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PRODUCTION, FRACTIONATION AND PURIFICATION OF GALACTO-OLIGOSACCHARIDES FROM WHEY LACTOSE

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NORMAN JOSEPH MATELLA

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PRODUCTION, FRACTIONATION AND PURIFICATION OF GALACTO-OLIGOSACCHARIDES FROM WHEY LACTOSE

By

Norman Joseph Matella

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ABSTRACT

PRODUCTION, FRACTIONATION AND PURIFICATION OF GALACTO-OLIGOSACCHARIDES FROM WHEY LACTOSE

By

Norman Joseph Matella

The following study aimed to optimize the production, fractionation and purification of galacto-oligosaccharides (GOSs) from whey lactose. Also, a market analysis was done to determine whether the GOS end product would be competitive in the prebiotic market. The first phase of the study involved optimization of GOS production from β-galactosidase treatment of lactose. These optimized conditions were then applied to develop equivalent ultrafiltration (UF) free-enzyme and immobilized-enzyme systems. The effects of fluid pressure on enzyme performance were studied for the UF free-enzyme systems. In immobilized-enzyme systems, the effects of immobilizing agents on enzyme performance were studied. The two systems were made equivalent based on enzyme activity and compared as recycle-batch and continuous systems. The effect of residual monosaccharides on GOS production was also studied. In phase 2 of this study, model sugar solutions were investigated under various nanofiltration (NF) temperature, pressure, pH and concentration conditions for maximal mono-/disaccharide removal and minimal oligosaccharide loss. Optimum conditions were then applied to develop a NF diafiltration process to fractionate GOSs from the monosaccharides and lactose contained in the enzymatic sugar mixture. In phase 3 of the study, oligosaccharide model

solutions were applied to ion exclusion chromatography (IEC) columns using high performance liquid chromatography (HPLC). Model solutions were studied at various flow rates, column temperatures and particle sizes to determine optimal resolution. These optimal conditions were then used to separate individual GOSs contained within the GOS fractionate from phase 2. Finally, the overall process and final product were studied for process economics and marketability. A continuous UF free-enzyme system with 42.3 U/mL of initial enzyme and 270 g/L of initial lactose was determined to achieve optimal GOS production, with relatively high maximum GOS yields (22%) and product throughput (~140 g/h) within relatively short start-up time (15 min). Fluid pressures did not inactivate enzymes in UF free-enzyme systems, while immobilizing agents severely inactivated enzymes within immobilized systems (50-90% inactivation). During the NF GOS fractionation phase, pH and concentration showed the strongest effect on sugar retention. The two variables were manipulated in a three-step diafiltration process that permeated >90% monosaccharides and ~50% lactose with <19% loss of GOSs. During chromatography experiments, optimum oligosaccharide separation was achieved with IEC at 0.4 mL/min flow rate, ambient column temperature and 7-μm particle size. However, lactose separation from the GOSs was difficult and required the use of ion moderated partition chromatography. In an economic assessment, the production cost for a GOS syrup was only $0.23 per pound solid, which was proven to be competitive in the prebiotic market.

DEDICATION

To my parents Joseph and Ann Matella for their perpetual love, support and sacrifice. And to my younger siblings Theresa, Joanne, Christina and Andrew for their support and love. To my little niece and Goddaughter, Alyssa Spencer, who can brighten my day with a single smile.

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Key to symbols and abbreviations

ANOVA	Analysis of Variance
C	Concentration
C_O	Initial concentration
Cp	Permeate Concentration
Cf	Feed Concentration
df	Degrees of Freedom
DP	Degree of Polymerization
DVB	Divinylbenzene
FOS	Fructo-oligosaccharide
GA	Glutaraldehyde
GOS	Galacto-oligosaccharide
HPLC	High Performance Liquid Chromatography
IEC	Ion Exclusion Chromatography
IMPC	Ion Moderated Partition Chromatography
k	Rate constant
MOS	Malto-oligosaccharide
MWCO	Molecular Weight Cut-Off
n	Reaction order
NF	Nanofiltration
P	Probability
PEI	Polyethyleneimine

p.s.i.	Pounds per square inch
R	Solute retention
RO	Reverse Osmosis
RPM	Revolutions per minute
Rs	Resolution
SCFA	Short Chained Fatty Acid
SWOT	Strengths, Weaknesses, Opportunities and Threats
t	Time or retention time
UF	Ultrafiltration
$V_{1 \text{ or } 2}$	Reactor Volume
Vf	Feed Volume
Vp	Permeate Volume
$W_{A \text{ or } B}$	Peak A or B Width
$W_{1 \text{ or } 2}$	Cloth Weight
WPC	Whey Protein Concentrate
(w/v)	Weight per volume
(w/w)	Weight per weight
α	Alpha-level or Probability of Uncertainty
β	Upward orientation of hydroxyl group of galactose carbon 4

INTRODUCTION

U.S. consumer interest in nutraceuticals, or functional foods, is exponentially rising (1, 2). The Institute of Medicine's Food and Nutrition Board has defined functional foods as "any food or ingredient that may provide a health benefit beyond the traditional nutrients it contains" (3). The nutraceutical market is projected to grow to $60 billion by 2010 totaling to 10% of the U.S. food market with a 12% annual growth rate (4). The high market interest in functional foods as health-enhancers is directly related to consumers' concern over the increasing incidence of various health problems, including obesity, cancer, and immunology (3). One segment of this market is the prebiotic market, which was estimated to be approximately $800 million in Japan, $103 million in Europe and $15 million in the U.S. in 2003 (2, 5). Growth expectations for this market are very high with projections of $1.6 billion in Japan, $216 million in Europe and $103 million in the U.S. by 2010 (1, 2, 5).

Prebiotics are non-digestible components, usually oligosaccharides, which are preferentially consumed by beneficial microflora within the human intestinal tract (6, 7). This preferential consumption, in turn, can lead to enhanced nutritional and immune status, reduced risk of colon cancer, treatment of Chrone's disease and irritable bowel syndrome, reduced cardiovascular disease risk, and reduction of pathogenic bacteria in the gut (6, 7). Fructo-oligosaccharides are the predominant prebiotic sold in the market; however, evidence has shown that galacto-oligosaccharides (GOSs) can offer the same bioactive benefits (6-9). Furthermore, researchers have shown that GOSs can

be produced from whey lactose, a readily abundant and inexpensive substrate (10).

There has been a significant body of research done on the manufacture of GOSs from lactose. Previous research has shown that β-galactosidase treatment of lactose results in large concentrations of galactose, glucose, unreacted lactose and a significant concentration of GOSs ranging from 3 to 7 degrees of polymerization (DP) or chain-lengths (7, 10-17). Despite the quantity of research already done, GOS production has not been widely implemented in the U.S. One major reason is that enzymatic GOS production and manufacture has not been well optimized. Also, in these studies, GOSs were manufactured in the presence of large concentrations of non-prebiotic sugars (i.e. glucose, galactose and unreacted lactose). Furthermore, individual GOS DPs were not separated and purified to target a variety of markets that demand pure forms of these chemicals. Finally, GOS market potential has not been well elucidated. Therefore, the specific objectives of the dissertational research were:

1. To optimize GOS manufacture using either immobilized- or free-enzyme processes;
2. To optimize membrane filtration technology to preconcentrate or fractionate GOSs (DP3-7) from the large concentrations of non-prebiotic simple sugars;
3. To optimize separation of individual GOS chain-lengths with ion exclusion chromatography (IEC);
4. To devise a market analysis for the potential of GOS products.

Figure 1 schematically illustrates how Objectives 1-3 can be coordinated within a GOS manufacturing process. The following dissertation comprises four chapters that address the three proposed phases to manufacture, preconcentrate and purify galacto-oligosaccharides (GOSs) from whey lactose, and one chapter that addresses GOS market analysis. The chapters were written as independent entities intended for journal publication. As a result, there may be some redundancy from chapter to chapter.

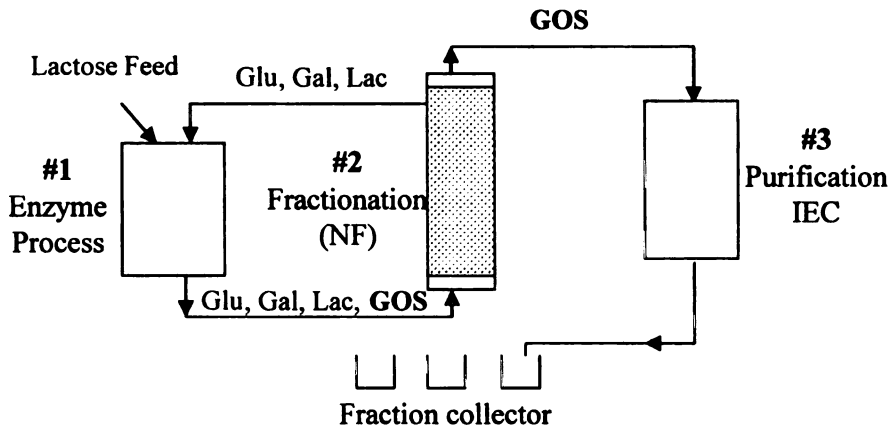

Figure 1 - Schematic of the overall GOS manufacture process. Objectives or phases are labeled as#1, #2, and #3.

Chapter 1 partly addresses Objective #1. The research in this chapter optimizes enzyme conditions for both free- and immobilized-enzyme processes. The chapter studies in detail the effects of fluid pressure on free enzyme behavior in ultrafiltration (UF) systems, and the effects of immobilizing agents in immobilized-enzyme systems. Furthermore, the chapter provides the first documented account in the literature surveyed of a comparison between equivalent free- and immobilized-enzyme systems.

Chapter 2 is a supplement of Chapter 1 and addresses the remainder of Objective #1. The study in this chapter uses the optimum process conditions from Chapter 1 to develop free- and immobilized-enzyme processes that continuously produce GOSs. The chapter details how start-up time and GOS throughput were improved in comparison to reports made in the technical literature. Also, the chapter shows how residual monosaccharides returned to phase #1 from phase #2 (see Figure 1 above) would affect GOS production. Also, the chapter describes which process (free- or immobilized-enzyme) is more advantageous for commercial application.

Chapter 3 directly addresses Objective #2. The study in this chapter first investigates the effects of fluid pressure, temperature, initial concentration and pH during nanofiltration (NF) on retention of various sugars (mono-, di- and oligosaccharides) within model solutions. Using the optimum parameters, a novel NF process was developed to remove non-bioactive sugars from the GOS fraction.

Chapter 4 directly addresses Objective #3. The study in this chapter investigates the influence of flow rate, column temperature and resin size on resolution of saccharide peaks (2-7 DP) using IEC. The study determines optimum conditions and attempts these conditions for GOS separation. Ion moderated partition chromatography (IMPC) was also studied for its enhanced separation between lactose and DP3 peaks. The chapter provides the first account of IEC separation of oligosaccharides in the literature surveyed. Future work and recommendations for this process were also provided.

Chapter 5 directly addresses Objective #4 with a market analysis. GOS process economics were provided in this chapter. Also, prebiotic markets were defined along with their size and growth potential. Potential target markets were also provided and defined. A SWOT analysis was conducted and general conclusions were made on market potential of the GOS products.

CHAPTER 1

THE EFFECT OF FLUID PRESSURE IN FREE-ENZYME ULTRAFILTRATION SYSTEMS AND THE EFFECT OF IMMOBILIZING AGENTS IN IMMOBILIZED-ENZYME SYSTEMS DURING ENZYMATIC GALACTO-OLIGOSACCHARIDE PRODUCTION

1.1 INTRODUCTION

Galacto-oligosaccharides (GOSs) are non-digestible, short-chain polysaccharides (DP3-6) known to promote and sustain the growth of beneficial bacteria, esp. *Bifidobacteria*, within the colon (18). GOSs are considered prebiotics and important functional food ingredients linked to numerous health benefits, including reduced colon cancer risk and enhanced immunity (8). The functional food market is estimated to be $20 billion in the U.S., $15 billion in Europe and $12 billion in Japan, and is growing at an annual rate of 7.5% and is expected to be 10% of all food choices in 10 years (2). The prebiotic market alone is expected to reach $103.2 million in the U.S. by 2010 (5, 19). As a result, GOSs have high value in U.S. and global markets and may be added to a variety of products including breads, fermented dairy foods and beverages (7). Furthermore, research communities in food science, nutrition, medicine and pharmacology desire large quantities of pure GOSs to further study the mechanisms of their benefits (7, 9, 20-25).

Whey lactose surplus has been increasing steadily in recent years due to the increased demand and production of cheese and whey protein concentrate. This surplus is further compounded by the low demand and limited applications for lactose; as a result, the market value for lactose is very low (26-28). In recent years, GOS manufacture from whey lactose has been suggested within the

published literature (10-13); however, commercially feasible processes have yet to be well established.

GOSs could be produced by β-galactosidase treatment of high concentrations of lactose in a transgalactosylation reaction (APPENDIX 1). This transgalactosylation reaction is an intermediary step where galactose units are polymerized to a glucose end unit to form GOSs of varying DP (29). However, as the reaction continues all sugars, including GOSs, will be hydrolyzed to the glucose and galactose monosaccharides. Though β-galactosidase can originate from a variety of microbial sources, *Aspergillus oryzae* has been shown to be the most promising enzyme source for commercial application (12, 13).

In any commercial enzyme process, it is crucial to separate valuable enzyme from the product stream for re-use with fresh substrate. Ultrafiltration (UF) is a process where fluid containing enzyme and product flows at high rate across a membrane surface at a certain fluid pressure. Depending on membrane pore size, enzyme is retained while smaller chemicals (i.e. sugars, GOS) are permeated. Under these conditions, shearing and resultant inactivation may increase with increasing fluid pressure (30, 31). To date, there have been no studies that have investigated the effect of fluid pressure on β-galactosidase. Research in this area may elucidate how other enzymes would perform within UF systems. Foda and Lopez-Leiva (10) applied UF technology (10,000 MW CO) in a continuous free-enzyme system that produced GOSs from whey permeate. However, their conditions were not commercially feasible, and the effect of fluid pressure was not studied.

Immobilizing enzyme on an inert stationary phase (i.e. cotton cloth) is another approach to separate the enzyme from the product stream. In this method, substrate is fed through a reactor containing the stationary material with the immobilized enzyme. This method is highly desirable by industries for a diverse array of enzyme processes; however, enzyme inactivation occurs during the immobilization procedure. Albayrak and Yang (12) performed an elegant study where they immobilized *A. oryzae* β-galactosidase on cotton cloth and studied GOS production from continuous lactose feed. Their study showed little loss of enzyme activity with immobilization by polyethyleneimine (PEI) and subsequent glutaraldehyde (GA) cross-linking. They even showed relatively high GOS yields and productivity rates. Though their immobilized system showed great commercial promise, it has yet to be compared to an equivalent UF free-enzyme system.

The present study was designed in three parts. The first aimed to optimize conditions for a free-enzyme UF system and to determine fluid pressure effects on the free enzyme in a UF free-enzyme system. The second part aimed to design an optimum immobilized-enzyme system using PEI and GA on cotton cloth and to study the effects of PEI and GA on enzyme activity. The third part aimed to compare equal UF free-enzyme and immobilized-enzyme recycle batch systems.

1.2 MATERIALS AND METHODS

1.2.1 Free-Enzyme Studies

Preliminary Free-Enzyme Experiments. The following experiments were designed to estimate optimal GOS production within batch solutions using various initial lactose concentration (43, 133 and 270 g/L), β-galactosidase (EC 3.2.1.23, *A. oryzae*, 9,400 U/g, Sigma, St. Louis, MO) concentrations (0, 4.5, 11.8 and 23.6 g/L) and incubation times (15, 30 and 50 min). All solutions were prepared in 0.1 M sodium acetate buffer, pH 4.5, and incubated at 40°C in a water bath shaker (150RPM). After incubation, samples were analyzed in triplicate for total GOSs (DP3-6) as well as lactose, glucose and galactose using high performance liquid chromatography (HPLC) procedures outlined by Albayrak and Yang (11, 12). In these procedures, solutions containing the sugars were measured using a HPLC system with refractive index detection and with a Rezex RNM™ Carbohydrate anion exchange column (7.8 x 300 mm) and a Rezex RNM™ Carbohydrate guard column (7.8 x 50 mm) (Phenomenex, Torrance, CA). HPLC-grade water was used as mobile phase at 0.4 mL/min flow rate. Column and detector temperatures were maintained at 85 and 45 °C, respectively.

Once optimum lactose and enzyme concentrations were determined, these conditions were fixed within another batch system to study continuous GOS production over time. In this system, a 250 mL solution (n=3) containing the optimum concentrations of lactose and enzyme in 0.1 M acetate buffer (pH 4.5) was incubated at 40°C in a water bath shaker (150 RPM). Sample aliquots

(0.5 mL) were collected at various times from 0 to 120 min. A 0.5-mL aliquot of 0.1 N NaOH was immediately added to sample aliquots; this amount was shown in preliminary studies as well as by other researchers to effectively terminate enzyme activity (12). Samples were analyzed for total GOSs (DP3-6) and other sugars using HPLC procedures.

Fluid Pressure Effect. Enzyme inactivation due to UF fluid pressure was studied in the following experiments. A 3.9-L lactose solution containing 200 g of lactose was made within 0.1 M sodium acetate buffer (pH 4.5) and was circulated through a continuous cross-flow membrane unit (custom-made by APV, Co., Lake Mills, WI) with two tubular polyethersulphone UF membranes in series with 4,000 MWCO and 0.024 m^2 total surface area (ES404, PCI Membrane, Milford, OH). In these experiments, permeate and retentate were recycled to the batch tank. Feed or retentate flow rate was set at 18.9 L/min, an industry standard (31, 32), and temperature was maintained at 40 °C with a water jacket. Separate runs were done at one of the following fluid pressures: 100, 200, 300 and 400 p.s.i. Once all conditions were set, a 100-mL enzyme solution containing 18.0 g of β-galactosidase (EC 3.2.1.23, *A. oryzae*, 9,400 U/g, Sigma, St. Louis, MO) in 0.1 M acetate buffer (pH 4.5) was added at time zero to the 3.9 L of solution within the batch tank of the membrane filtration unit. The resulting mixture gave 4-L of a 5% (w/v) lactose solution containing 4.5 g/L of enzyme or 42.3 U/mL enzyme concentration. (Note: In preliminary tests, Bradford assays showed 100% retention of this enzyme at the various fluid pressures studied (APPENDIX

2)). Samples (0.5 mL) were collected from the batch tank at various times from 0 to 190 min and immediately added to 0.5 mL of 0.1 N NaOH to deactivate any enzyme (11, 12). Three trials were conducted at each pressure.

