooligosaccharides (GOS) are indicated with arrows. Results are representative for two independent experiments.

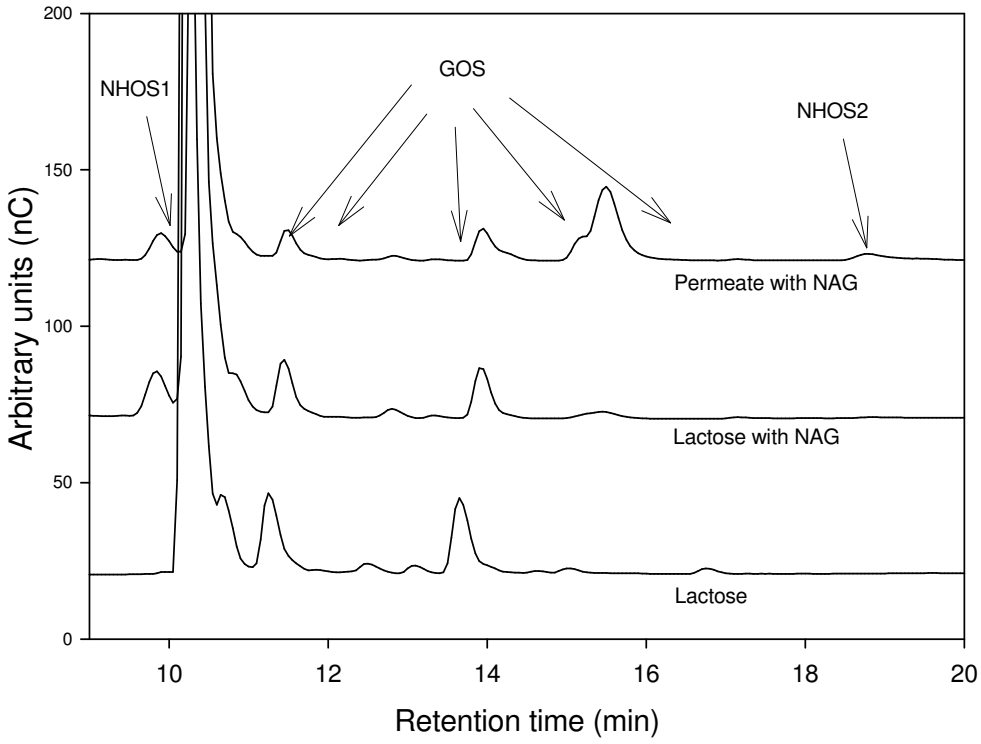

Figure 10. Separation of galactooligosaccharides and heterooligosaccharides produced by crude cell extracts of *L. bulgaricus* ATCC 11842 in solutions of 19% (w/w) lactose, ultrafiltration whey permeate with 19% (w/w) lactose, and 19% (w/w) lactose with 19% (w/w) N-acetylglucosamine incubated at 37ºC for 24 hours, with GOS, HOS (NHOS1, NHOS2), lactose, and glucose and galactose are labelled with arrows. Separations were performed with High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection in nanocoulombs (nC). Results are representative for two independent experiments.

3.5. Separation of oligosaccharide mixtures with High Performance Liquid Chromatography

Mixtures of sugar solutions produced with CCE of *L. bulgaricus* ATCC 11842 were applied to an amino-bonded column with an initial water concentration of 11 to 23% for OS separation shown in Figure 11. HPLC separation was used in the separation of OS from monosaccharides and lactose for further testing by Brenna Black using LC-MS. HPAEC-PAD is not a suitable method as its limits of separation are in picomoles which will be too dilute to collect as fractions for further analysis. In addition, HPAEC-PAD analysis involves elution with sodium hydroxide and sodium acetate, which are incompatible with LC-MS analysis. A HPLC method was developed to allow for collection of larger quantities of OS fractions and the use of a neutral eluent which is easily removed. OS were fractionated in microgram quantities, and the acetonitrile-water solvent was removed by evaporation under nitrogen gas and lyophilisation prior to LC-MS analysis.

3.6. Structural identification of oligosaccharides with Gas Chromatography – Mass Spectrometry

OS mixtures were analyzed with GC-MS analysis to confirm the presence of fucose and NAG in HOS. Chromatograms of OS mixtures produced by CCE of *L. bulgaricus* ATCC 11842 in lactose with NAG and lactose with fucose are shown in Figure 12 and 13, respectively.