A 0 p.s.i. control was prepared as a 1 L batch solution, by the following procedure. A 900-mL solution containing 50 g of lactose in 0.1 M acetate buffer (pH 4.5) was maintained at 40°C in a water bath shaker (150 RPM). A 100-mL enzyme solution containing 4.5 g of β-galactosidase (Sigma, 9,400 U/g) in 0.1 M acetate buffer (pH 4.5) was added at time zero. Samples (0.5 mL) were collected at various times from 0 – 190 min and immediately added to 0.5 mL of 0.1 N NaOH.

Lactose concentrations for all samples at each pressure level were determined using HPLC methods. Concentrations were plotted against reaction time. Curves were fit using n^{th}-order models with JMP IN ™ (Cary, NC) software using **Eq. 1.1**, where C is lactose concentration (mM), C_O is initial lactose concentration (mM), k is the rate constant ($mM^{1-n}\ min^{-1}$), n is the order of the reaction, and t is the time (min) (33). C and t were the dependent and independent variables, respectively. Using JMP IN™ software, k, n and C_O parameters were estimated through non-linear regression. In this study, n^{th}-order models were employed because the data did not fit the Michaelis-Menten model.

$$C = \left[(n-1)(kt) + C_O^{\,1-n}\right]^{\frac{1}{1-n}} \qquad \textbf{Eq. 1.1}$$

1.2.2 Immobilized-Enzyme Studies

Immobilization on Cotton Cloth. β-Galactosidase immobilization on cotton terry cloth was done according to procedures outlined by Albayrak and Yang (12). The procedure was done with both β-galactosidase from Sigma (EC 3.2.1.23, *A. oryzae*, 9,400 U/g, Sigma, St. Louis, MO) and the more concentrated and pure Bio-Cat enzyme (EC 3.2.1.23, *A. oryzae*, 100,000 U/g, Bio-Cat, Troy, VA) as outlined below:

1. PEI solution (1 mL of 2.2 mg/mL) was allowed to adsorb to 0.2 g of cloth after 5-10 min or until cloth appeared completely wet.
2. β-galactosidase enzyme (50 mg or 10 mL of 5 mg/mL enzyme solution) was added to the PEI-containing cloth in a flask and shaken at 450 RPM for 5-10 min or until solution turned from cloudy white to clear.
3. The clarified solution was slowly decanted and PEI-enzyme-coated cotton cloth pieces were immersed in a GA solution (0.2% (w/v), pH 7.0) for cross-linking. The solution was kept cool in an ice bath for at least 5 min or until a slight yellow color was observed on the cloth, which indicates cross-linking.
4. The GA solution was carefully decanted and the cross-linked cotton cloth containing the immobilized enzyme was washed extensively with distilled water and then with acetate buffer (0.1 M, pH 4.5).

Active Enzyme Assay. The following procedure describes how active enzyme was determined from immobilized cloth:

1. A piece of cloth (0.2-2g initial wt.) containing the immobilized enzyme was put in 12.5 mL of 0.1 M of sodium acetate buffer (pH 4.5, 40°C) kept in a water bath incubator shaker set at 40°C and 250 RPM.
2. Lactose solution (12.5 mL of 20%) made in 0.1 M sodium acetate buffer (pH 4.5, 40°C) was added at time zero to the flask containing the immobilized enzyme cloth and allowed to incubate for 5 min at 40°C and 250 RPM in the water bath shaker.
3. After 5 min, 0.5 mL of solution was removed, added to 0.5 mL of 0.1 N NaOH, and analyzed for glucose liberated with HPLC methods.
4. Amount of active immobilized enzyme was determined from a standard curve of glucose concentration (mM) after 5 min versus free enzyme concentration (mg/mL).

Immobilization Yield. Immobilization yield or the amount of active enzyme immobilized on the cloth versus initial loaded enzyme amount was adapted from procedures outlined by Albayrak and Yang (12). In this method, free-enzyme solutions made with 0.1 M acetate buffer (pH 4.5) containing various amounts of known free enzyme (0 to 10 mg/mL) were assayed for active enzyme (25 mL, 10% (w/v) initial lactose, 40°C, pH 4.5, 5 min incubation). A standard curve of free enzyme concentration (mg/mL) versus glucose liberated from the assay (mM) was plotted and used to determine the amount of active enzyme immobilized on cotton cloth. Immobilization yield was calculated as a percentage

of active enzyme immobilized on cloth (mg/mL) versus the initial enzyme amount (mg/mL).

To verify whether active enzyme was lost during the immobilization process, fractions from steps 1-4 of the immobilization procedure (i.e. immobilized-enzyme cloth, enzyme solution with residual PEI, GA solution, water wash, acetate wash) were assayed for active enzyme and each compared with a free-enzyme control. The effect of combining the PEI-enzyme solution with the GA solution was also assayed for active enzyme in a separate experiment and compared with a free-enzyme control. This experiment was done with both the Sigma and Bio-Cat enzymes in three separate trials.

Equating Immobilized- and Free-Enzyme Systems. In immobilized enzyme systems, cloth (0 to 2g of initial cloth weight before immobilization, 2.5 cm width), which contained immobilized Bio-Cat enzyme were assayed for active enzyme (25 mL, 10% (w/v) initial lactose, 40°C, pH 4.5, 5-min incubation). Initial cloth weight before immobilization was plotted against glucose concentration. In a separate assay, three solutions containing optimum free enzyme concentration (as determined from preliminary experiments) were assayed (25 mL, 10% (w/v) initial lactose, 40°C, pH 4.5, 5-min incubation), and mean glucose concentration was recorded. The initial cloth weight that gave the closest concentration of glucose to the optimum free enzyme concentration solutions was used for the remaining experiments as the equivalent immobilized-enzyme amount.

Recycle Batch Reactors. Once free- and immobilized-enzymes were equated, compatible UF free-enzyme and immobilized-enzyme recycle batch reactors were constructed. Recycle batch systems return unreacted substrate and products back to the batch tank. Figure 1.1A shows the free-enzyme system where 4 L of 270 g/L of solution with optimum initial lactose concentration (pH 4.5) and free enzyme (Sigma) concentration was recycled in the cross-flow UF membrane system at 100 p.s.i., 40°C, 18.9 L/min feed rate and 97.5 L/m^2 h flux for 90 min.

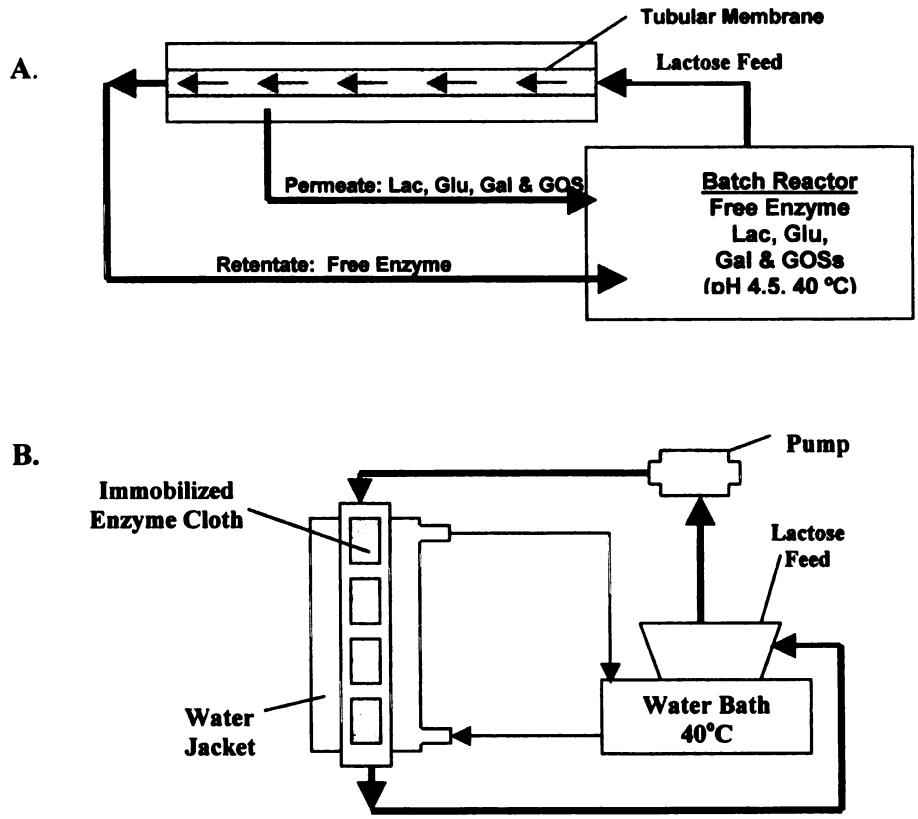

Figure 1.1 - A) A recycled UF membrane free-enzyme system that contains optimum initial β-galactosidase and lactose concentrations at pH 4.5, 40 °C; B) A recycled immobilized-enzyme system, which uses optimum immobilized cloth amount and initial lactose concentration at pH 4.5, 40°C.

Figure 1.1B shows the immobilized system where cotton cloth strips of appropriate initial weight were spiraled within a glass column reactor (9 mm i.d. x 60 cm, 25 mL reactor volume). The immobilized system was also a recycle batch process where 500 mL of 270 g/L of lactose (pH 4.5) was circulated through the reactor at 100 mL/min with a peristaltic pump, and temperature was maintained at 40°C with a water jacket (Figure 1B). Sample aliquots (0.5 mL) for both the free- and immobilized-systems were collected at various times from 0 to 90 min and added to 0.5 mL of 0.1 N NaOH to be analyzed by HPLC procedures. Three trials were conducted.

1.3 Results and Discussion

1.3.1 Free Enzyme Studies

Preliminary Free-Enzyme Experiments. Table 1.1 shows that the largest amount of total GOSs (54.84 g/L) was seen with the 270 g/L lactose solution incubated for 30 minutes with 4.5 g/L or 42.3 U/mL of Sigma enzyme. These results concur with numerous researchers that have also shown that increasing lactose concentrations increases GOS yields (11-13, 16). A 270 g/L or 27% (w/v) lactose solution was used as an upper limit, since higher concentrations would be too viscous for membrane filtration (31). Our results also indicated that extending incubation time did not necessarily increase GOS amount. This result is mainly because GOS formation is an intermediary step of β-galactosidase hydrolysis and is dominant near the beginning of the reaction (29). Additionally, these results demonstrate that higher enzyme concentration does not necessarily

increase GOS yield. This result is primarily true because higher enzyme levels would increase GOS hydrolysis to glucose and galactose (13, 16).

Table 1.1 - Effect of initial lactose concentration, enzyme concentration and incubation time on total GOS (g/L) production.*

Time (min)	0	15			30			50		
Enzyme (g/L) / Lactose (g/L)	0	4.5	11.8	23.6	4.5	11.8	23.6	4.5	11.8	23.6
43	1.37	4.63	15.08	16.03	10.44	10.92	17.58	13.87	11.78	22.84
133	1.51	13.87	13.85	19.13	22.80	16.37	18.05	18.55	30.30	17.83
270	1.45	46.56	51.83	43.83	54.84	50.17	42.08	43.38	29.62	28.28

*GOS values represent the means of triplicate sets, coefficient of variance percents ranged from 0.052 to 0.89%

Figure 1.2 shows the progress curves of the free-enzyme batch system containing 42.3 U/mL or 4.5 g/L of enzyme and 270 g/L of initial lactose. The "total GOS" curve represents DP3-6 oligomers and shows an increase to ~20% of total sugars within 30 min (See APPENDIX 3 for Chromatogram). This corresponds to the decline in lactose from 100 to 50% within 30 min. After 30 min, lactose and total GOSs begin a slow decline while glucose and galactose begin increasing steadily. Similar patterns were discovered by other investigators (11-13). These results show that GOS formation precedes lactose hydrolysis as the dominant reaction from 0 to 30 min. However, after 30 min (or 50% lactose conversion) hydrolysis to glucose and galactose becomes the predominant reaction. Furthermore, various researchers have noted that GOS production is inhibited by the increasing levels of glucose and galactose (11-13,

16, 17). This inhibition would further explain the decline in GOS production seen in this study after 30 min.

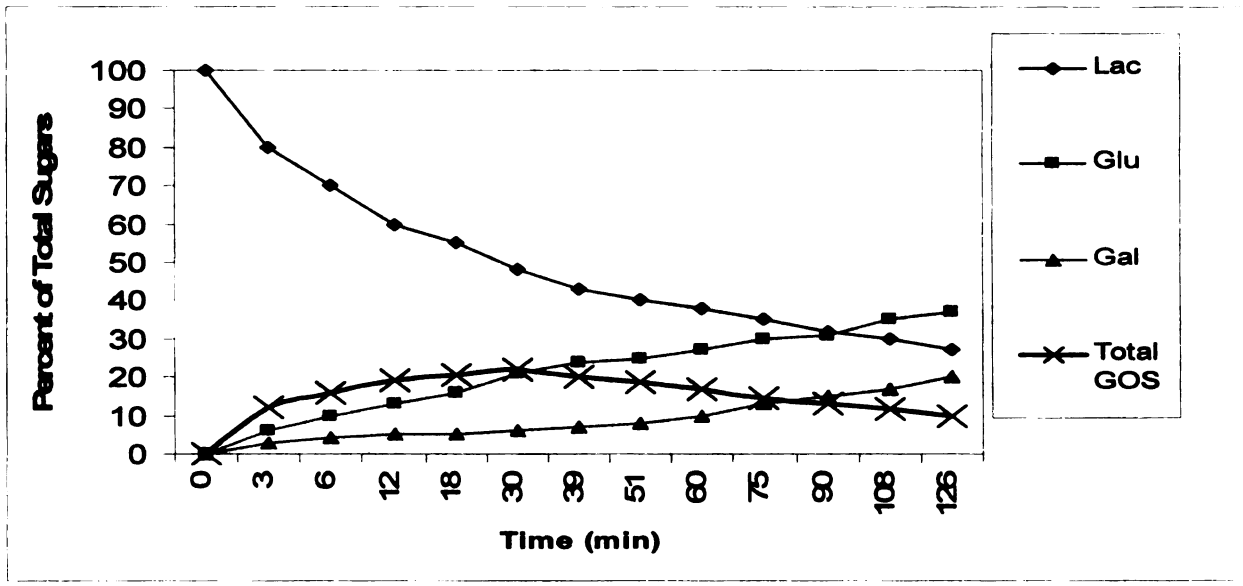

Figure 1.2 - Mean percent of total sugars for lactose, glucose, galactose and total GOS over time within a batch solution of 27% (w/v) initial lactose and 42.3 U/mL of enzyme (250 mL, pH 4.5) incubated at 40°C and 150 RPM (CV% range of 2.4 to 5.2%).

Fluid Pressure Effect. Plots of lactose concentration over time at each fluid pressure showed near equal curvature (APPENDIX 4). To determine any statistical difference, rate constants, *k*, were determined by n^{th} order modeling using **Eq. 1**. A typical Michaelis-Menten model was not used since β-galactosidase even with 5% initial lactose concentration would cause transgalactosylation intermediate reactions. Michaelis-Menten kinetics would only be valid if hydrolysis was the only reaction. Because both lactose hydrolysis and polymerization are occurring simultaneously, the Michaelis-Menten model was not appropriate (33). Thus, n^{th} order modeling, often used for multiple-

substrate reactions, provided a simple way to evaluate rate constant, *k*, at each pressure level using lactose concentration over a fixed period of time (0-190 min).

Table 1.2 shows *k*, *n* and C_o parameter estimates with their standard errors. The mean reaction order, *n*, was estimated to be 1.81 for 100 to 400 p.s.i. fluid pressures. This value represented an overall reaction order that accounts for the multiple hydrolysis and polymerization reactions occurring simultaneously during enzyme reaction (33). Since *n* values were not significantly different for pressures of 100-400 p.s.i. (P=0.6), *k*'s for each of these pressure levels could be statistically compared. Using analysis of variance (ANOVA) ($\alpha = 0.05$, df = 12), no significant difference was found among *k*'s for 100, 200, 300 and 400 p.s.i. (P=0.6). These results indicate that, unexpectedly, pressure from 100 to 400 p.s.i. did not have an effect on enzyme performance. These results indicate that shearing differences are minimal within UF systems possibly because flow at high speeds (18.9 L/min) may create almost equal enzyme shearing regardless of pressure (34). Furthermore, only a small percentage of enzymes actually occupy the layer near the membrane surface while the majority of enzymes remain in the bulk solution (34).

Table 1.2 - Parameter estimates of lactose hydrolysis rate constant (*k*), reaction order (*n*) and initial lactose concentration (*Co*) using nth order modeling (5% (w/v) initial lactose, 42.3 U/mL enzyme, pH 4.5, 40°C).

Pressure (p.s.i.)	k ($mM^{1-n}min^{-1}$)	k std error ($mM^{1-n}min^{-1}$)	n	n std. error	Co (mM)	Co std. error (mM)
0 (batch control)	0.00164	0.000379	1.81*		114*	
100	0.00415	0.00560	1.79	0.056	119	1.81
200	0.00418	0.00144	1.80	0.0916	117	2.65
300	0.00397	0.00136	1.81	0.0909	105	13.9
400	0.00408	0.00155	1.83	0.0892	116	6.10

* Denotes average value determined from pressure levels 100, 200, 300 and 400 p.s.i.

The $k_{0p.s.i.}$ (0.00157 mM^{1-n} min^{-1}) was much lower than *k* values at the other pressure levels. However, an appropriate comparison between $k_{0p.s.i.}$ and the other *k* values could not be made since the batch control $n_{0p.s.i}$ (2.06) was significantly greater than the *n* values for the other pressure levels ($n_{avg.}$ = 1.81). To appropriately compare $k_{0p.s.i.}$ with *k* values at the other pressure levels, $n_{0p.s.i}$ and $C_{o0p.s.i}$ parameters were held constant at the mean *n* and C_o of the other pressure levels during nth order modeling with JMP IN™ software (Table 1.2) (33). With this adjustment, the $k_{0p.s.i.}$ increased only slightly and was significantly less than *k*'s at the other pressure levels ($P<0.001$). One explanation for this difference may be the enhanced agitation in the UF system compared to the control batch solution stirred at 150 RPM. A higher degree of agitation inputs more energy and convective flow into the enzyme reaction system, thereby increasing particle dispersion and enzyme and sugar solubility, especially at the beginning of the reaction (34). This agitation, in turn, would

increase how quickly the reaction progresses. Another explanation may be a direct result of pressure. Some researchers have suggested that pressure can directly affect enzyme activity by favorably or unfavorably altering the shape of the enzyme active site (35). This altered shape could also explain the difference in rate constants seen in our experiments.

1.3.2 Immobilized-Enzyme Studies

Immobilization Yield. Surprisingly, immobilization yield was <0.2% with Sigma enzyme (<0.01 mg/mL of active enzyme versus 5 mg/mL of initial enzyme). The purer and more concentrated Bio-Cat enzyme showed a slightly greater immobilization yield at ~8% (0.39 mg/mL of active enzyme versus 5 mg/mL of initial enzyme). These values were much lower than the 90-95% immobilization yields reported by Albayrak and Yang (12).