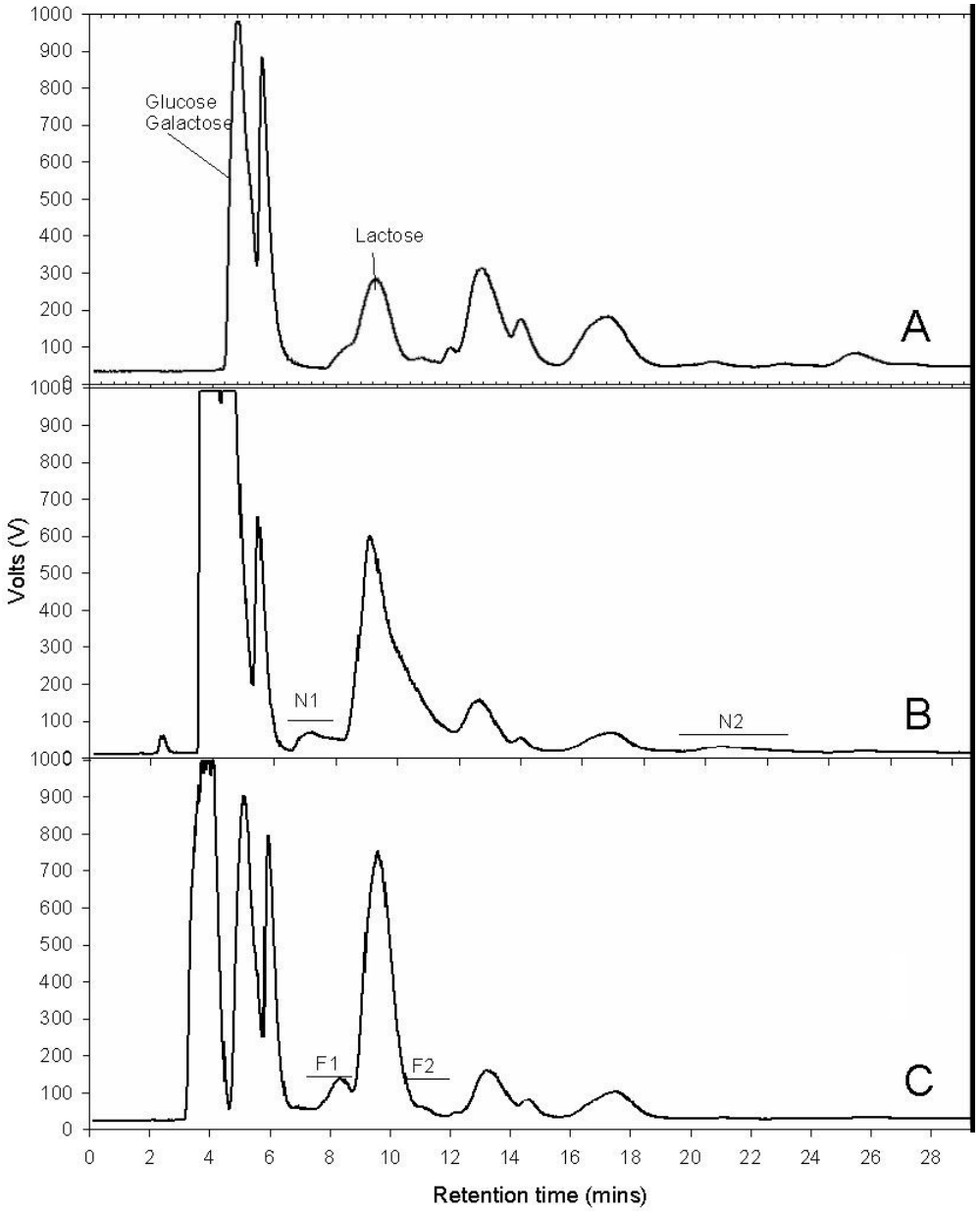

Figure 11. Separation of oligosaccharides produced by crude cell extracts of *L. bulgaricus* ATCC 11842 incubated in solutions of A) 19% (w/w) lactose , B) 19% (w/w) lactose with 19% (w/w) N-acetylglucosamine and C) 19% (w/w) lactose with 19% (w/w) fucose at 37ºC for 24 hours using High Performance Liquid Chromatography with a water:acetonitrile gradient and Electron Light-Scattering Detection. Results are representative for two independent experiments.

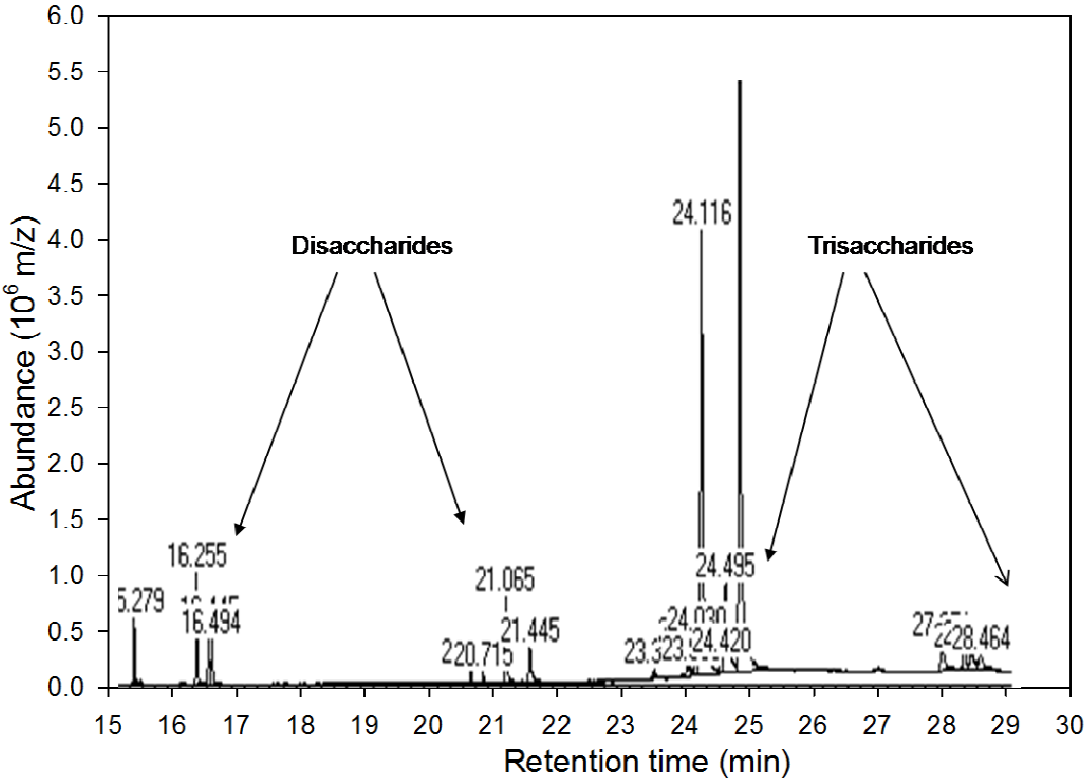

Figure 12. Gas chromatogram of a mixture of oligosaccharides produced with crude cell extracts of *L. bulgaricus* ATCC 11842 in solutions of 19% (w/w) lactose with 19% (w/w) N-acetylglucosamine at 37ºC for 24 hours via permethylation using Gas Chromatography-Mass Spectrometry with a temperature gradient from 70 to 320 ºC on a HP-5 column. Data from mass spectra of peaks indicated elution of disaccharides between 15.28 and 21.46 minutes, and trisaccharides between 23.30 to 28.46 minutes. Results are representative for two independent experiments.

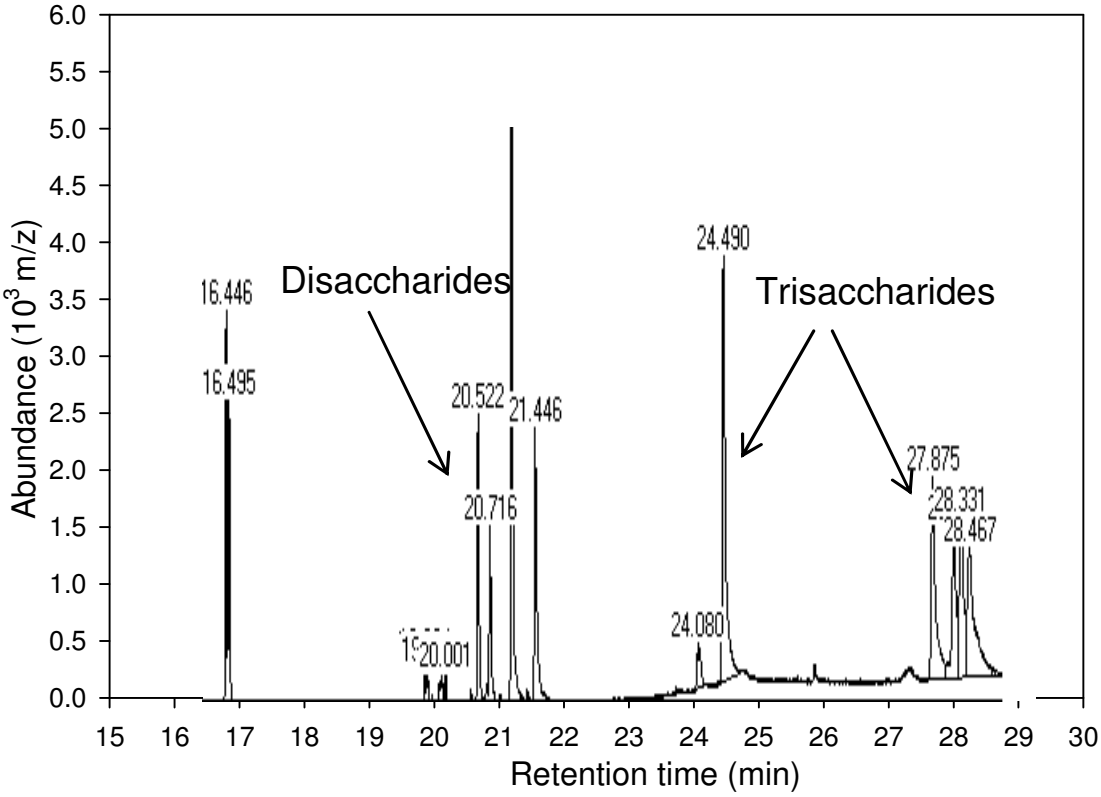

Figure 13. Gas chromatogram of a mixture of oligosaccharides produced with crude cell extracts of *L. bulgaricus* ATCC 11842 in solutions of 19% (w/w) lactose with 19% (w/w) fucose at 37ºC for 24 hours via permethylation using Gas Chromatography-Mass Spectrometry with a temperature gradient from 70 to 320 ºC on a HP-5 column. Data from mass spectra of peaks indicated elution of disaccharides between 20.00 and 21.45 minutes, and trisaccharides between 24.08 to 28.47 minutes. Results are representative for two independent experiments.

Figures 12 and 13 show the permethylated structures from OS mixtures appearing as clusters of peaks on gas chromatograms following a temperature gradient from 70 to 320ºC. Mass spectra data of peaks indicated elution of monosaccharides, disaccharides, and trisaccharides. Figure 12 indicates elution of

two clusters of disaccharides from 15.28 – 16.49 minutes and 20.72 – 21.45, trisaccharides from 23.30 – 24.71 minutes, and higher OS from 27.00 – 28.46 minutes. Figure 13 indicates elution of monosaccharides from 16.45 – 16.50 minutes, disaccharides from 20.00 – 21.45, trisaccharides from 24.08 – 24.49 minutes, and higher OS from 27.88 – 28.47 minutes. Mass spectra of peaks from the above gas chromatogram are shown in Figures 14 and 15.