To determine where enzyme was lost or inactivated, various fractions collected during the immobilization process were assayed for active enzyme versus a free-enzyme control (Table 1.3). The cloth immobilized with Sigma enzyme showed little to no detectable active enzyme, while the enzyme solution with residual PEI contained ~86% of the active enzyme. GA solution (9%) and water wash (2%) showed lower but significant active enzyme amounts. Each percentage when added totaled to near 100% activity.

These results suggest that Sigma enzyme did not adhere to the PEI-cloth during the first step of the immobilization process. This lack of initial adsorption was observed during the immobilization experiments when the enzyme solution

failed to turn from cloudy white to clear. Since initial adsorption of enzyme (negatively charged for β-galactosidase and most enzymes) to PEI (positively charged) depends on electrostatic attraction, our results suggest that the less pure Sigma enzyme may contain other compounds that prevent initial adsorption. The manufacturer disclosed that the enzyme contained significant amounts of dextrin (neutral charge), which may sterically interfere with electrostatic attraction of enzyme to the PEI treated cloth.

Table 1.3 - Relative enzyme activities of various fractions during immobilization of two enzyme purities on to cotton cloth with PEI and GA.

Enzyme Type	Fraction	Relative Activity*
Less Pure Enzyme (Sigma) (9,400 U/g)	Cloth	<0.2%
	PEI-Enzyme solution	86%
	GA solution	9%
	Water wash	2%
	Acetate buffer wash	ND
	Combined PEI-Enzyme and GA solutions	33%
More Pure Enzyme (Bio-Cat) (100,000 U/g)	Cloth	8%
	PEI-Enzyme solution	<0.1%
	GA solution	ND
	Water wash	ND
	Acetate buffer wash	ND
	Combined PEI-Enzyme and GA solutions	ND

*Relative activity is based on fraction enzyme activity vs. free-enzyme control.

Since nearly 100% of active enzyme was recovered in all fractions, the enzyme was not inactivated by either PEI or GA solutions individually. However,

when the fraction of enzyme solution with residual PEI was combined with GA solution, active enzyme was severely reduced from 86% to 33% (Table 1.3). This suggests that the PEI-enzyme-GA complex inactivates enzyme by greater than 50%. There may be a two-step mechanism that explains this. In the first step, the highly branched and highly charged PEI electrostatically aggregates a large amount of enzyme. In the second step, GA reacts with various enzyme functional groups (i.e. amino, sulfhydryl, etc.) to alter enzyme shape, or block active sites.

Unlike Sigma enzyme, active Bio-Cat enzyme was mostly found on the cloth fraction (~8%) while other fractions contained little or no active enzyme (Table 1.3). These results suggest that more concentrated enzymes have less interfering components and would thus almost entirely adsorb during the first step of immobilization. However, ~90% inactivation of enzyme was seen once the enzyme had been immobilized on cloth. This result confirms that inactivation occurs within the PEI-enzyme-GA complex. However, the degree of inactivation at the cloth surface is greater than within a solution containing PEI, GA and enzyme. These results indicate that since large amounts of enzyme aggregate onto the PEI-cloth layer, the subsequent GA and enzyme functional group reactions may be more extensive due to the higher localization of enzyme at the cloth surface.

In general, we showed that inactivation due to PEI and GA is extensive (~50% to ~90%). Our results disagree with Albayrak and Yang (12), who showed no apparent inactivation of free-enzyme solutions that contained 1 mL of

PEI solution and 1 mL of GA solution. However, repeating their experiment with both the Sigma and Bio-Cat β-galactosidase enzymes still showed very strong inactivation (69 ± 3% inactivation). Mateo et al. (36) found that GA cross-linking showed activity losses ranging from 51 to 100% for various enzymes (oxynitrilase, nitrilase, aldehydrogenase, *Penicillin* G acylase). These results concur with our analyses and suggest that, in general, valuable active enzyme is severely lost during immobilization with PEI and GA.

Equating Immobilized- and Free-Enzyme Systems. Figure 1.3 shows a linear plot of initial cloth weight used for immobilization of Bio-Cat enzyme versus glucose liberated from the enzyme activity assay with R^2 of 0.97 when cloth weights of 0.2, 0.5, 1.0, 1.5 and 2.0 g were assayed in triplicate. The mean glucose amount (108.8 ± 2.40 mM, n=3) liberated from active enzyme assays of 42.3 U/mL or 4.5 g/L Sigma free-enzyme solution was then inserted in the plot's equation to determine approximate cloth weight needed. Using the equation, it was found that ~2 g of initial cloth weight would be needed. This finding was confirmed by assaying for active immobilized enzyme on four cloths of 0.5 g initial weight before immobilization. Their mean glucose concentration (106.4 ± 3.4 mM, n=3) was not significantly different from that of 42.3 U/mL or 4.5g/L of free Sigma enzyme (108.8 ± 2.40 mM glucose, n=3) (P=0.5). As a result, this amount of cloth was used for immobilized-enzyme recycle batch studies.

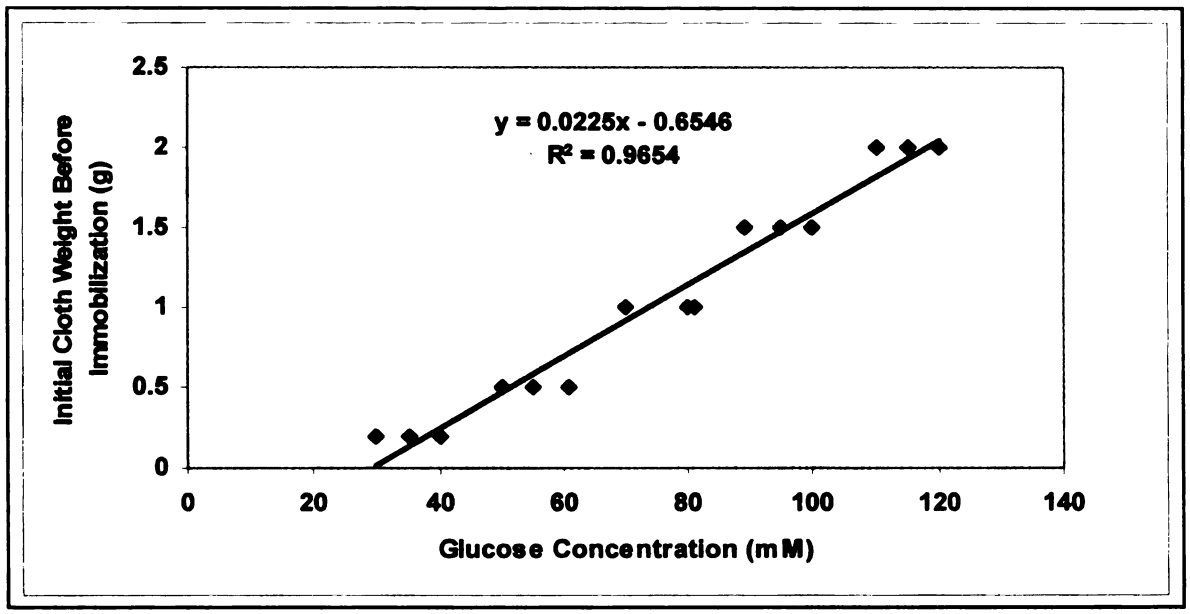

Figure 1.3 - Standard curve of cloth weight before enzyme immobilization (g) versus glucose concentration (mM) liberated during active enzyme assays.

Recycled Batch Reactors. Figures 1.4 and 1.5 show rapid decrease of lactose concentration until about 50% conversion for both free- and immobilized-enzyme recycled batch reactors. This rapid decrease in lactose corresponded to the rapid increase of total GOS (DP3-6) formation, which reached maximum levels at 50% lactose conversion. The flattening and gradual decline of the lactose and the GOS curves after 50% lactose conversion corresponded with the steady increase of glucose and galactose, which both have been shown by researchers to inhibit transgalactosylation (11, 12). These results confirm that GOS formation truly is an intermediary stage to the overall hydrolysis reaction to monosaccharide units during β-galactosidase treatments of highly concentrated lactose solutions.

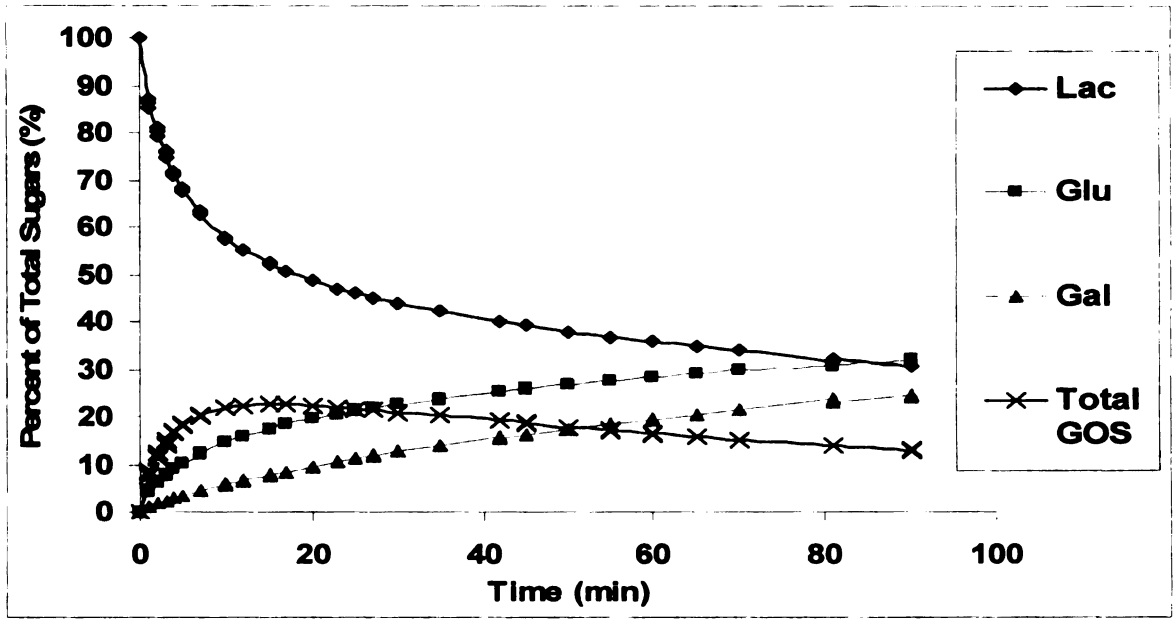

Figure 1.4 - Mean percent of total sugars for lactose, glucose, galactose and total GOS over time during a recycled free-enzyme UF reactor (CV% range of 0.097 to 2.86%).

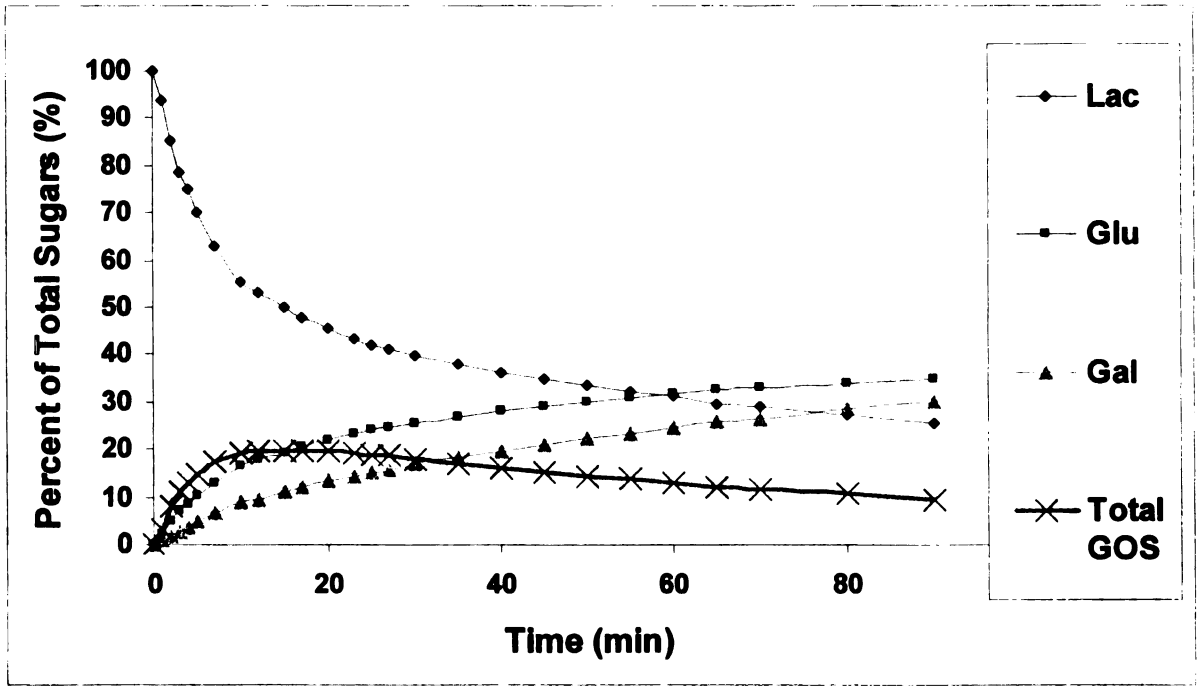

Figure 1.5 - Mean percent of total sugars for lactose, glucose, galactose and total GOS over time within a recycled immobilized-enzyme reactor (CV% range of 1.7 to 4.9%).

Total GOSs reached ~22% maximum yield for the free-enzyme system (Figure 1.4), and ~20% maximum yield with the immobilized-enzyme recycle batch reactor (Figure 1.5). The free-enzyme UF system may have produced slightly better yields because of higher feed and flux rates that may have enhanced agitation and dissolving of enzyme and sugars compared to the less agitated immobilized-enzyme system. Both systems achieved their maximum GOS yields within 15-17 min. Interestingly, preliminary batch results showed maximum GOS yield within twice as much time (30 min) (See Table 1.1 and Figure 1.2). As mentioned previously, this enhanced agitation may have increased the dispersion and dissolution of enzymes and sugars early in the reaction, thus 50% lactose conversion is approached faster. This result may especially be important within highly concentrated solutions (i.e. 27% (w/v) sugars), where compounds are less soluble unless energy (i.e. in the form of agitation) disperses particles thus enhancing dissolving capabilities (37, 38).

The general trends of all sugars (lactose, total GOS, glucose and galactose) were very similar to previous reports (10-12). Foda and Lopez-Leiva (10) found similar maximum GOS yields (~22%) within a *K. lactis* β-galactosidase free-enzyme UF system using whey permeate solution containing 23% (w/v) initial lactose. However, their maximum yield occurred after 4.5 hours of incubation. Albayrak and Yang (12) found in their immobilized cotton cloth *A. oryzae* β-galactosidase studies that they could attain a maximum of ~26% (w/v) total GOSs in 30 min using a very high initial lactose concentration (40% (w/v)). The present study showed that GOS yields similar to those reported in literature

could be achieved within a much shorter start-up time (15-17 min) using only 27% (w/v) of initial lactose. Though this 15-17-min start-up time is feasible for commercial use, more work is needed to construct systems that continuously collect product rather than a recycle batch system that would cause a steady decline of valuable product after 15-17 min, or 50% conversion. However, this study provides the framework for the development and comparison of future continuous free- and immobilized-enzyme systems.

1.4 Conclusions

The present study showed that fluid pressure effects on enzyme are negligible in free-enzyme UF systems. However, high fluid pressures and high agitation may enhance enzyme performance within free-enzyme UF systems compared to batch solutions. In immobilization procedures with PEI and GA, a highly concentrated and pure enzyme form (i.e. 100,000 U/mg) was required for adequate immobilization. Also, the combination of PEI and GA immobilizing agents causes severe enzyme inactivation possibly due to active site blockage or alteration during cross-linking. Compatible free- and immobilized-enzyme recycle batch systems showed relatively high maximum GOS yields (22% and 20%, respectively) within a relatively short time; however, future work is needed to develop continuous systems.

1.4.1 Novel Contributions

This study has five worthy contributions to science and technology:

1. A better understanding of the importance of agitation and high fluid pressures in UF systems in enhancing ß-galactosidase behavior.
2. A better understanding of the PEI and GA effects on enzyme activity.
3. Development of new systems that produce high GOS yields in shorter times than previously reported (2- to 20-fold shorter time).
4. The first true comparison of equivalent free- and immobilized-enzyme systems to produce GOSs.
5. Provides the groundwork for further research using continuous systems.

CHAPTER 2

COMPARISON OF BATCH AND CONTINUOUS FREE-ENZYME AND IMMOBILIZED-ENZYME SYSTEMS FOR GALACTO-OLIGOSACCHARIDE PRODUCTION FROM WHEY LACTOSE

2.1 INTRODUCTION

Galacto-oligosaccharides (GOSs) are non-digestible, short-chain polysaccharides (DP3-6) associated with a number of health benefits, including reduced colon cancer risk and enhanced immunity (18). GOSs along with monosaccharides can be produced by β-galactosidase treatment of lactose. Though β-galactosidase may originate from various microbial sources, *Aspergillus oryzae* has been shown to be the most promising enzyme source due to its high productivity (11-13, 39). Separating this valuable enzyme from the product stream is imperative for reuse of the enzyme and for commercial applicability. This can be accomplished with either ultrafiltration (UF) free-enzyme or immobilized-enzyme systems.

One type of UF commonly used in commercial practice is a cross-flow system, where substrate and free enzyme held in a feed tank is pumped through a hollow membrane tube with pore size of 1,000 to 20,000 molecular weight cut-off (MWCO) at high feed rates and pressures. The retentate stream (containing the enzyme) is sent back to the tank and continually recirculated through the membrane. The permeate (containing smaller compounds, such as sugars) can either be sent back to the tank for further reaction, as in a recycle batch system, or collected, as in a continuous system. A number of researchers have investigated GOS production within UF free-enzyme systems, and GOS yields

have been reported between 10 and 55% of total sugars. However, start-up time, or the time to reach maximum GOS amount, is very high, typically within the range of 0.5 to 5 h (10, 12, 13, 40, 41). These conditions are not practical for commercial systems that must run for a short time (i.e. 8-h day), thus further work is needed to reduce start-up time.

Enzyme immobilization on a solid support matrix (i.e. cotton cloth, chitosan, etc.) is another method that effectively separates product from enzyme. In this process, substrate is fed through a reactor containing the immobilized enzyme support matrix. The effluent can be recirculated back through the reactor in a recycle batch system. In a continuous system, the effluent is collected at a flow rate that would achieve optimum residence time of substrate within the reactor. Immobilized systems are attractive because they can easily be made continuous with minimal start-up time (39). Also, they can be stable for days or months at a time (12). Research groups have immobilized β-galactosidase on cotton cloth, chitosan and membrane surfaces with some success and have reported GOS yields of ~25% of total sugars. However, many of these immobilized-enzyme systems showed very low product throughput (10-15 g GOS/h) (11, 12, 39, 40), severely limiting the cost-effectiveness of these systems in a commercial setting. Furthermore, immobilization typically requires large quantities of expensive pure and concentrated enzymes, since immobilizing agents can severely inactivate enzymes (50 to 90% inactivation) (36, 42). This extensive inactivation is another significant cost barrier for processors.