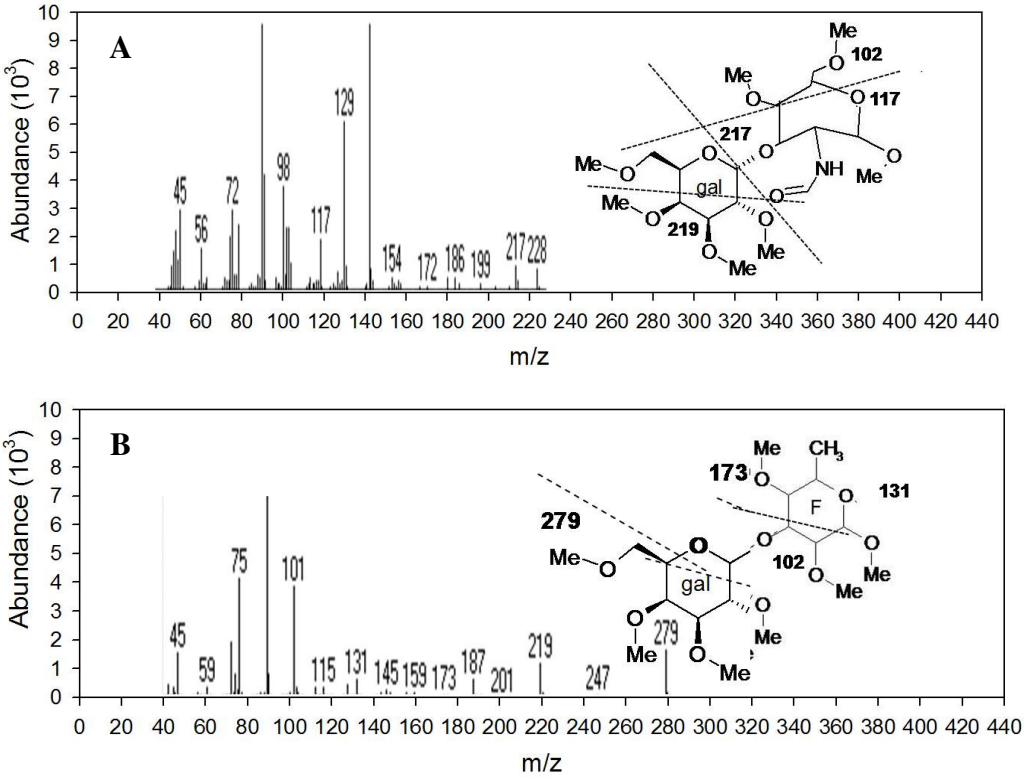

Figure 14. Mass spectra of peaks from gas chromatogram of disaccharides produced with crude cell extracts of *L. bulgaricus* ATCC 11842 in (A) 19% (w/w) N-acetylglucosamine with a retention time of 24.71 minutes from Figure 12A and (B) 19% (w/w) fucose with a retention time of 24.49 minutes from Figure 12B at 37ºC for 24 hours via permethylation using Gas Chromatography-Mass Spectrometry with a temperature gradient from 70 to 320 ºC on a HP-5 column. Results are representative for two independent experiments.

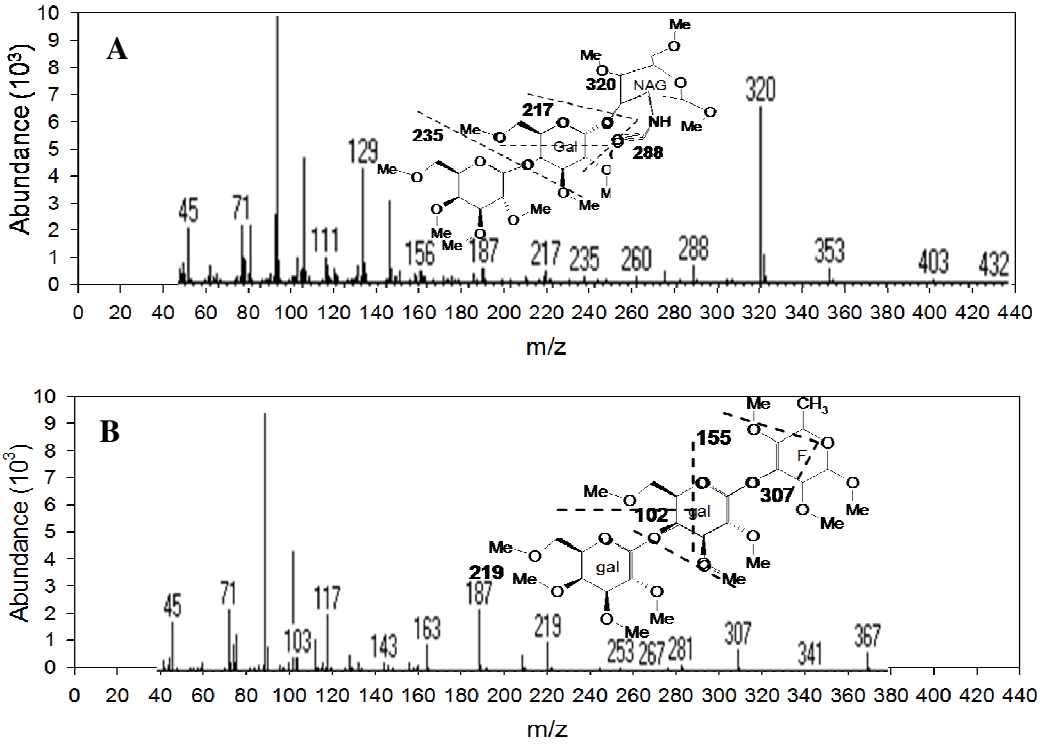

Figure 15. Mass spectra of peaks from gas chromatogram of trisaccharides produced with crude cell extracts of *L. bulgaricus* ATCC 11842 in 19% (w/w) lactose with (A) 19% (w/w) N-acetylglucosamine with a retention time of 24.71 minutes from Figure 12A and (B) 19% (w/w) fucose with a retention time of 24.49 minutes from Figure 12B at 37ºC for 24 hours via permethylation using Gas Chromatography-Mass Spectrometry with a temperature gradient from 70 to 320 ºC on a HP-5 column. Results are representative for two independent experiments.

The structures based on the molecular weight fractions of the mass spectrum in Figures 14A, 14B, 15A, and 15B were assembled using ISISDRAW software. The mass spectrum of Figure 14A of peak from Figure 12 with a retention time of 16.25 minutes had corresponding molecular weight fractions of 102, 117, 217, and 219. These molecular weight fractions were unique to a compound with

hexose and NAG. The mass spectrum of Figure 15A of peak from Figure 12 with a retention time of 24.71 minutes had corresponding molecular weight fractions of 217, 235, 288, and 320. The structure that was tentatively identified with the mass spectra of Figures 14A and 15A were a combination of hexose and NAG.

The mass spectra of Figure 14B for a peak from Figure 13 with a retention time of 21.06 minutes had corresponding molecular weight fractions of 101, 131, 173, and 279, which were exclusive for a compound with a molecule of hexose and fucose. The mass spectrum of Figure 15B for a peak from Figure 13 with a retention time of 24.49 minutes had molecular weight fractions with molecular weights of 102, 155, 219, and 307, which were exclusive for a compound with two hexoses and a molecule of fucose.

3.7. Structural identification of oligosaccharides with Liquid Chromatography – Mass Spectrometry

To confirm the presence of HOS with lactose and fucose or NAG, LC-MS analysis of OS fractions was conducted by Brenna Black (2009) as part of her graduate research project. Molecular weight fractions of OS samples containing NAG and fucose are shown in Figures 16 and 17, respectively. The monosaccharide composition acquired from molecular weight fragments in Figures 16 and 17. While the standards of galactose and glucose could not be differentiated from each other by way of tandem MS fragmentation patterns (data not shown), the β-galactose backbone structure is a common feature formed by βgal (Splechtna et al., 2007). Thus, galactose is the reducing terminal of OS. The

presence of NAG or fucose on the terminal galactose given a distinct molecular mass and could be distinguished as HOS.

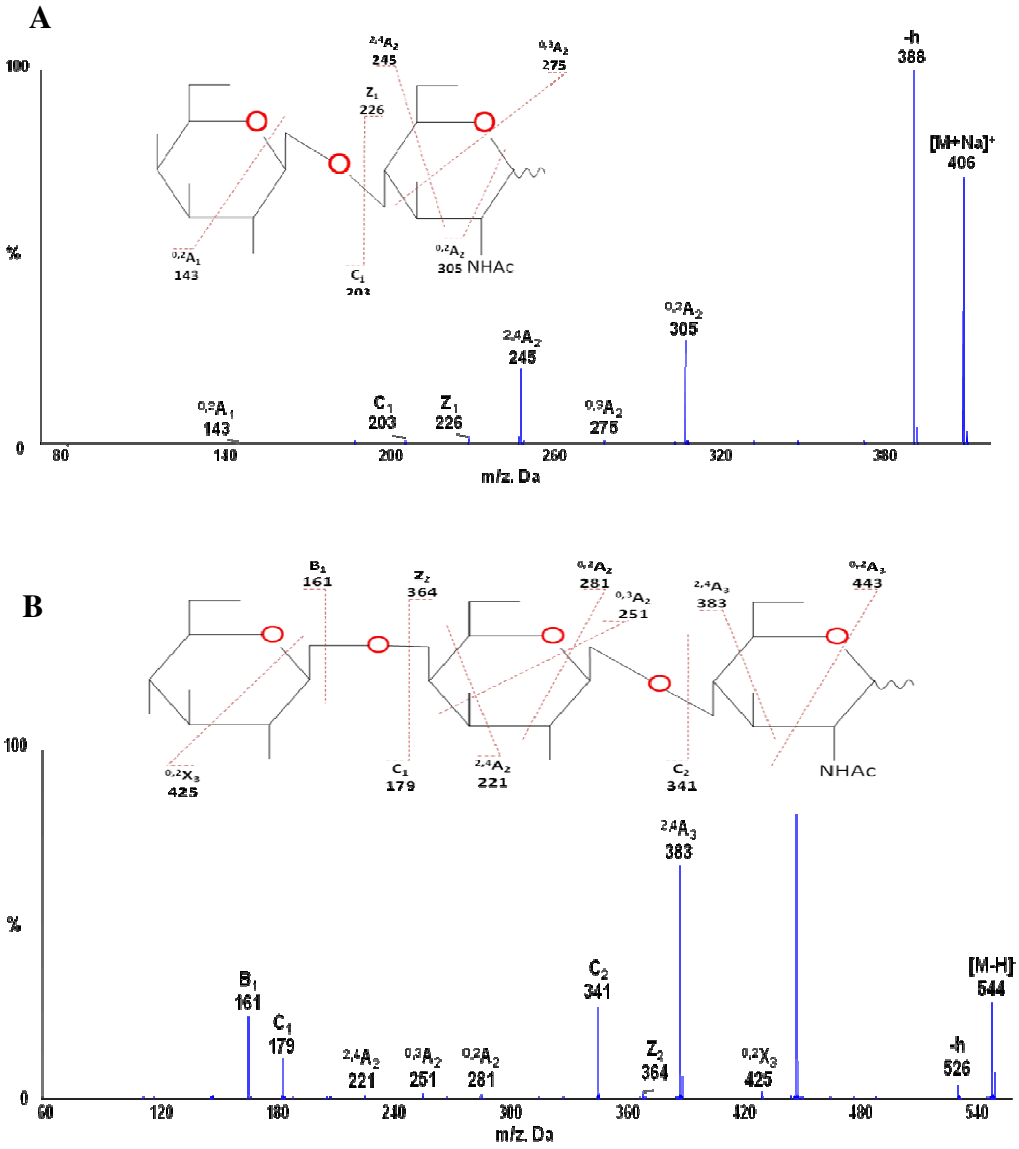

Figure 16. Mass spectra of peaks from liquid chromatograms of disaccharide (A) and trisaccharide (B) produced with crude cell extracts of *L. bulgaricus* ATCC 11842 in solutions of 19% (w/w) lactose with 19% (w/w) N-acetylglusoamine at 37ºC for 24 hours using ElectroSpray Ionization Collision-Induced Dectection tandem Mass Spectrometry. This figure was provided by Brenna Black (2009).