GOS production by β-galactosidase is a kinetically controlled reaction (40, 43). Therefore, higher substrate (lactose) concentrations would lead to greater GOS formation, which has been shown by a number of researchers to increase GOS yield considerably (10-13, 17, 29, 40, 43). Enzyme concentration also has an effect on GOS formation. Research groups have shown that increasing enzyme concentration decreases the GOS yield but may significantly improve start-up time (40). Lower enzyme concentrations may increase yield, but start-up time is significantly extended (40). Thus, an optimal balance is required to achieve high GOS yield and short start-up time.

Several researchers have investigated continuous free- and immobilized systems. However, our research group was unable to find a study in the published literature that developed equivalent UF free-enzyme and immobilized-enzyme systems for comparison. Such a study would enable an unbiased comparison to show the advantages or disadvantages of each system.

GOS formation occurs in a transgalactosylation reaction with a minimum of three steps as shown below (29):

1. LACTOSE + ENZYME → LACTOSE-ENZYME
2. LACTOSE-ENZYME → GALACTOSYL-ENZYME + GLUCOSE
3. GALACTOSYL-ENZYME + ACCEPTOR → GALACTOSYL-ACCEPTOR + ENZYME

In the first step, lactose attaches to the β-galactosidase active site at the galactose end. Then, glucose is hydrolyzed leaving the galactosyl-enzyme complex. This complex will then react with an acceptor. If the acceptor is water, as in dilute solutions, then galactose is formed. If the acceptor is a sugar (i.e. lactose or GOS), as in more concentrated solutions, then GOSs are formed.

This transgalactosylation reaction is an intermediate step of lactose hydrolysis. Thus, as the enzyme reaction continues, more of the monosaccharides are formed (29).

Galactose and glucose significantly inhibit the transgalactosylation reaction as their concentration increases within the latter stages of β-galactosidase hydrolysis (9-13, 29, 43, 44). Galactose competitively inhibits GOS formation, while glucose non-competitively inhibits GOS formation (11, 12, 29, 40, 44). Albayrak and Yang (11) showed significant inhibition by galactose and glucose concentrations at >3% (w/w) and >7% (w/w), respectively (11). To optimize enzyme kinetics and GOS production, removal of these monosaccharides from the reactor is important. Furthermore, from a thermodynamic standpoint, GOS removal is also important, since continual removal of product with simultaneous continual feed of substrate would drive transgalactosylation forward to continuously produce maximum GOSs (12, 43).

The present study was designed with three objectives. The first was to compare GOS production in equivalent free- and immobilized-enzyme recycle-batch reactors. The second was to compare GOS production in equivalent free- and immobilized-enzyme continuous reactors. The third was to determine residual monosaccharide effect on GOS production in both free- and immobilized-enzyme continuous reactors.

2.2 MATERIALS AND METHODS

2.2.1 Free and Immobilized Enzymes

Free Enzyme. Optimum conditions for free enzyme systems were determined to be 270 g/L (or 27% (w/v)) of lactose solution and 4.5 g/L or 42.3 U/mL of β-galactosidase (EC 3.2.1.23, *A. oryzae*, 9,400 U/g, Sigma Co., St. Louis, MO), and 30 min incubation at 40°C and pH 4.5. These conditions were determined in preliminary batch studies where various lactose concentrations (43, 133 and 270 g/L), enzyme concentrations (0, 4.5, 11.8 and 23.6 g/L) and incubation times (0, 15, 30 and 50 min) were analyzed for optimum GOS production. Lactose concentration of 27% (w/v) was used as an upper limit, since higher concentrations would be too viscous for membrane filtration (31, 32). Sugar analysis was done using HPLC procedures outlined by Albayrak and Yang (12). In these procedures, solutions containing the sugars were measured using an HPLC system with refractive index detection and with a Rezex RNM™ Carbohydrate anion exchange column (7.8 x 300 mm) and a Rezex RNM™ Carbohydrate guard column (7.8 x 50 mm) (Phenomenex, Torrance, CA). HPLC-grade water was used as mobile phase at 0.4 mL/min flow rate. Column and detector temperatures were maintained at 85 and 45 °C, respectively. All sugar standards were purchased from Sigma.

Immobilized-Enzyme. β-Galactosidase (EC 3.2.1.23, *A. oryzae*, 100,000 U/g, Bio-Cat Co., Troy, VA) was immobilized on cotton terry cloth (2.5 cm width) according to procedures outlined by Albayrak and Yang (12). Immobilized-

enzyme amount was equated to 42.3 U/mL of free enzyme by the following procedure. First, amount of active enzyme on the cloth was determined by assaying various cloth weights (0 to 2 g wt. before immobilization) in 25 mL of 10% (w/v) lactose in 0.1 M acetate buffer (pH 4.5) incubated at 40°C in a water bath shaker (450 RPM, 5 min) (n=3). Weight of cloth before immobilization was plotted versus glucose concentration from the enzyme assay. The mean glucose concentration from the free enzyme assay was then inserted in the plot's equation to approximate the weight of cloth needed to equate to 42.3 U/mL of free enzyme.

2.2.2 Recycle-Batch Reactors

Free Enzyme. The following experiment was designed to determine GOS production in a recycle-batch UF membrane unit (Figure 1.1A). A 3.9-L lactose solution containing 1080 g of lactose was made within 0.1 M sodium acetate buffer (pH 4.5, 40°C) and was circulated through a continuous cross-flow APV membrane unit with a centrifugal pump and two tubular polyethersulphone UF membranes in series with 4000 MWCO and 0.024 m^2 total surface area (ES404, PCI Membrane Co., Milford, OH). In these experiments, permeate and retentate were recycled to the batch tank. Feed or retentate flow rate was set at 18.9 L/min, an industry standard (31, 32). The fluid pressure was set between 100-150 psi. Flux was maintained at 97.5 L/m^2 h, and temperature was kept at 40 °C with a water jacket. Once all conditions were set, a 100-mL enzyme solution containing 18.0 g of β-galactosidase (EC 3.2.1.23; 9,400 U/g) in 0.1 M acetate

buffer (pH 4.5) was added at time zero to the 3.9 L of solution within the batch tank of the membrane filtration unit. The resulting mixture gave 4-L of a 27% (w/v) lactose solution containing 4.5 g/L or 42.3 U/mL of enzyme. Samples (0.5 mL) were collected from the batch tank at various times from 0 to 90 min and immediately added to 0.5 mL of 0.1 N NaOH to deactivate any enzymatic activity (12). Three replicate runs were completed. Samples were analyzed for the various sugars using HPLC methods.

Immobilized-Enzyme. A column reactor was prepared by spiraling immobilized-enzyme cotton strips (2.5 cm width) that equate to the optimum weight determined from the method above within a glass column reactor (25 mL volume; 9mm i.d. x 60cm) (Figure 1B). In a separate container, 27% (w/v) lactose in 0.1 M acetate buffer (pH 4.5) was kept at 40°C in a water bath and periodically stirred. The water from the water bath was also circulated through the column reactor's water jacket to maintain sample temperature at 40°C (Figure 1.1B). The lactose solution was pumped through the immobilized-enzyme reactor at 90-100 mL/min and recycled back to the batch container using a peristaltic pump. Samples (0.5 mL) were collected at various times (0 to 90 min) and 0.5 mL of 0.1 N NaOH was immediately added to samples to deactivate any possible enzymatic activity. Sugars in these samples were then tested using HPLC procedures. Three separate trials were done.

2.2.3 Continuous Enzyme Systems

Free Enzyme. Lactose solution (27% (w/v)) containing 42.3 U/mL of free enzyme was circulated through the UF membrane filtration unit as was done with the free-enzyme recycle-batch reactor. However, instead of permeate being recycled back to the batch tank; it was continuously collected while fresh 27% (w/v) lactose was continuously fed (Figure 2.1). Since permeate flow rate was 38 mL/min (97.5 L/m²h flux), the fresh lactose solution was fed into the batch tank at the same flow rate using a peristaltic pump (Figure 2.1). Permeate samples (0.5 mL) were collected at various times from 0 to 120 min and immediately added to 0.5 mL of 0.1 N NaOH to deactivate enzyme. Sugars in these samples were then tested using HPLC procedures. Three trials were done.

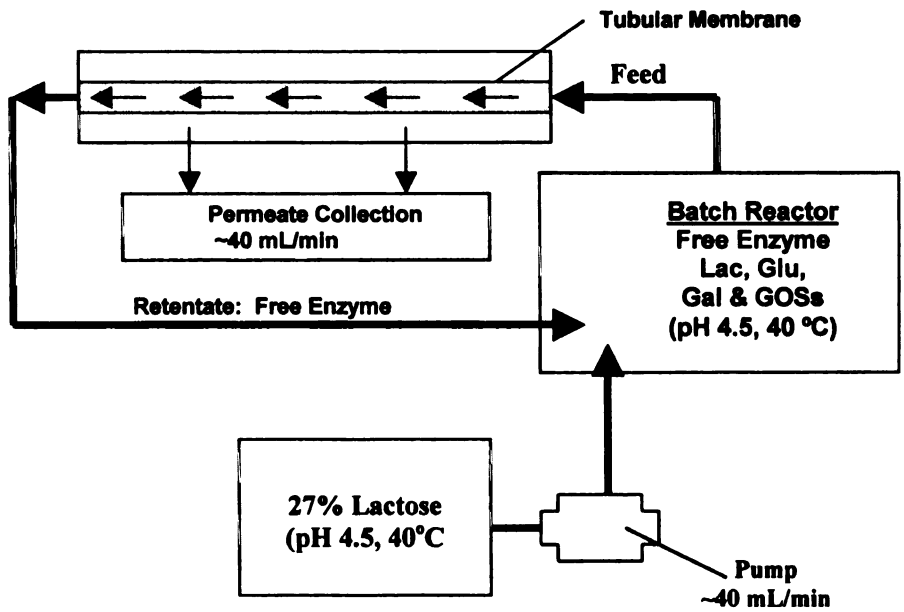

Figure 2.1 - Continuous free-enzyme system using UF membrane filtration to separate sugars from enzyme with 27% (w/v) lactose feed at pH 40°C and pH 4.5.

Immobilized Enzyme. An immobilized-enzyme reactor (25 mL volume) was prepared similarly to the recycle-batch reactor described in Figure 1.1B; however, rather than effluent being recycled back to the reactor, it was passed once through the reactor and collected. Optimal lactose feed rate for the single-pass immobilized-enzyme reactor was first determined to allow for sufficient residence time for optimum GOS production. The optimum was determined by feeding 27% (w/v) lactose (pH 4.5) through the reactor at various flow rates from 0.7 to 11 mL/min using a peristaltic pump. At each flow rate, six to ten 0.5-mL samples of effluent were collected and immediately added to 0.5 mL of 0.1 N NaOH. Samples were analyzed for lactose and total GOSs using HPLC methods, and values were plotted versus flow rate. Lactose feed (27% w/v) was continually fed at the determined optimum flow rate by a peristaltic pump (Figure 2.2). Aliquots (0.5 mL) of effluent were collected at various times (0 min to 148 min) and 0.5 mL of 0.1 N NaOH was immediately added to deactivate any possible enzymatic reaction. Sugar concentrations were measured using HPLC procedures. Three separate trials were done.

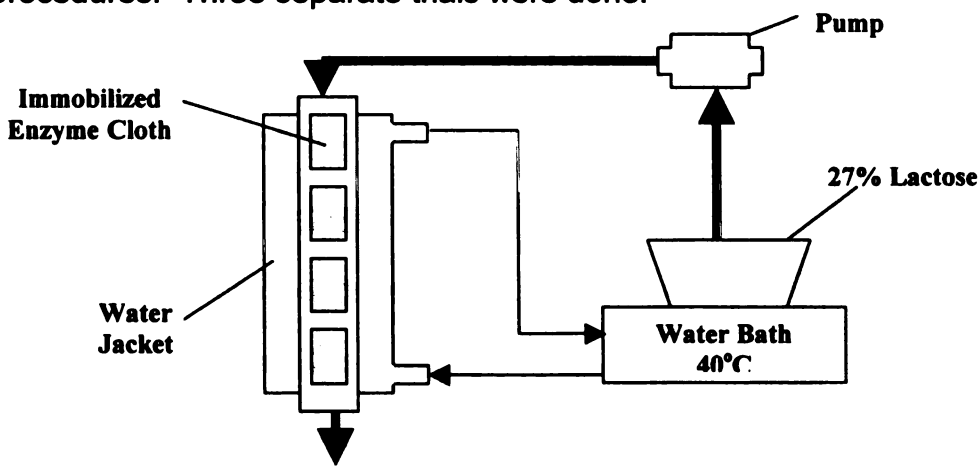

Figure 2.2 - Single-pass immobilized enzyme system with continuous 27% (w/v) lactose feed at 40°C, pH 4.5 and optimum flow rate.

2.2.4 Monosaccharide Effect

Recently, researchers have shown 80% removal of monosaccharides from disaccharides and GOSs using nanofiltration (NF) (45-47). This is an important finding, since unreacted lactose containing only residual monosaccharide levels can be recycled as feed. However, there has been no report of the literature surveyed on the inhibitory effect on GOS formation due to residual monosaccharide levels. To study any inhibitory effect on GOS formation due to residual monosaccharide levels, continuous free-enzyme membrane filtration and single-pass immobilized-enzyme experiments were repeated with a feed solution containing 27% (w/v) lactose, 0.87% (w/v) glucose and 0.43% (w/v) galactose. The concentrations of glucose and galactose were determined by calculating 80% removal of the mean glucose and galactose amounts in free-enzyme solutions at optimum GOS level or 50% lactose conversion (See calculation below). In this experiment, GOS production over time was plotted and compared with experiments where the feed did not contain any monosaccharides. Three trials were done.

Glucose and galactose concentrations were determined by the following sample calculations:

Glucose Calculation

Mean Glucose Concentration at maximum GOS level: 0.2402 mol/L

Initial Volume: 4L

Glucose Molecular Weight: 180.2 g/mol

0.2402 mol/L x 4 L x 180.2 g/mol = 173 g of glucose

After 80 % removal:

173.13 g x (1 – 0.8) = 34.6 g of glucose

Percent Glucose in 4 L solution

(34.6 g / 4000 mL) x 100 = 0.87% (w/v)

Galactose Calculation:

0.12036 mol/L x 4 L x 180.2 g/mol = 86.76 g

86.758 g x (1 – 0.8) = 17.35 g

(17.35 g of galactose / 4000 mL) x 100 = 0.43% (w/v)

2.3 Results and Discussion

2.3.1 Free and Immobilized Enzymes

A plot of cloth weight before immobilization versus glucose liberated from the active enzyme assay showed a linear relationship with R^2 of 0.96 when cloth weights of 0.2, 0.5, 1.0, 1.5 and 2.0 g were assayed in triplicate (Figure 1.3). The mean glucose amount liberated from activity assays of 42.3 U/mL of free-enzyme (108.8 ± 2.40 mM of glucose, n=3) was substituted into the plot's equation to determine approximate cloth weight needed. Using the equation, ~2 g of cloth would be needed to equate to 42.3 U/mL of free enzyme. This finding was confirmed by assaying four 0.5-g cloths immobilized and comparing their mean glucose concentration (106.4 ± 3.4 mM, n=3) with that of the 42.3 U/mL free enzyme (108.8 ± 2.40 mM glucose, n=3). For all remaining immobilized-enzyme

reactor experiments, four 0.5-g cloths immobilized with Bio-Cat β-galactosidase were used.

2.3.2 Recycle-Batch Reactors

The general trends of all sugars (lactose, total GOSs, glucose and galactose) shown in Figures 1.4 and 1.5 were very similar to previous studies. They are characteristic of the β-galactosidase reaction occurring, where transgalactosylation is dominant early in the reaction, but as glucose and galactose concentrations increase to certain levels, GOS formation is reduced. As a result, hydrolysis of the GOS and lactose becomes the dominant reaction after 50% lactose conversion. Total GOS reached ~22% maximum yield at 15 min or at ~50% lactose conversion for the free-enzyme system (Figure 1.4). The immobilized-enzyme recycle batch reactor showed slightly less GOS yield (~20%) with slightly longer start-up time (17 min) (Figure 1.5). The free-enzyme system may have given slightly better GOS yields and start-up times because of higher flow and flux rates, which may have enhanced particle dispersion and dissolving of enzyme and substrate. This is an important factor to consider with high sugar concentration (27%) solutions that can easily precipitate solutes (37, 38, 48). Interestingly, both recycle batch systems had much better start-up times than the preliminary batch studies that showed maximum GOS yield at 30 minutes when solutions were stirred at only 150 RPM. This result provides further evidence that agitation is an important factor for start-up time, since the agitation may effectively increase enzyme or substrate concentration in solution.

Table 2.1 compares batch system results of this study with other studies. To accommodate for differences in enzyme origin, enzyme concentration (U/mL) was provided as a basis of comparison. The results indicate that enzyme concentration had the greatest effect on start-up time rather than yield. In general, increases in enzyme concentration (1.5 to 42.3 U/mL) showed significant improvement in start-up time (5 to 0.25 h), where our enzyme concentration (42.3 U/mL) showed the best start-up time. Interestingly, our start-up time (0.25 h) was ~2-fold better than that reported by Albayrak and Yang (12) despite their higher enzyme concentration (10,000 U/mL) for the batch solution. Their findings may be a result of high sugar concentrations (40% lactose) and less agitation in their system, which may have caused precipitation of enzyme or substrate out of solution (38). Though our recycle-batch systems showed a better start-up time than what has been reported in the literature, batch systems, in general, are not ideal for commercial use. The main reason is the steady decline of GOSs after 17 min; therefore, maximum GOS cannot be continuously collected.

Table 2.1 - Comparison of various batch, continuous free-enzyme and immobilized-enzyme systems.