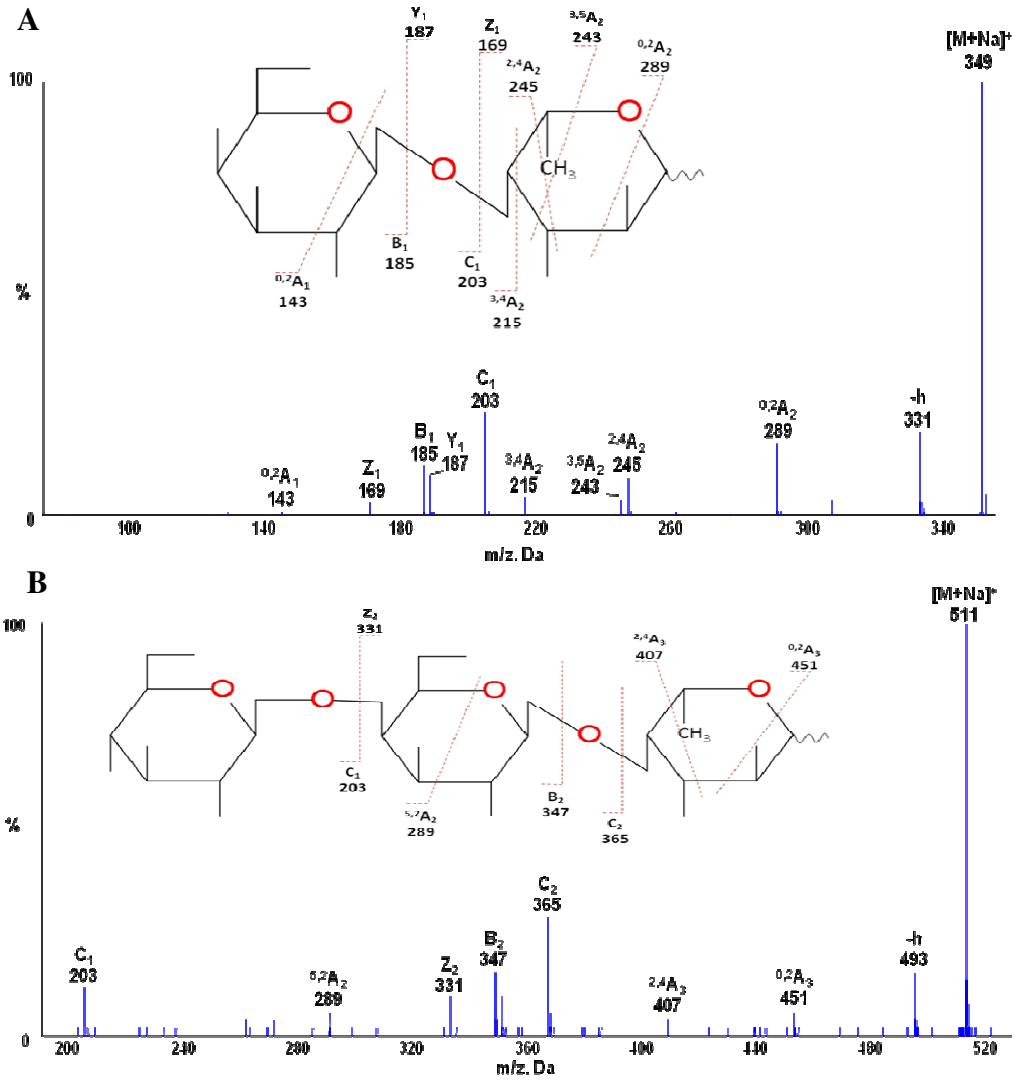

Figure 17. Mass spectra of peaks from liquid chromatograms of disaccharide (A) and trisaccharide (B) produced with crude cell extracts of *L. bulgaricus* ATCC 11842 in solutions of 19% (w/w) lactose with 19% (w/w) fucose at 37ºC for 24 hours using ElectroSpray Ionization Collision-Induced Dectection tandem Mass Spectrometry. This figure was provided by Brenna Black (2009).

4. Discussion

4.1. Screening of β-galactosidase activity in crude cell extracts of lactic acid bacteria

Five of the 17 strains that had shown both hydrolase and transferase activity were of dairy or intestinal origin. *L. bulgaricus* and *S. thermophilus* are used in the manufacture of fermented dairy foods and are therefore well adapted to utilize lactose. Conversion of lactose into digestible sugars produces a variety of fermented dairy foods, such as yogourt, that can be consumed by people who lack intestinal β-gal for digesting lactose in foods (Occhino et al., 1986). Lactose fermentation occurs in the intestinal tract of humans and animals, which are the origins of *L. reuteri*, *L. animalis*, and *B. breve*. Lactose is fermented into glucose as an energy source for the growth of these microorganisms in the intestinal tract. (Axelsson, 2004). Microbial strains from cereal sources, such as *L. fermentum* FUA 3095 and meat sources, such as *C. maltaromaticum* ATCC 14931 did not show β-gal activity. Cereal and meat do not contain lactose, which explains the reason that those strains did not β-gal activity (Axelsson, 2004, Carr et al., 2002).

4.2. Optimization of transferase activity of β-galactosidase

4.2.1. Buffer optimization

The improvement of GOS production via transferase activity of β-gal enzymes in CCE with the addition of KCl and $MgCl_2$ was in agreement with Garman et al. (1996). As GOS production was still occurring after 24 hours of incubation at 37 ºC, the reaction conditions could still be improved to enhance transferase activity of β-gal enzymes in CCE.

4.2.2 Glucose removal

Formation of GOS is limited by the accumulation of glucose during fermentation. While Cheng et al. (2006) and Sheu et al. (2001) observed that incorporation of *S. cerevisiae* and glucose oxidase in reaction mixtures resulted in more GOS production by β-gal, there was no difference in transferase activity of CCE from LAB in this study.

Selective purification of OS mixtures was performed by Goulas et al. (2007) by yeast fermentation with food-grade *S. cerevisiae* during transgalactosylation of lactose by *B. bifidum*. Goulas et al. (2007) observed that *S. cerevisiae* had consumed glucose that was formed upon lactose hydrolysis, with no losses in the OS content. Goulas et al. (2007) had used whole cells of *B. bifidum* that was optimized for β-gal by treatment with toluene to increase cell permeability. Whole cells were lyophilized and directly applied to solutions of 450 and 500 mg mL^{-1} lactose in phosphate (pH 6.8) and citrate (pH 6.2) buffers incubated at 40 ºC (Goulas et al., 2007).

Cheng et al. (2006) incorporated *K. marxianus* and glucose oxidase to remove glucose that was formed upon lactose hydrolysis by β-gal of *A. oryzae, K. lactis and Bacillus spp*. in 330 mg mL^{-1} of lactose. Cheng et al. (2006) observed that the combination of glucose removal with *K. marxianus* and glucose oxidase with β-gal had produced higher concentrations of GOS in a shorter reaction time.

Sheu et al. (2001) observed that enzyme activity was also enhanced after purification with glucose oxidase in FOS production with *β*-fructofuranosidase from *A. japonica.* Addition of 1 gram per 100 mL of glucose oxidase dissolved in

0.1 M sodium acetate buffer (pH 5.4) produced a pure solution of FOS from a solution of 300 mg mL^{-1} sucrose at sodium acetate buffer (pH 5.4) in a shorter reaction time (Sheu et al., 2001).

By comparing with studies by Goulas et al. (2007), Cheng at al. (2006), and Sheu et al. (2001), reasons for discrepancies between the lack of effect of *S. cerevisiae* and glucose oxidase in this study is the difference in the source of β-gal and the concentration of lactose. Goulas et al. (2007), Cheng at al. (2006), and Sheu et al. (2001) had used whole cells, and purified enzymes bacterial and fungal enzymes, respectively. This is study is the first experiment using β-gal from LAB. A second reason may be that the initial sugar concentrations used by Goulas et al, Cheng et al., and Sheu et al. (2001) were higher compared to the 19% (w/w) used in this study.

4.2.3. Effect of incubation temperature and incorporation of glycerol as a compatible solute

The increase in stability of β-gal of *K. lactis* at 40, 50, and 70 ºC in its higher activity in solutions of lactose with glycerol was due to the effect of glycerol as a compatible solute. Despite a decrease in the enzyme activity at higher temperatures, β-gal activity at 70ºC was higher for β-gal of *K. lactis* in the buffered lactose containing glycerol. Glycerol had protected the enzymes are from osmotic induced dehydration and thermal denaturation (Santos and da Costa, 2002, Martınez-Villaluenga et al., 2008). The presence of potassium ions in the buffered lactose solution had also maintained enzyme activity of CCE at 70 ºC.

The increase in transferase activity of β-gal in CCE of *L. bulgaricus* ATCC 11842 in solutions of lactose with glycerol at 40, 50, and 70 ºC was because of the higher incubation temperature that allowed a higher initial lactose concentration to further favour OS formation (Huber et al., 1976). Prior experiments with lower lactose concentration indicated incomplete lactose removal and enzyme denaturation when CCE of *L. bulgaricus* ATCC 11842 was used (Vasiljevic and Jelen, 2002).

4.3. Use of ultrafiltration whey permeate on β-galactosidase activity

The impact of UF whey permeate on β-gal activity was studied by Splechtna et al. (2007) who found that enzyme activity was 1.8-fold higher than in pure lactose. In this study, the β-gal of CCE of *L. bulgaricus* ATCC 11842 had utilized UF whey permeate as effectively as a buffered lactose solution with added potassium and magnesium salts in GOS production. The presence of monovalent and divalent cations, namely potassium and magnesium, in UF whey permeate acted as mineral cofactors on β-gal, which enhance its activity in lactose hydrolysis and GOS formation (Wong et al., 1978, Garman et al., 1996). While the mineral content of the reconstituted UF whey permeate was not tested, it is assumed to be of an equivalent concentration as the 100 mmol L^{-1} of KCl and 2 mmol L^{-1} $MgCl_2$ in solutions of 19% (w/w) lactose, because of the equivalent lactose hydrolysis and GOS production by CCE of *L. bulgaricus* ATCC 11842 in both solutions of buffered lactose and UF whey permeate. As UF whey permeate contains calcium, sodium, manganese, along with trace minerals zinc and copper,

the β-gal activity of CCE of *L. bulgaricus* ATCC 11842 was enhanced due to the synergy in the mineral content in the solution of UF whey permeate (Wong et al., 1978).

4.4. Oligosaccharide production from the application of acceptor carbohydrates

The formation of HOS with mannose, fucose, and NAG using CCE shows that these sugars are galactosyl acceptors for the synthesis of novel products by β-gal of LAB. This discovery expands the list of β-gal found in Table 3 that are capable of using mannose and NAG as galactosyl acceptors. Synthesis of novel HOS with NAG and fucose by CCE also provides an opportunity to use food-grade LAB.