Process	Enzyme Source	Enzyme Conc. (U/mL)	Initial Lactose Conc. (g/L)	GOS Yield (%)	Start-up Time (h)	Total GOS Throughput (g/h)	Reference
Batch Systems	*T. maritima*	1.5	500	18	5	***	(41)
	K.lactis	2	230	22	4.5	***	(10)
	K.lactis	~6	280	22	1.5-2	***	(40)
	A. oryzae	10,000	400	25	0.5	***	(12)
	A. oryzae	42.3	270	22	**0.25**	***	This study
Continuous Free-Enzyme Systems	*K. lactis*	2	230	22	4.5	121	(10)
	K. lactis	8	250	24	1.5	10	(40)
	A. oryzae	42.3	270	22	0.25	**139**	This study
Continuous Immobilized-Enzyme Systems	*A. oryzae*	10,000	400	25	***	13.5	(12)
	B. singularis	~1	300	~55	***	12.8	(15)
	A. oryzae	42.3	270	20	0.07	**17.8**	This study

*** Not applicable or not reported.

2.3.3 Continuous Enzyme Systems

UF free-enzyme. Figure 2.3 shows the progress curves for the continuous UF free enzyme system. Maximum GOS yield and start-up time were very similar to UF recycle batch systems; however, after 15 min start-up, maximum GOS level was maintained at 22% through the duration of the 2 h run. The reaction mixture also contained 50% lactose, 18% glucose and 9% galactose. The GOS amount was ~60 g/L with a 139-g/h throughput. Table 2.1 shows that similar continuous UF free-enzyme systems reported in the technical literature had similar GOS yields; however, start-up time in our study was 6- to 18-fold better, once again suggesting the importance of enzyme concentration. Moreover, our study

showed better throughput than the other systems. This may be a result of the greater pressures, flux rates and higher speeds through the cross-flow membrane system used in this study. Also, enzyme origin (*A. oryzae*) may be a factor, but this is unclear, and further investigation is required to elucidate this.

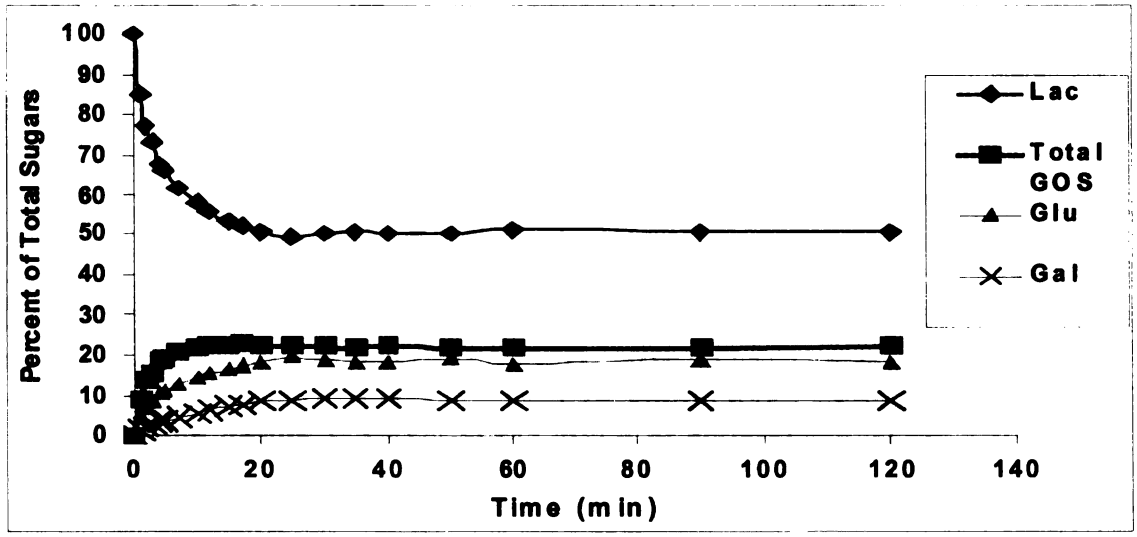

Figure 2.3 - Percent of total sugars over time during free-enzyme ultrafiltration system with constant feed of 27% (w/v) lactose. Each datum represents the mean of three replicates with CV% from 0.26 to 2.7%.

Immobilized-enzyme. Figure 2.4 was used to determine the optimum flow rate and residence time for the continuous immobilized enzyme system. The results showed that maximum GOS yield (20%) was achieved with a 5.5 mL/min feed rate or 4.5 min residence time in a single-pass immobilized-enzyme reactor. This yield had occurred at near 50% lactose conversion. Low flow rates gave residence times that were too long; as a result, more GOSs were hydrolyzed to monosaccharides. High flow rates gave residence times that were too short within the reactor; as a result, there was little time for optimum GOS formation.

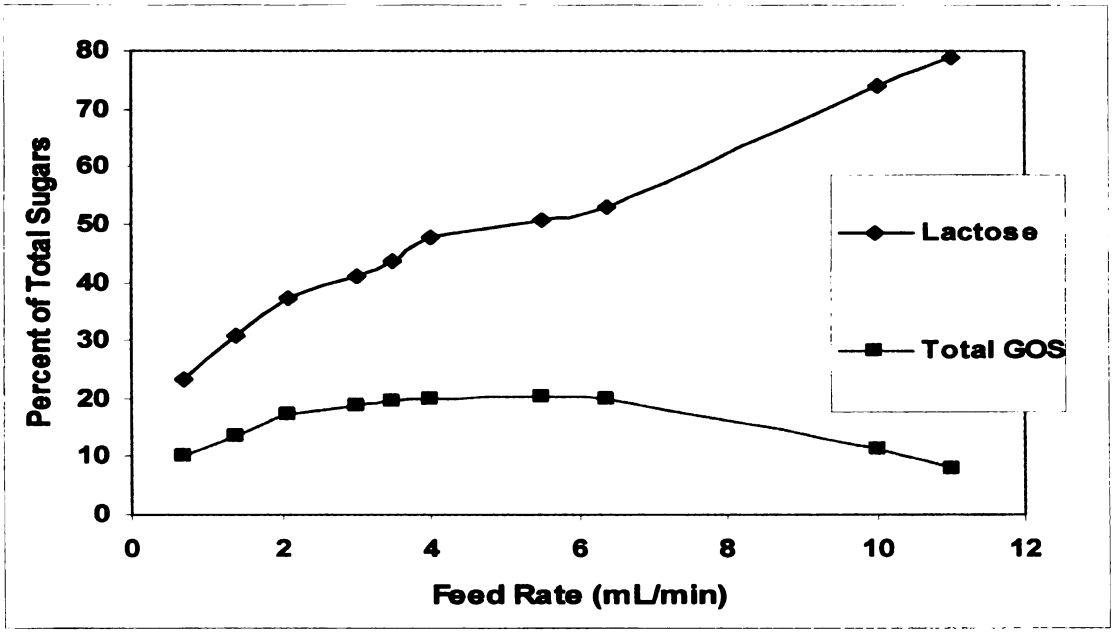

Figure 2.4 - Percent of lactose and total GOS at various feed rates of 27% (w/v) lactose solution (pH 4.5, 40°C) in single-pass immobilized-enzyme reactor. Each datum represents the mean of several replicates, CV% from 1.3 to 4.8%.

Figure 2.5 shows that ~20% maximum GOS production was achieved within a very short start-up time (4.5 min) and was maintained for the 2 h experimental run. Low start-up time is characteristic of continuous immobilized enzyme systems, since start-up time is dictated by the optimum flow rate or residence time within the reactor. The effluent also contained ~50% lactose, ~20% glucose and ~10% galactose. Maximum concentration of GOS was 54 g/L with a 17.8 g/h throughput. In Table 2.1, the yield in our continuous immobilized enzyme system was less than that reported by Albayrak and Yang (12). This may be because of their much higher initial lactose concentration (400g/L), which probably led to higher GOS yield. Also, the 240-fold greater concentration of

enzyme used by Albayrak and Yang (12) could have contributed to the yield differences. Shin et al. (15) reported a much higher yield (55%) with a very low enzyme concentration (1 U/mL) from *B. singularis*. However, their yield included disaccharides along with higher chain-lengths whereas our yield included only DP3 to DP6 GOS. In a final comparison, throughput was better in our study than those reported within the literature. This may be a result of the higher feed rates used in our study.

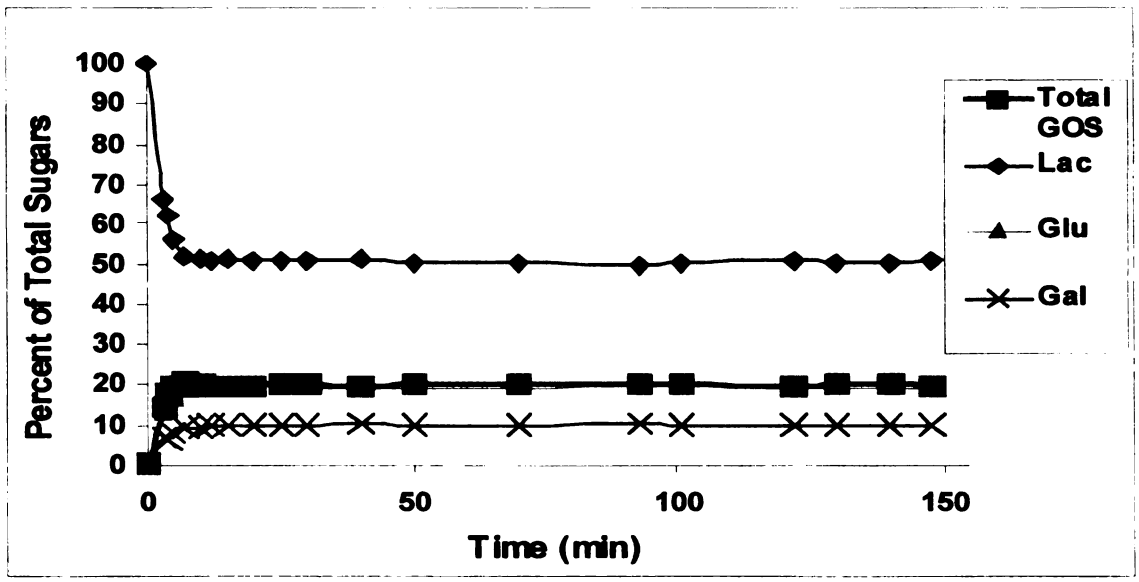

Figure 2.5 - Percent of total sugars over time within an immobilized-enzyme system with constant feed of 27% (w/v) lactose. Each datum represents the mean of three replicates with CV% from 0.20 to 5.3%.

Comparison. The continuous UF free-enzyme and immobilized-enzyme systems had the same enzyme concentration (U/mL) and were consequently considered equivalent in this study. The results show that GOS yield was slightly better with the UF free-enzyme systems, but start-up times were ~3-fold higher than the immobilized-enzyme system (Table 2.1). However, the free-enzyme system showed ~8-fold better throughput than the immobilized-enzyme

counterpart. The reason for this stark difference is the much faster permeate flow rates in the UF system (38-40 mL/min or 2.3-2.4 L/h) compared to the immobilized-enzyme equivalent feed rates (5.5 mL/min or 0.330 L/h). Though scaling up immobilized enzyme systems would allow for faster feed rates and thus higher throughput, continuous UF free-enzyme systems could likewise be scaled up to achieve even greater throughputs than those observed in this experiment.

Albayrak and Yang (12) found that the immobilized enzyme was 20 times more stable; however, in a separate study, researchers showed almost 60-90% inactivation of enzyme during immobilization (42). Also, the amount of enzyme required and cost were much greater than the free-enzyme system (42). Microbial contamination with both systems was minimal. In fact, our lab found no growth for both systems when plated after 24 hours of operation (APPENDIX 5). Furthermore, our lab showed no significant activity loss after 2 weeks in UF free-enzyme systems. However, a commercial processor should consider some other important issues when choosing a process, including: amount and cost of enzyme, half-life of enzyme, existing equipment and maintenance cost.

2.3.4 Monosaccharide Effect

Figures 2.6 and 2.7 show no apparent inhibitory effect by glucose and galactose at 0.87% and 0.43%, respectively for both free- and immobilized-enzyme reactors. To compare whether adding monosaccharides had an effect on maximum total GOS production, a Student's t-test was conducted on mean

total GOS after 15 min (Table 2.2). Since variances between control groups and monosaccharide-added groups were determined to be equal using F test (α=0.05), pooled variance was used to determine t values (49). In both the free- and immobilized enzyme studies, the Student's t-test showed no significant difference in mean total GOS after 15 min (P = 0.15 and 0.18, respectively). Though Albayrak and Yang (11, 12) showed inhibition by both glucose and galactose, their glucose and galactose concentrations were much greater (7 and 3%, respectively). Our results show great promise for industry application that would implement a system that could fractionate lactose from monosaccharides. Various researchers (45-47) have already shown 80% removal of monosaccharides from lactose using thin film trilaminate nanofiltration membrane composed of polyethersulphone with 50% sodium chloride rejection with four filtration steps. Using such a system would allow recycling of lactose feed containing residual amounts of glucose and galactose. Also, such a system would lend a manufacturer the opportunity to use the removed glucose and galactose fractions as substrate for alcohol fermentation for fuel production.

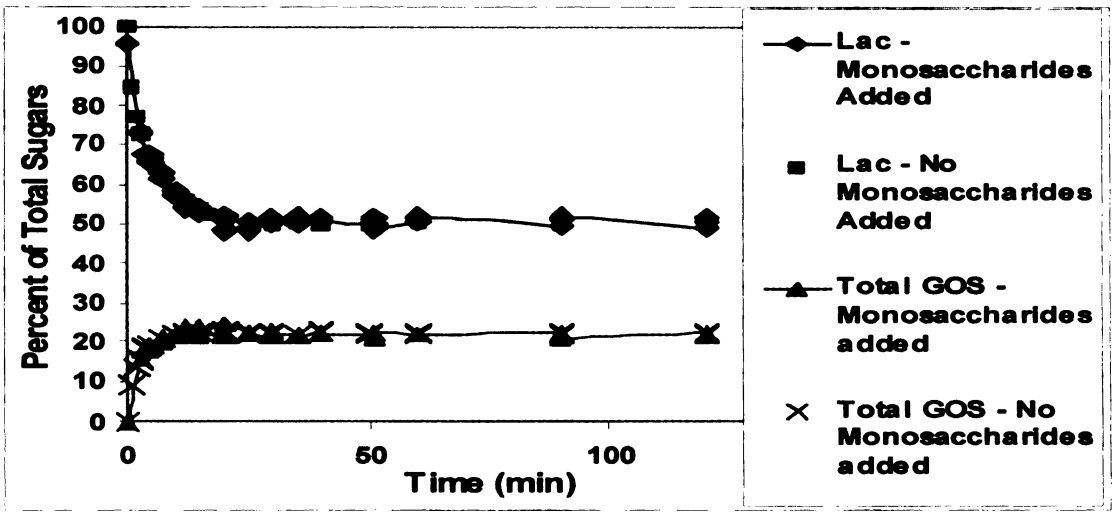

Figure 2.6 - Lactose and total GOS percent over time during continuous free-enzyme UF system where 27% (w/v) lactose feed either contained no monosaccharides or 0.87% (w/v) glucose and 0.43% (w/v) galactose. Each datum represents the mean of three trials (CV% from 0.13 to 5.1%).

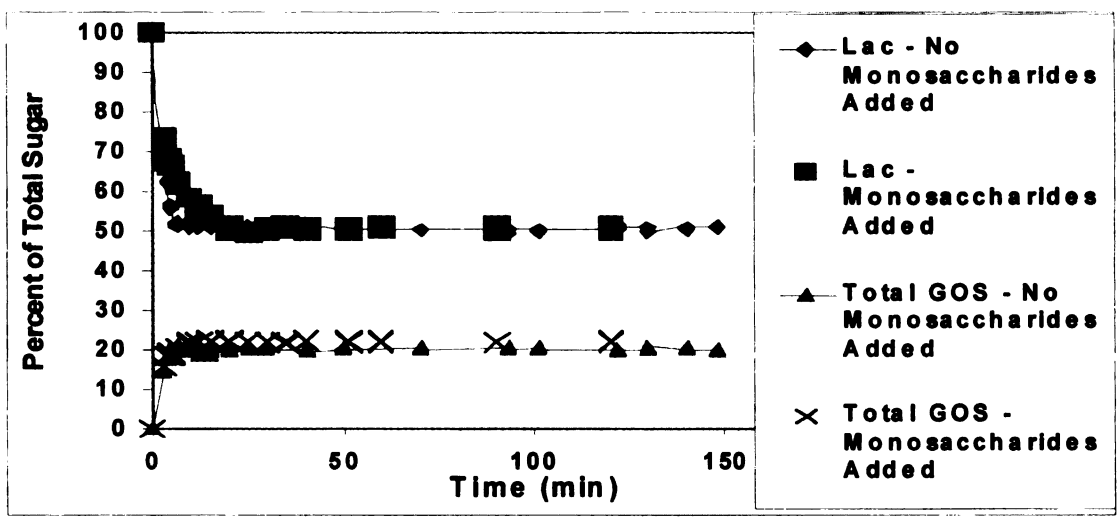

Figure 2.7 - Lactose and total GOS percent over time during continuous immobilized-enzyme system where 27% (w/v) lactose feed either contained no monosaccharides or 0.87% (w/v) glucose and 0.43% (w/v) galactose. Each datum represents the mean of three trials (CV% from 0.21 to 3.9%).

Table 2.2 - Student t-test comparing the effect of adding monosaccharides on the mean total GOS after 15 min within free- and immobilized-enzyme systems (α=0.05).

	Total GOS Percent			
Continuous Free-Enzyme	**Mean**	**Std Dev.**	**n**	**P-value**
No Monosaccharides Added	22.3	0.651	33	0.15
Monosaccharides Added	22.1	0.816	30	
Continuous Immobilized-Enzyme				
No Monosaccharides Added	20.1	0.99	39	0.18
Monosaccharides Added	20.3	0.72	30	

2.4 CONCLUSIONS

High enzyme concentrations and enhanced agitation within free- and immobilized-enzyme systems greatly improved start-up time. In general, the continuous UF free enzyme system showed considerably better product throughput (6 times greater) and yield (22% vs. 20%) than the immobilized-enzyme equivalent as well as most other systems reported in the technical literature. Furthermore, since there was no significant monosaccharide inhibitory effect at residual concentrations (0.87% and less), new technologies that remove 80% of monosaccharides from lactose can be implemented in a commercial GOS process that would recycle unreacted lactose.

2.4.1 Novel Contributions

This study has four worthy novel contributions to science and technology:

1. A comparison of equivalent free- and immobilized-enzyme systems.

2. A novel free-enzyme system that showed shorter start-up time (0.25 vs. 1.5-5 h) feasible for commercial application.
3. A novel enzyme system that showed high product throughput for commercial application.
4. Evidence that low monosaccharide concentrations do not significantly inhibit GOS production.

CHAPTER 3

FRACTIONATION OF GALACTO-OLIGOSACCHARIDES FROM DI- AND MONOSACCHARIDES USING NANOFILTRATION

3.1 INTRODUCTION

Galacto-oligosaccharides (GOSs) are known prebiotics that have been associated with a number of health benefits, including reduced colon cancer risk and enhanced immunity (50-52). Additionally, GOS structure lend them a wide range of functional properties in foods and beverages, including their use as lower calorie sweeteners, fat replacers and bulking agents (53).