HOS were also produced when NAG was incorporated into UF whey permeate as an acceptor carbohydrate. The mineral content of UF whey permeate had also shifted the degree of β-gal activity, lowering the hydrolyase activity and increasing transgalactosylation in forming more GOS and HOS than in solutions of lactose.

Mannose was previously demonstrated to act as a galactosyl acceptor for β-gal from *Bacillus circulans* (Miyasato and Ajisaka, 2004). The OS ß-Gal-(1-3)-Man, ß-Gal-(1-4)-Man, and ß-Gal-(1-6)-Man, but no trisaccharides were formed by transgalactosylation of β-gal from *B. circulans* (Miyasato and Ajisaka, 2004).

NAG was previously demonstrated to act as a galactosyl acceptor for β-gal from bovine testes and Lac-Z β-gal of *E. coli* (Hedbys et al., 1989a). The OS ß-Gal-(1-3)-ß-GlcNAc and ß-Gal-(1-6)-ß-GlcNAc, but no trisaccharides were formed by transgalactosylation of β-gal from bovine testes and Lac-Z β-gal of *E. coli*. While acceptor products have not been extensively studied with β-gal of LAB, novel OS were produced using mannose, NAG, and fucose with CCE.

4.5. Separation of crude mixtures with High Performance Liquid Chromatography

HOS with NAG and fucose were identified via HPLC, because of the difference of their retention times from monosaccharides, lactose, and GOS on the HPLC column. The difference in their retention times, were due to the size, structure, and orientation of functional groups from the composition of their constituent monosaccharides. This was in agreement with Blanken et al. (2000), as NAG has an acetamido group and fucose lacks a hydroxyl group, which affects their retention time on the HPLC column (Blanken et al, 2000).

4.6. Structural analysis of oligosaccharides with Gas Chromatography – Mass Spectrometry and Liquid Chromatography – Mass Spectrometry

The mass spectra generated by GC-MS and LC-MS had indicated the presence of NAG and fucose. The presence of an acetoamido group in NAG, which contains nitrogen and an acetyl group, generates even-numbered mass fractions in GC-MS analysis (Hedbys et al., 1989a).

GC-MS analysis with permethylation of HOS tentatively identified the composition of sugars containing fucose and NAG. The hexose sugar connected to the NAG or fucose is also assumed to be a molecule of galactose, as it is a common feature formed by β-gal (Splechtna et al., 2007). Subsquent analysis of permethylated structures by hydrolysis, reduction, and a secondary derivatization step of acetylation is required to determine the position of the glycosidic linkages of the constituent monosaccharides in HOS (McGinnis et al., 1989).

The composition of HOS with fucose and NAG produced by CCE of LAB is comparable to the 2' fucosyllactose, 3' fucossyllactose, lactose-N-tetraose, and lactose-N-neotetraose found in human milk OS, which are shown in Table 7.

Table 7. Comparison of heterooligosaccharides produced from β-gal of lactic acid bacteria with selected human milk oligosaccharides with comparable composition

Oligosaccharides in human milk	
2' fucosyllactose [a,b]	Fuc(α1-2)Gal(β1-4)Glc
3' fucosyllactose [a,b]	Gal(β1-4)Glc | Fuc(α1-3)
Lacto-N-tetraose [a,b]	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
Lacto-N-neotetraose [a,b]	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Heterooligosaccharides produced by β-galactosidases of lactic acid bacteria	
Galactosyl-fucose	Fuc-Hex Fuc-Hex-Hex
Galactosyl-N-Acetylglucosamine	GlcNAc-Hex GlcNAc-Hex-Hex

Fuc: fucose, Gal: Galactose, Glc: Glucose, GlcNAc: N-acetylglucosamine, Hex: hexose

a: Dua and Bush, 1983 ; b: Thurl et al., 1991

Conclusions

GOS were produced by CCE from *L. reuteri* 100-23, *L. animalis* FUA 3045, *S. thermophilus* FUA 3068 and *L. bulgaricus* ATCC 11842 in 19% (w/w) lactose and HOS were produced with 19% (w/w) lactose and 10% (w/w) mannose, NAG, and fucose as acceptor sugars. Optimal buffer conditions were determined as a pH of 6.8 for *S. thermophilus* FUA 3068 and *L. bulgaricus* ATCC 11842, pH of 5.4 for *L. reuteri* 100-23 and *L. animalis* FUA 3045 with 2 mmol L^{-1} $MgCl_2$ with 100 mmol L^{-1} KCl. Incorporation of 6% (w/w) glycerol had shifted the β-gal activity of CCE of *L. bulgaricus* ATCC 11842 toward transgalactosylation at incubation temperatures of 40, 50, and 70 ºC. UF whey permeate with 19% (w/w) lactose is a suitable source of lactose for production of GOS using CCE of *L. bulgaricus* ATCC 11842. Three types of HOS with mannose, and two types of HOS with each NAG and fucose. The composition of a trisaccharide containing NAG and a di- and/or trisaccharide containing fucose that was produced by *L. bulgaricus* ATCC 11842 was determined with GC-MS and LC-MS following HPLC separation. This is the first public report indicating the production HOS with NAG and fucose as galactosyl acceptors by CCE from LAB.

While the composition of HOS produced by CCE of β-gal from LAB differ from human milk OS, the presence of galactosyl-fucose and galactosyl-NAG may nevertheless yield OS structures that are recognized by pathogens. These novel HOS containing NAG and fucose need to be tested for effectiveness compared with human milk OS, which could provide future opportunities of applying to pathogens to determine its effectiveness against pathogens.

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