GOSs of 3-7 degrees of polymerization (DP) are formed during transgalactosylation within solutions of high lactose concentration using β-galactosidase. Various researchers have optimized GOS production to over 20% of the weight of total sugars (11, 12, 54, 55); however, glucose and galactose monosaccharides are also formed in large quantities (over 25%) within the same solution (11, 12, 54, 55). In addition to these products, a large quantity of unreacted lactose (~50%) remains. Fractionation of GOSs from these non-prebiotic smaller chained sugars is highly desirable by the food industry as well as research communities in nutrition, pharmaceutics and medicine. However, despite growing interest and improving technologies, GOS fractionation or preconcentration remains a serious and costly problem for large-scale production. As a result, research and applications with these bioactive agents in human nutrition, medicine and food have been severely impeded. To date, fractionation of GOSs from an enzymatic reaction

mixture is achieved using chromatography (56). This current process is very expensive and limits wide commercial use.

In recent years, nanofiltration (NF) has been investigated as a fast and inexpensive alternative for oligosaccharide fractionation from lower molecular weight sugars (57-59). NF uses a membrane of a specific polymer and pore size to separate compounds based on molecular size, charge or both. Mass transport across the membrane may be influenced by fluid pressure, temperature, feed concentration and pH or ion strength (34).

Sarney et al. (60) showed that bioactive oligosaccharides from human milk could be fractionated from other sugars using NF; however, this only was accomplished when the milk was pretreated with β-galactosidase for several hours to fully hydrolyze lactose to glucose and galactose. Goulas et al. (45) showed that 80% of initial monosaccharides could be permeated with 18% of di- and oligosaccharide loss in a dead-end NF system in 4-6 diafiltration steps. Each diafiltration step involved the addition of solvent to the recycled retentate for removal of the lower molecular weight sugars (34). Grandison et al. (47) demonstrated the effect of membrane type, pressure, feed concentration and temperature on separation of oligosaccharides from di- and monosaccharides in a dead-end and cross-flow NF system. Their results showed an optimum of 80% removal of initial monosaccharides could be achieved in four to six diafiltration steps using lower pressures, lower feed concentrations and higher temperatures (58).

The majority of these studies did not remove adequate amounts of lactose along with monosaccharides; whereas, an ideal system would need to remove large quantities of lactose in addition to the monosaccharides from the reaction mixture. Also, these studies did not investigate the effects of pH on retention. Studies have shown that changing pH can improve selectivity of certain solutes by changing the charge characteristics of the solute and/or membrane surface (61, 62); however to date, there is no report on sugar/oligosaccharide selectivity using pH. Furthermore, many of the aforementioned oligosaccharide fractionation studies used commercially processed oligosaccharide mixtures that had been pre-fractionated to contain lower quantities of monosaccharides (45, 47). Oligosaccharide fractionation directly from an enzyme-produced sugar mixture is still required and has not been fully studied.

Beyond the few studies mentioned, there has been very little work done in this area. There is still a great need to fractionate the GOSs from the lactose and monosaccharides. Thus, the objectives of this study were two-fold. The first was to determine optimum pressure, temperature, feed concentration and feed pH for maximal mono- and disaccharide removal with minimal oligosaccharide loss, within a cross-flow NF system using model solutions containing mono-, di- and trisaccharides. The second was to use these optimum cross-flow NF conditions to fractionate GOSs directly from an enzymatically prepared mixture of GOSs, lactose, glucose and galactose.

3.2 MATERIALS AND METHODS

3.2.1 Membrane Filtration Unit and Membrane Types

Membrane filtration was operated on a continuous cross-flow APV membrane unit with two tubular membranes in series, with a water jacket and centrifugal pump system. Throughout the study, AFC30 polyamide NF membranes with ~500 molecular weight cut-off (MWCO) and 0.024 m^2 surface area (PCI Membrane, Milford, OH) were used to separate sugars. Before and after each membrane filtration trial, membranes were rinsed thoroughly with double distilled water at 500 p.s.i. and 25 °C. To assure uniform compaction as well as no fouling between runs, the flux, or the permeation flow rate per membrane surface area, of the water before and after each run was verified to be the same.

3.2.2 Sugar Analysis

Sugars were analyzed using HPLC procedures previously established (55). In these procedures, solutions containing the sugars were measured using an HPLC system with a refractive index detector. A Rezex RNM™ Carbohydrate anion exchange column (7.8 x 300 mm) (Phenomenex, Torrance, CA) with a guard column (7.8 x 50 mm) (Phenomenex, Torrance, CA) was used to separate oligosaccharides and simple sugars. HPLC-grade water was used as mobile phase at 0.4 mL/min. Column and detector temperatures were maintained at 85 and 45 °C, respectively.

The concentrations of sugars (lactose, glucose, galactose, and oligosaccharides (DP3-6)) were proportional to their peak areas (55). Sugar concentrations were normalized as weight percentages of total sugars (55). Due to limited availability of GOS standards, malto-oligosaccharides were used. Our laboratory found that malto-oligosaccharides (DP3-7) had the same retention times and detection responses as GOSs with the same DP. Thus, standard curves for each GOS were determined using malto-oligosaccharides.

3.2.3 Temperature Effect

A model 10% (w/v) sugar solution (0.1 M sodium acetate buffer, pH 4.5, 5.67L) comprised of 20% total GOSs (DP3-6), 20% total monosaccharides (glucose + galactose) and 60% lactose was made using a single-pass immobilized-enzyme system as described in published procedures (12). This solution was run through the NF unit at a feed rate of 18.9L/min, fluid pressure of 500 p.s.i. and various temperatures (30, 50 and 60 °C). The system was equilibrated by running the solution for 30 min at the appropriate temperature. Also, flux was determined at each temperature level by measuring permeate flow rate (L/h) and dividing by membrane surface area (m^2). After equilibration and flux determination, sample permeate (2L) was collected at each temperature level. Samples of permeate, retentate and initial solutions were analyzed for sugars using HPLC procedures. Three independent trials were conducted. Retention values (R) were calculated (**Eq. 3.1**) and plotted against temperature to study the effects of temperature on retention as well as to determine optimum

temperature for maximal mono- and disaccharide removal with minimal GOS loss. Temperature effect on retention values as well as flux was studied using ANOVA ($\alpha = 0.05$).

$$R = 1 - \frac{Cp}{Cf} \qquad \text{Eq. 3.1}$$

Where *R* is Retention, and *Cp* and *Cf* are permeate and feed concentration, respectively.

3.2.4 Pressure Effect

At optimum temperature, a model 10% (w/v) sugar solution (0.1 M acetate buffer, pH 4.5, 5.67L) comprising of ~20% total GOSs (DP3-6), ~20% total monosaccharides (glucose + galactose) and ~60% lactose was made using a single-pass immobilized-enzyme reactor according to published procedures (12). The solution was run through the membrane unit at a feed rate of 18.9L/min, optimum temperature (determined from the previous section) and fluid pressures of 300, 500 and 700 p.s.i. Sample permeate (2L) was collected at each pressure regime. However, before sample was collected, the system was equilibrated by running the solution for 30 min at the appropriate pressure. Also, flux was determined before sample collection (n=3). Samples of permeate, retentate and initial solutions were analyzed for sugars using HPLC procedures. Three independent trials were conducted. Retention value versus pressure was plotted to study the effects of pressure on retention as well as to determine optimum pressure for maximal mono- and disaccharide removal with minimal GOS loss.

Pressure effect on retention values as well as flux was studied using ANOVA (α = 0.05).

3.2.5 Concentration and pH Effect

Model solutions used previously in this study for temperature and pressure effect were at pH 4.5, which is the optimum pH for GOS production with β-galactosidase. However, to study the pH effect, model solutions were made using glucose, lactose and raffinose sugar standards within 0.1 M acetate buffer of varying pH. Since raffinose and lactose could not be resolved in HPLC chromatograms during preliminary analyses, two separate model solutions were required. One model solution contained ~7 mM of lactose and glucose, while the other contained ~7 mM raffinose (i.e. same molecular weight (504 MW) as DP3 GOS) was made. Model solutions were prepared in 0.1 M sodium acetate buffer solutions of pH 4.5, 6.0, 7.0 and 8.0. The pH of 4.5 was selected as a lower limit since it is the optimum pH of the enzyme process (11, 12). A pH of 8.0 was selected as an upper limit since higher pH levels would damage the membrane, according to the manufacturer. In duplicate trials, model solutions (5.67L) were run through the membrane filtration unit at 18.9 L/min feed rate as well as optimum temperature and pressure conditions. Equilibration for 30 min and flux determination were done before permeate collection. Sample permeate (3L) was collected for each pH. Permeate, retentate and initial samples were analyzed using HPLC procedures, and pH effect on retention and flux was studied using plots and ANOVA (α = 0.05).

The study was repeated at various pH levels using model solutions (5.2L) containing ~200 mM and ~330 mM of glucose and lactose, respectively in one solution and ~70 mM of raffinose in another model solution. These higher concentrated sugar model solutions were made to mimic sugar concentrations similar to those found in a reaction mixture that would have resulted from an optimum enzymatic process (42). The effect of initial concentration as well as pH was studied and compared with lower concentration solutions.

3.2.6 Nanofiltration Trials with GOS Sugar Mixture

In the following trials, a sugar mixture that had been made from continuous 27% (w/v) lactose feed (0.1 M acetate buffer, pH 4.5, 40 °C) through a single-pass immobilized-enzyme reactor was used in a discontinuous diafiltration process (12, 42). This solution contained 20, 60 and 20% of total monosaccharides, lactose and total GOSs, respectively. The sugar mixture (~5.2L) was adjusted to pH of 7.0 using ~3mL of 17 N NaOH). This relatively small volume of concentrated NaOH was assumed to not change the initial concentration significantly. The solution was then fed through the membrane filtration unit at 18.9L/min feed rate and optimum temperature and pressure. The solution was recycled for 30 min at these conditions to equilibrate the membranes. Flux was also determined. After equilibration and flux determination, sample permeate (3L) was collected and analyzed along with initial and retentate samples.

A second diafiltration step was done by adding 3L of 0.1 M acetate buffer (pH 4.5) in the membrane filtration batch tank to replenish the volume that had been permeated. The pH was adjusted to 4.5 using ~3mL concentrated HCl (17.4N). This relatively small volume was not enough to change flux rate and was assumed to not change the initial concentration significantly. The solution was then recycled in the membrane filtration unit for 30 min under optimum parameters to achieve equilibrium. Flux was determined. After equilibration and flux determination, permeate (3L) was collected for analysis along with samples of retentate. Diafiltration with 3L of 0.1 M sodium acetate buffer (pH 4.5) was repeated. All initial, permeate and retentate samples were analyzed and accounted for mass balance and percent permeation (**Eq. 3.2**) (32, 34). Three independent trials were done to determine reproducibility and validity of results.

$$Percent\ Permeation = \frac{Cp \times Vp}{Cf \times Vf} \times 100 \qquad \textbf{Eq. 3.2}$$

Cp and *Vp* are permeate concentration and volume, repectively

Cf and *Vf* are feed concentration and volume, respectively

3.3 Results and Discussion

3.3.1 Temperature Effect

As expected, monosaccharides showed the lowest retention, while DP3 showed the highest across all temperatures (Figure 3.1). Figure 3.1 shows that

retention for each sugar was steady from 30 and 50 °C with a slight decrease at 60 °C. Despite this slight decline, there was no significant temperature effect on retention of glucose, lactose and DP3 (P=0.15, 0.26 and 0.51, respectively). Also, there was no apparent difference between lactose and DP3 retention; both remained very high, while monosaccharides readily permeated. As expected, flux at 500 p.s.i. did increase significantly with temperature in Figure 3.2 (P<0.0001).

These results concur with the general theory of mass transport across membrane systems. Increasing temperature will increase the rate of solvent and solute transport across the membrane due to a lowering of viscosity. This would explain the steady increase of flux seen in this and other studies (47). Despite the increase in flux, solvent and solute are theorized to transport equally across the membrane, causing no change in retention factor (34).

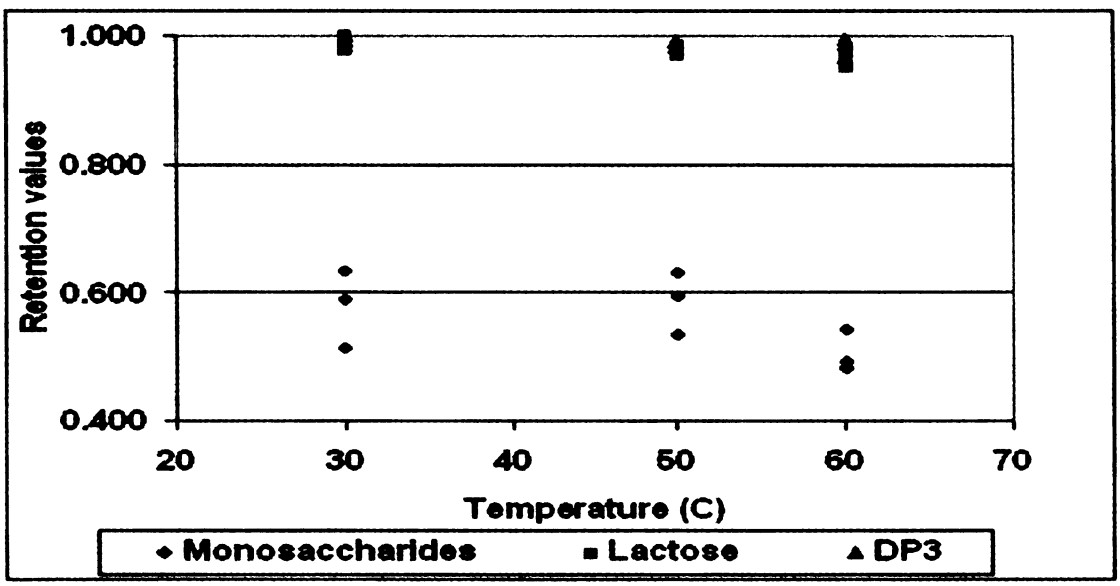

Figure 3.1 - Retention values of sugars in a nanofiltration system at various temperatures at 500 p.s.i.

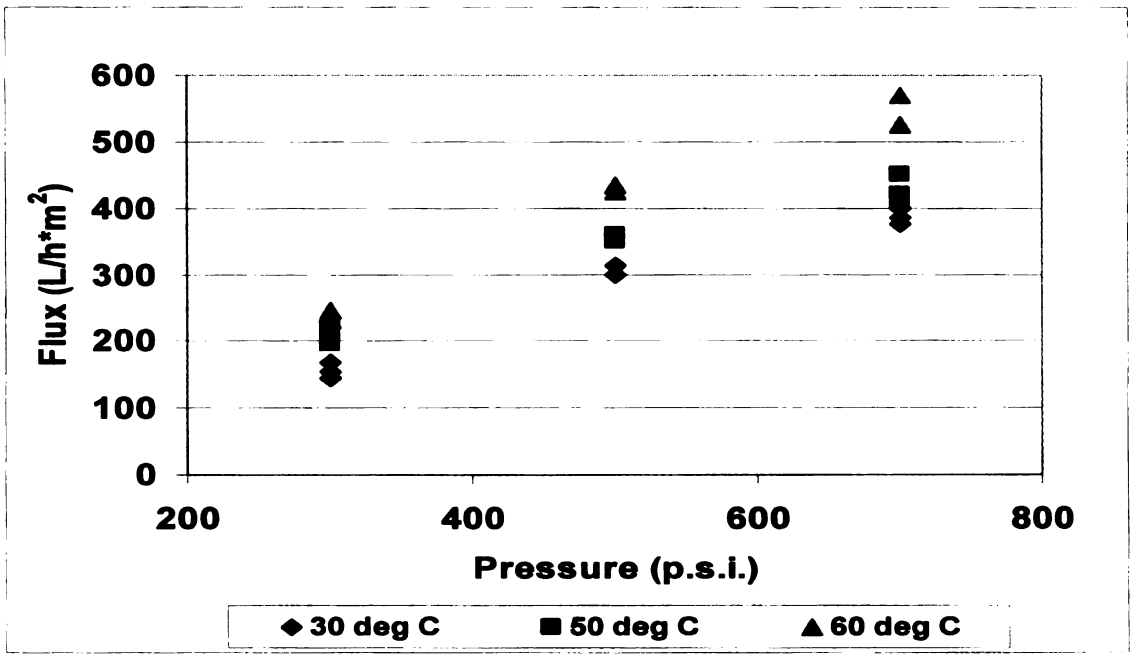

Figure 3.2 - Flux of sugar solution at various pressures and temperatures.

Interestingly, Grandison et al. (47) found a similar yet more pronounced trend of declining retention with increased temperature for monosaccharides and disaccharides within model solutions containing fructose, sucrose and raffinose run in NF systems at various temperatures from 20 to 60°C (47). Their rationale was that higher kinetic energy from increased temperature caused an increase in convective flux. This increase in convection had preferentially facilitated diffusion of smaller sugars (i.e. fructose) more than larger sugars (i.e. raffinose) since convective forces would more easily affect lower molecular weight compounds. This may explain why glucose showed a more pronounced effect (P=0.15) than lactose and DP3 (P=0.26 and 0.51) in our studies.

Since no significant temperature effect on retention was found in this study, a temperature of 50°C was used as the temperature of operation. It was

thought that 50°C would offer adequate fluidity for higher sugar concentrations that would be used in later experiments. Also, 50°C was well within the recommended range of operation specified by the membrane manufacturer.

3.3.2 Pressure Effect

Figure 3.3 shows no significant change in retention of glucose, lactose and DP3 (P=0.15, 0.15 and 0.97, respectively) with pressure. These results concur with published findings (45, 60, 63). Though it has been theorized that higher pressures may cause a preferential permeation of solvent rather than solute, this is generally found with solutions containing solutes of very low retention (i.e. R=0.2) (34). However, the sugars in our study had relatively high retentions for the effects of this phenomenon to occur (34).

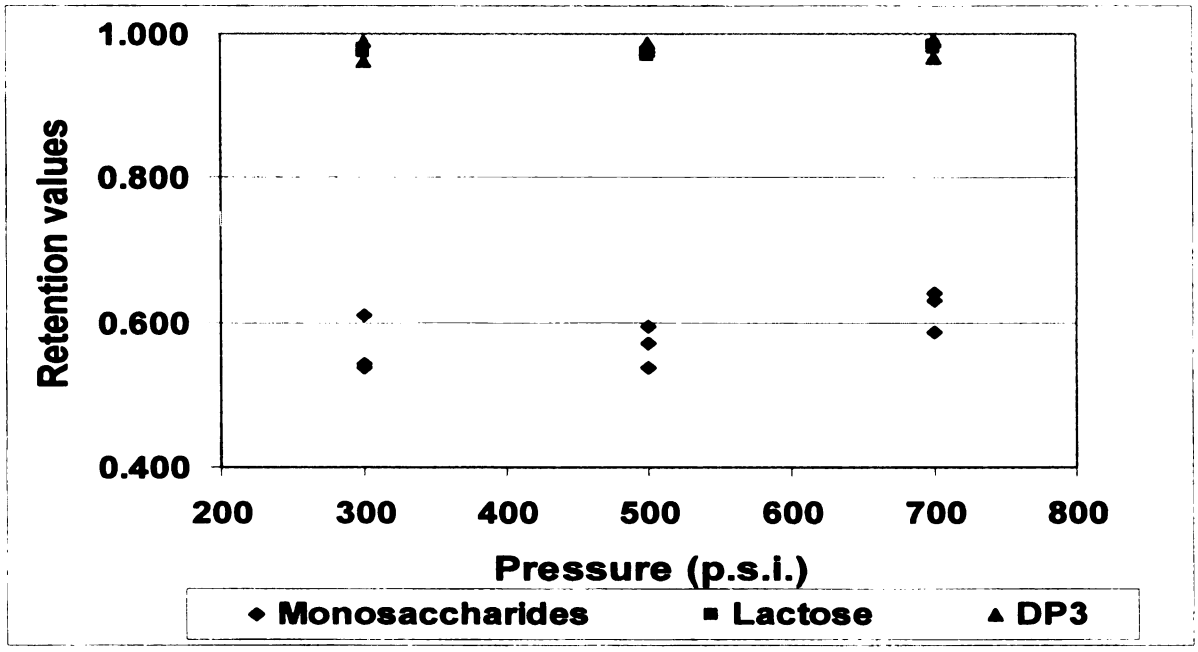

Figure 3.3 - Retention values of sugars in a nanofiltration system at various pressures at 50°C.

Flux increased linearly with pressure (Figure 3.2) at all temperatures (R^2=0.97, P<0.0001). This relationship is easily recognized, since increasing pressure proportionally increases solvent and solute transport across the membrane (34). Though preferential permeation of solvent may cause a deviation from this linear relationship, it was already noted that a model sugar solution with relatively high retentions would generally not show this effect.

3.3.3 pH Effect

7 mM sugar model solutions showed steady decrease in retention with increasing pH (Figure 3.4). Glucose showed the greatest response to pH, followed then by lactose. Raffinose seemed to show the greatest resistance to pH effect from 4.5 to 8; however, all glucose, lactose and raffinose showed a statistically significant declining relationship (all P values<0.001). The effect of pH on flux was also evident. As shown in Figure 3.5, flux increased significantly with increasing pH (P<0.0001).

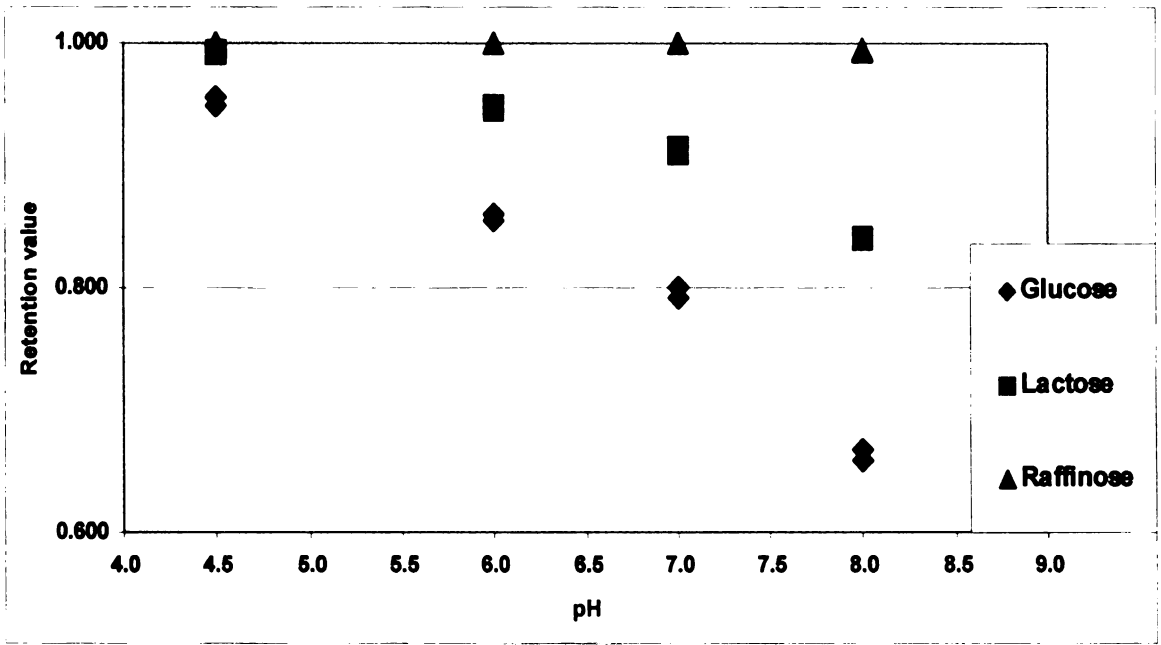

Figure 3.4. Effect of pH on retention values of model solutions containing ~7mM of glucose, lactose and raffinose.

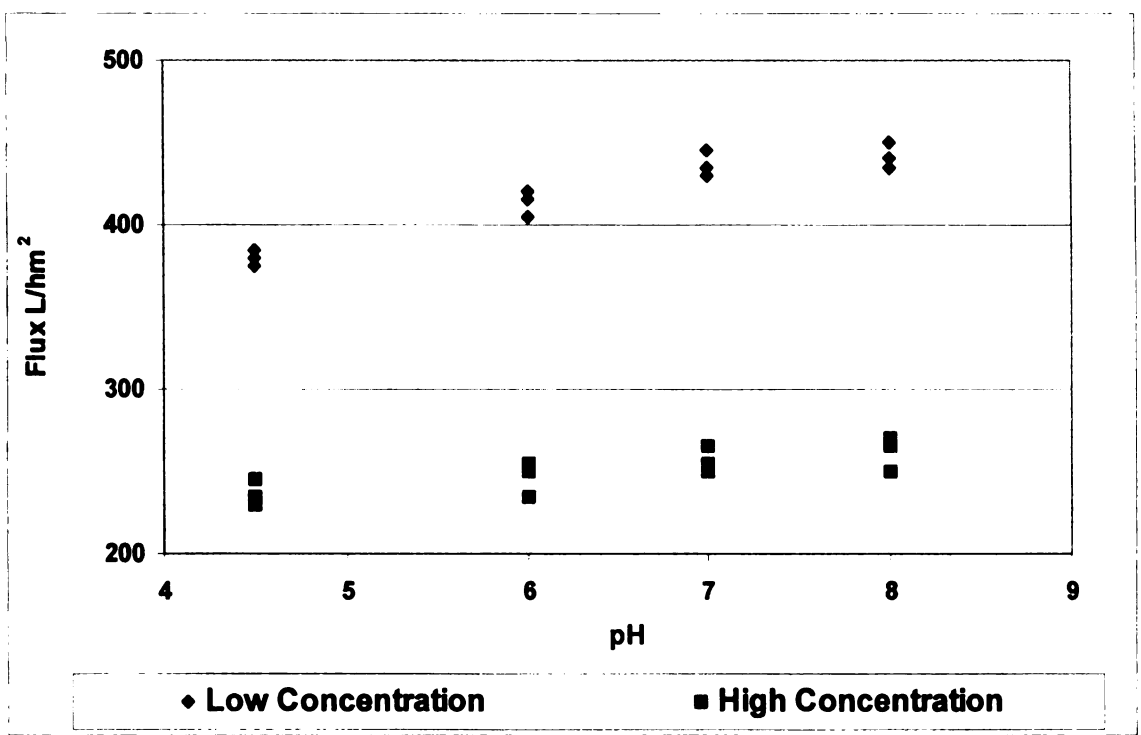

Figure 3.5. The effect of pH on flux of high and low concentrated oligosaccharide mixtures in NF.

This phenomenon may be partly explained through the changes in charge density on the polyamide membrane surface. Polyamide has carboxylic acid end groups with an isoelectric point between pH 4 and 5, depending on the length of the polymer (Figure 3.6) (64).

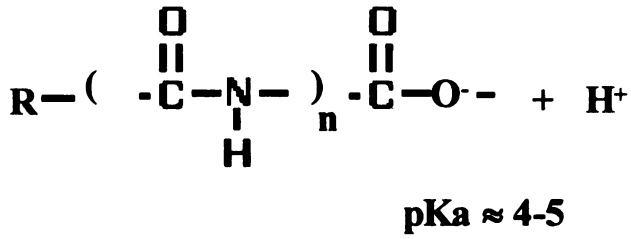

Figure 3.6. Structure of polyamide polymer with carboxylic acid end group.

At pH 4.5, the membrane has a neutral net charge, and sugar molecules (also with neutral charge) are transported across the membrane primarily based on convective forces and their steric properties (i.e. size and shape). Water and charged species within the solution also transport more slowly due to an increase in hydrophobicity of the membrane. However, at higher pH, the membranes increase in negative charge density and become easily hydrated by the water solvent which more readily passes through the membrane carrying any dissolved species (i.e. sugars) (32, 34). This phenomenon partly explains the decrease in retention and the increase in flux associated with increasing pH. In addition, higher pH regimens may also cause the sugars themselves to be more dissolved in the solvent thereby also improving their transport across the membrane. This

would explain why glucose, a more soluble monosaccharide, was more affected by pH than raffinose, a less soluble trisaccharide (Figure 3.4).

3.3.4 Concentration Effect

A similar effect of pH on retention was observed with higher concentrated model solutions (Figure 3.7). Though significant for all sugars ($P<0.01$ for all sugars), the trend was not as pronounced as with lower concentrated sugar model solutions and seemed to level off around pH of 7.

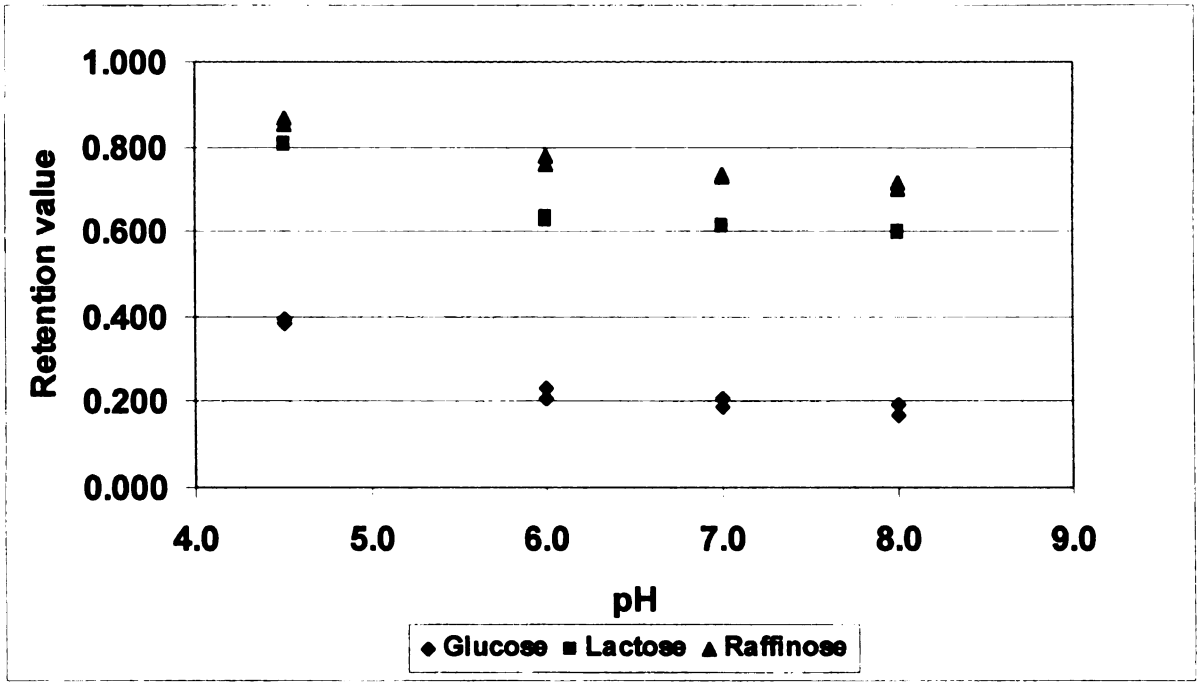

Figure 3.7. Effect of pH on retention values of model solutions containing ~200, 330 and 66 mM of glucose, lactose and raffinose, respectively.

Interestingly, the higher concentrated model solution had significantly lower retention with each sugar at each pH when compared with lower concentrated models ($P<0.0001$ for all sugars) (Figure 3.7 vs. Figure 3.4). This result suggests that higher bulk concentrations cause a higher concentration

polarization at the membrane surface. As a result, more compounds near the membrane pores will diffuse down their concentration gradient (34).

The flux at higher concentration showed a positive linear relationship with pH similar to lower concentrated models; however, the flux was significantly less with higher concentrated solutions ($P<0.0001$) (Figure 3.5). This effect is largely a result of increased viscosity impeding the rate of diffusion of solute and solvent. Furthermore, there are more solutes (i.e. sugars) competing with solvent for pores at the membrane boundary layer. Since the solutes diffuse more slowly across the pores than the solvent, flux is reduced (34).

High concentrations exceeding 30% total soluble solids may cause a high concentration polarization at the membrane surface. This could lead to the formation of a gel layer, or membrane fouling (34). Fouling, or the permanent blocking of membrane pores, was not found in this study since water flux before and after each run was the same. This ensured no significant fouling had occurred with the concentrations used in this study. Other studies similarly showed little fouling with similar concentrations of sugar in NF systems (45, 47).

3.3.5 Nanofiltration Trials with GOS Sugar Mixture

The model solution studies suggested that pH and concentration are strong determinants of sugar retention, where higher concentration and pH cause greater permeation of sugars. NF at higher concentrations and higher pH levels (i.e. 7 or 8) would be beneficial in removing large amounts of lactose and monosaccharides; however, this would result in greater loss of GOSs in

subsequent diafiltration steps. This has been a serious problem faced by researchers and manufacturers (34, 45, 47). A solution to this problem would be subsequent diafiltration at a lower pH (i.e. 4.5). This would allow a large transport of monosaccharides and lactose within the first filtration step and reduced transport in subsequent diafiltrations. This process was attempted in these experiments.

Table 3.1 shows the mass of sugars permeated at each diafiltration step. The first filtration step at pH 7 accounted for 56.6%, 31.7% and 12.6% permeation of monosaccharides, lactose and GOSs, respectively. Diafiltration 2 at pH 4.5 showed much lower percent permeation at 24.7%, 12.9% and 5.3% permeation of monosaccharides, lactose and GOSs, respectively. Finally, the third diafiltration step at pH 4.5 showed even lower percent permeations for monosaccharides, lactose and GOSs (10.2%, 3.5% and 1.0%, respectively).

Percent permeation significantly decreased with diafiltration steps 2 and 3 mainly because of the lower pH. However, removal of sugars at each diafiltration step (especially the first filtration step) caused a lower concentration of total sugars for the subsequent diafiltration steps. As the results from our model solutions suggest, lower concentrations may have also caused the reduced permeation of solute in diafiltration steps 2 and 3 (See Appendix 6 for initial and post-diafiltration chromatograms).

The total percent permeation of monosaccharides, lactose and GOSs after 3 diafiltration steps was 91.5%, 48.1% and 18.9%, respectively. Grandison et al. (47) and Goulas et al. (45) found that they could only achieve ~80% removal of

monosaccharides in 4-6 diafiltration steps. However, their process did not remove adequate amounts of lactose. We found that we could successfully accomplish this by manipulating pH and concentration as parameters for selection. Other researchers have applied the theory of changing pH in NF systems to fractionate proteins and minerals (61, 62, 65). However, this study demonstrates the first account of sugar fractionation using varying pH during NF.

Table 3.1. Mass and percent permeation of monosaccharides, lactose and galacto-oligosaccharides within 3L of permeate at each diafiltration step.

	Total Monosaccharides (g)	Lactose (g)	Total GOSs (g)
Initial pH 7.0	204.2 ± 5.1	714.4 ± 4.8	196.7 ± 1.6
Diafiltration 1 (pH 7.0)	115.6 ± 7.1	226.4 ± 1.6	24.79 ± 2.2
Diafiltration 2 (pH 4.5)	50.40 ± 4.7	92.05 ± 6.3	10.55 ± 1.3
Diafiltration 3 (pH 4.5)	20.74 ± 1.0	25.11 ± 1.9	1.97 ± 0.23
Mean Sugar Mass of Permeated Sugar	186.74	343.56	37.31
Percent Permeation Diafiltration 1	56.6%	31.7%	12.6%
Percent Permeation Diafiltration 2	24.7%	12.9%	5.3%
Percent Permeation Diafiltration 3	10.2%	3.5%	1.0%
Total Mean Percent Permeation	91.5%	48.1%	18.9%

3.4 CONCLUSIONS

The following study showed that pH and concentration had a strong effect on permeation of sugars of varying chain length in NF systems with polyamide

membranes. A unique process was developed to fractionate biologically active oligosaccharides from contaminant di- and monosaccharides present within sugar mixtures. The process showed ~92% removal of monosaccharides and ~48% removal of lactose with only ~18% loss of GOSs in 3 diafiltration steps. This was achieved by lowering pH from 7 to 4.5 after the first diafiltration step. This adjustment allowed for better fractionation of biologically active oligosaccharides than what has been reported within the published literature. This novel approach could easily be scalable for commercial application and has wide applicability for fractionation of other bioactive oligosaccharides within sugar mixtures.

3.4.1 Novel Contributions

This study has four worthy contributions to science and technology:

1. A better understanding of pH and charge effects on polyamide membrane and sugar interactions in NF systems.
2. A novel and superior process that fractionates GOSs and possibly other biologically active oligosaccharides from di- and monosaccharide contaminants in few steps.
3. Provides the groundwork for further research in manipulation of pH and/or concentration to fractionate other important oligomeric compounds (i.e. nucleotides, etc.) in NF systems.

4. Provides a more preconcentrated bioactive reagent to be used by the food industry as well as research communities in nutrition, medicine and pharmacology.

CHAPTER 4

SEPARATION OF GALACTO-OLIGOSACCHARIDES USING ANION-EXCLUSION CHROMATOGRAPHY

4.1 INTRODUCTION

Galacto-oligosaccharides (GOSs) are non-digestible, short-chain polysaccharides (3-7 degrees of polymerization (DP)) preferentially consumed by beneficial intestinal microflora in the colon. As a result, they have been characterized as prebiotics and have been associated with a number of important health benefits, including enhanced immunity and reduced colon cancer risk (7, 22, 24, 25, 66-69). Recent research has shown that oligosaccharides of certain DP have different important functions and benefits for the food, pharmaceutical and chemical industries as well as the medical and nutritional research communities (21, 23, 66, 70, 71). As a result, there has been a strong demand for pure forms of these compounds (7, 21, 23, 66, 70, 71). However, these compounds are expensive to produce, and as a result, have low commercial availability.

Ion exclusion chromatography (IEC) is a technique that combines the principles of ion exchange and reversed phase adsorption chromatography to separate neutral or near-neutral compounds from highly ionic species (72-75). IEC is often used as an effective analytical tool, especially to separate organic acids, alcohols or sugars within complex food matrices (i.e. beverages) (73, 74, 76). IEC has also become a widely attractive form of process chromatography for commercial-scale up due to a number of advantages over ion exchange and

reversed phase chromatography, including reduction of pollution load, robust nature of the resin, highly resolved separations and decreased energy consumption (75). In the sugar industry, IEC is commonly used to separate glucose from fructose while eluting contaminant ionic species (i.e. Cl-). In the pharmaceutical and chemical industries, IEC is used to separate and purify bioactive or chemical reagents from other contaminant compounds formed within complex reaction matrices (75).

Most IEC resins are composed of polystyrene divinylbenzene (DVB) with a charged ionic species (77). Styrene polymer behaves as the fundamental support resin, while the DVB is added for cross-linking between the polymers of styrene (77). Less cross-linking (i.e. 2%) enhances diffusion of compounds within the stationary phase and through the column; however, the resin is less robust and has a tendency to swell and/or shrink. High cross-linking offers better mechanical stability, but diffusion is impeded as a result. Approximately 6-8% cross-linking is ideal for most applications (77).

Polystyrene DVB with 8% crosslinking is usually made by mixing 92 mol of styrene and 8 mol of DVB with a catalyst that would initiate polymerization (i.e. benzoyl peroxide). As polymerization continues, the liquid matrix is poured in warm water containing surfactant. Based on the surfactant concentration and degree and manner of agitation, the liquid breaks up into drops or spheres of certain size (77). The spheres are usually then treated with a strong acid or base to incorporate charged groups in the benzene rings (77). This step distinguishes IEC manufacture from conventional ion exchange manufacture. Usually, larger

concentrations of strong acids, bases or other charged compounds are used to contribute to the very important ion exclusion separation properties (77).

The principles and mechanisms of IEC are quite complex, combining the principles of a number of other types of chromatography (i.e. ion exchange, reversed phase, etc.). The highly charged resins are first hydrated at the surface and within their pores with a water layer (73, 76). This hydrated layer serves as a liquid stationary phase much like a semi-permeable membrane. Thus, all species of the solution are freely exchanged through this liquid membrane layer. Usually, highly charged species with "like" charge to the stationary phase will be repelled from the stationary phase and will elute first from the column (73, 76), while highly charged species of opposite charge will readily transport across the liquid membrane and within the pores of the resin to neutralize net charge of the stationary phase, as well as be highly retained. Neutral or near-neutral species (i.e. sugars or protonated organic acids) will also transport across the membrane but will be less retained. These compounds will separate based on adsorption and steric properties (73, 76).

Negative ions, especially Cl^- and SO_4^{-2}, can be highly undesirable contaminants in food or pharmaceutical products because they are potentially reactive and are perceived to be dangerous by the public (75, 78, 79). As a result, anion exclusion chromatography is commonly used to separate these compounds without large elution volumes. Polystyrene DVB with sulfonated negative charged groups is one of the most common anion exclusion resins used to remove these contaminants (75). As a result, they are commonly used in the

sugar and pharmaceutical industries and have proven to be a cost-effective form of chromatography.

With any type of laboratory or large-scale chromatography process, resolution and peak broadening are important aspects to optimize. Resolution is a measure of a column's ability to separate two or more compounds (74). Column resolution is defined as (74):

$$Rs = \frac{2(t_B - t_A)}{W_A + W_B} \qquad \textbf{Eq. 4.1}$$

t_A and t_B are retention times for peaks A and B, respectively.

W_A and W_B are peak base widths of peaks A and B, respectively.

As **Eq. 4.1** suggests, high resolution is when the retention times between two peaks are large and the peak base widths are small. Peak broadening occurs when the base widths of the peaks are very wide and the peak points are flat or broad. This is associated with low resolution and usually occurs as an effect of several mass-transfer processes during solute migration (74). Low resolution and extensive peak broadening are undesirable for analytical purposes. In commercial-scale processes, low resolution makes it difficult to fractionate compounds with similar retention times, resulting in low purity. Also, peak broadening causes collection of a more diluted compound, resulting in increased post-process energy expenditure to concentrate the sample (i.e. evaporation, freeze-drying, etc.). Resolution and peak broadening in IEC can be optimized through solvent pH, column temperature, mobile phase flow rate and stationary phase particle size (74, 76).

The aim of this study was to separate lactose and oligosacchardes of 3 to 7 degrees of polymerization (DP) using anion exclusion chromatography. Optimized conditions were first determined by studying resolution under various column temperatures, solvent flow rates and resin particle sizes using standard sugar mixtures. Then, a sugar mixture containing lactose and GOSs (DP3-6) was studied under the optimum conditions.

4.2 Materials and Methods

In the following studies, flow rate, column temperature and resin particle size were investigated to achieve optimum resolution with minimal peak broadening. Since sugars have high pKa values (12-14) and would thus remain essentially uncharged within a large pH range, it was not necessary to study mobile phase pH as a possible variable. Therefore, all HPLC procedures were carried out using HPLC-grade water as mobile phase. The two columns used in this study were 250 x 4.1 mm, 7 µm particle size and 150 x 4.1 mm, 3 µm particle size (Hamilton Co. Reno, NV). Both columns had polystyrene DVB polymeric support with 6% cross-linking and negatively charged sulfonate groups. Refractive index was used for detection of sugars. Standards (>99% purity) of lactose and malto-oligosaccharides (DP3-7) were used to construct standard curves for quantitation of lactose and GOSs (DP3-7), respectively. Malto-oligosaccharides were also used in model solutions during initial optimizing procedures. GOS standards were not used due to high expense and low commercial availability. Nevertheless, researchers have shown that peak area in

refractive index detection corresponds to the weight of the sugar. Since GOSs and malto-oligosaccharides of similar DP have the same molecular weight, they would elicit the same detection response (11).

4.2.1 Flow Rate

With the 3 and 7 μm particle size column and at ambient temperature, the following flow rates were studied: 0.2, 0.3, 0.4, 0.5, 0.6, 0.75 and 1 mL/min (n=3). For each flow rate, the system was equilibrated until a steady baseline was achieved. After equilibration, a 10-μL injection of a standard solution mixture of 0.02 mM of lactose and malto-oligosaccharides (DP3-7) was analyzed. Each flow rate level was done in three independent trials. Resolution between peaks was determined using the HPLC computer program and **Eq. 4.1**. ANOVA effect tests with α=0.05 were conducted at each flow rate level.

4.2.2 Column Temperature

At optimum flow rate, the following column temperatures were studied: ambient, 30, 40, 50 and 60 °C (n=3). The 3 and 7 μm particle size columns were heated using a column incubator interfaced with a computer program that monitors and maintains temperature. For each temperature, the system was equilibrated until a steady baseline was achieved. After equilibration, the standard solution mixture was injected at 10 μL and analyzed. Each temperature level was done in three independent trials. Resolution between peaks was

determined using the HPLC computer program and **Eq. 4.1**. ANOVA effect tests with α=0.05 were conducted at each temperature level.

4.2.3 Particle Size

The optimum resolutions from both particle size columns were compared to each other using ANOVA (α=0.05). Based on the statistical analysis, an optimum particle size was selected.

4.2.4 Separation of GOS Mixture

GOSs were produced using 27% lactose solution (0.1 M acetate buffer, pH 4.5, 40°C) reacted through an immobilized β-galactosidase reactor according to procedures outlined in previous experiments in our laboratory (42). The resultant mixture contained approximately 60% lactose, 20% total GOSs and 20% total monosaccharides. About 92% of total monosaccharides and 48% of lactose were removed with only 19% loss of GOSs using nanofiltration procedures established within our laboratory (80). The end result was a sugar mixture containing ~290 g of lactose, 225 g of GOSs and 20 g of monosaccharides in ~5 L of 0.1 M acetate buffer (pH 4.5).

Under optimum flow rate, column temperature and particle size, the sample mixture containing GOSs and lactose was injected (10μL) and analyzed using HPLC procedures. Resolution was determined using the computer program and **Eq. 4.1**.

4.2.5 Other Chromatographic Procedures

The optimum IEC procedure was then compared to established ion moderated partition chromatography (IMPC) methods (11, 12). IMPC uses the principles of ion exchange and size exclusion chromatography. IMPC of a 0.02 mM standard mixture of lactose and malto-oligosaccharides (DP3-6) at 0.4 mL/min flow rate of HPLC-grade water at 85°C was conducted using a Phenomenex™ IMPC column with the following properties: Na^+ counter-ion, Polystyrene DVB, 7 µm particle size, 7.8 x 300 mm, 8% cross-linking.

4.3 Results and Discussion

4.3.1 Flow Rate

Figure 4.1 shows the effect of flow rate on average resolution for 3 and 7 µm particle size column resins. The results clearly show a significant decline in resolution as flow rate increases ($P<0.001$). The optimum resolution was observed at 0.4 mL/min for both resin sizes. Though there was not a significant difference between 0.4 mL/min and 0.3 mL/min ($P=0.5$), the faster flow rate was considered better for analytical and commercial applications.

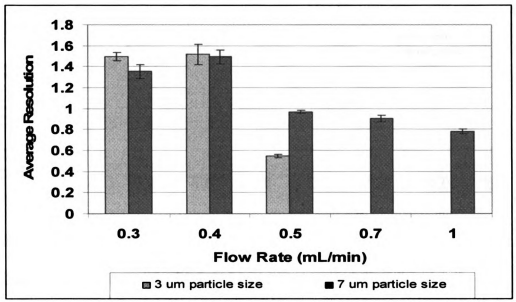

Figure 4.1. Average resolution for anion exclusion chromatography of 3- and 7-μm particle size at various mobile phase (HPLC grade water) flow rates.

It appears that the 3-μm particle size resin is more sensitive to flow rate, such that resolution was zero at flow rates greater than or equal to 0.7 mL/min (Figure 4.1). Chromatograms in Figure 4.2 demonstrate this further, where the six peaks (lactose, DP3-7) all co-eluted together at higher flow rate. This may be explained by the denser column packing with a smaller particle size (74). This would result in higher backpressure when flow rate is increased. A higher backpressure would cause a fluid movement against the direction of flow rate, which is desirable to sharpen peaks. However, if this backpressure is high enough it would cause a slower migration of the fastest peaks thus leading to severe co-elutions as shown in Figure 4.2 and illustrated in Figure 4.3 (74).

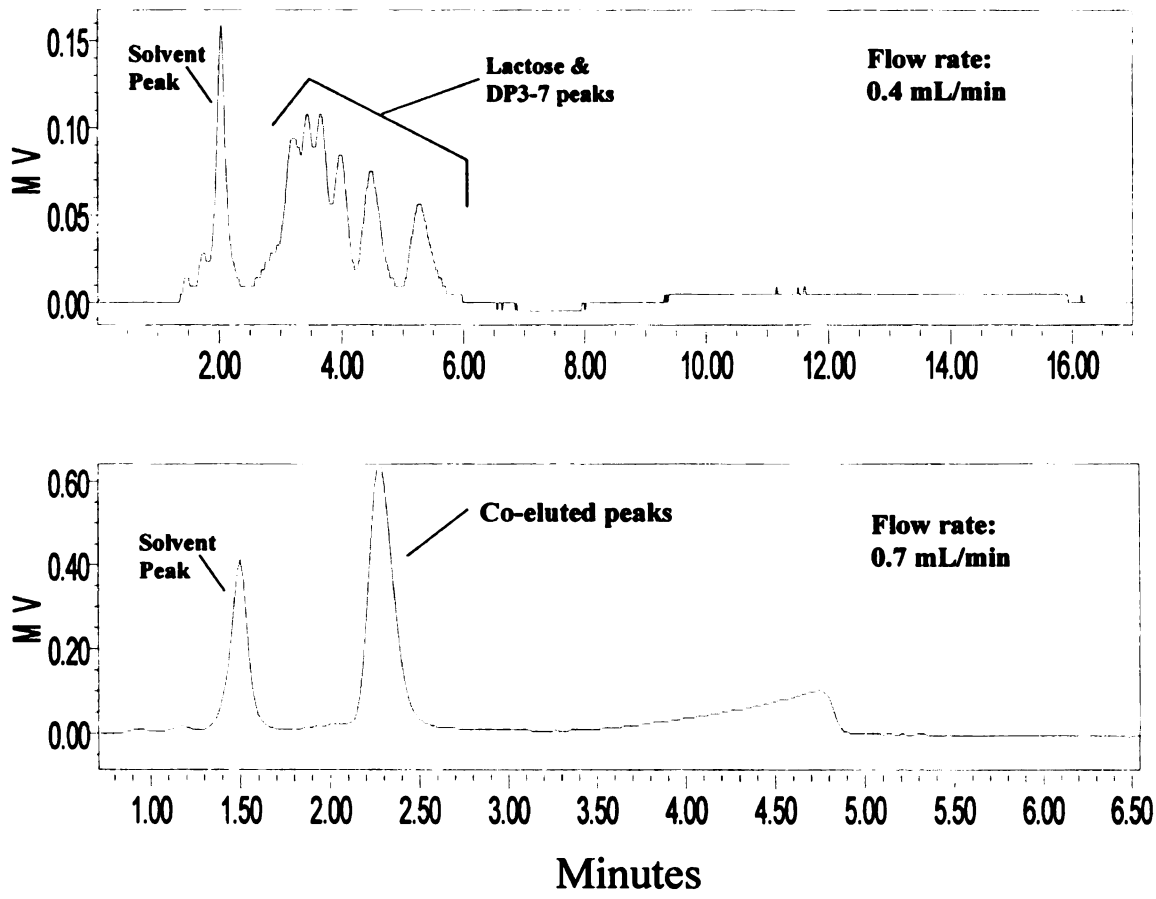

Figure 4.2. Chromatograms of lactose and malto-oligosaccharide (DP3-7) standard (0.02mM) at 0.4 mL/min (top) and 0.7 mL/min (bottom) flow rate showing the co-elution of peaks. HPLC-grade water mobile phase, 3 µm anion exclusion particle size resin and ambient column temperature.

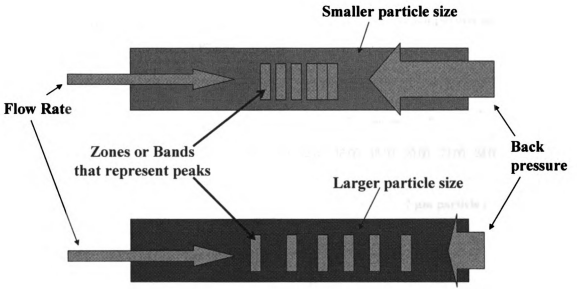

Figure 4.3. Illustration of backpressure effect on peak co-elutions within small and large particle size resins in HPLC columns.

Figure 4.4 shows the chromatograms for both particle sizes at optimum flow rate. Though the sharper peaks and shorter retention times observed with the 3-µm particle size would offer better resolution, this was offset by the severe co-elutions as a result of high backpressure. There appeared to be better separation of peaks with the 7 µm particle size; however, at the expense of some peak broadening. Despite this, better separation with the larger particle size may be preferred.

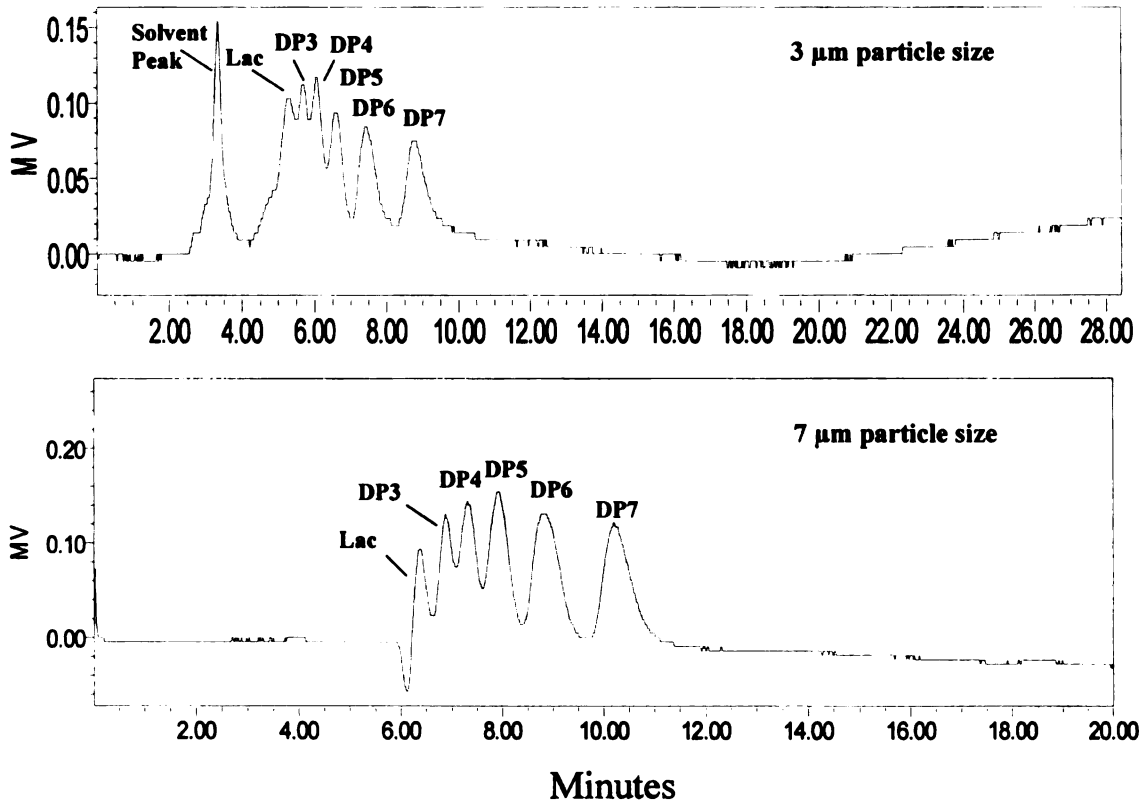

Figure 4.4. HPLC chromatograms of 0.02mM sugar mixture of lactose and malto-oligosaccharides (DP3-7) at ambient column temperature, HPLC-grade water mobile phase and 3 (top) and 7 (bottom) µm particle size resin.

4.3.2 Column Temperature

Figure 4.5 shows a significant temperature effect on average resolution for both resin sizes ($P<0.001$), where increases in temperature caused a severe increase in peak broadening and co-elution (Figure 4.6). The smaller particle size resin showed more sensitivity to temperature. This result was not surprising, since temperature would decrease fluid viscosity thereby increasing convective fluidity and the influence of backpressure (74). In general, higher temperatures were undesirable in an IEC system, and ambient column temperature proved to have the best resolution.

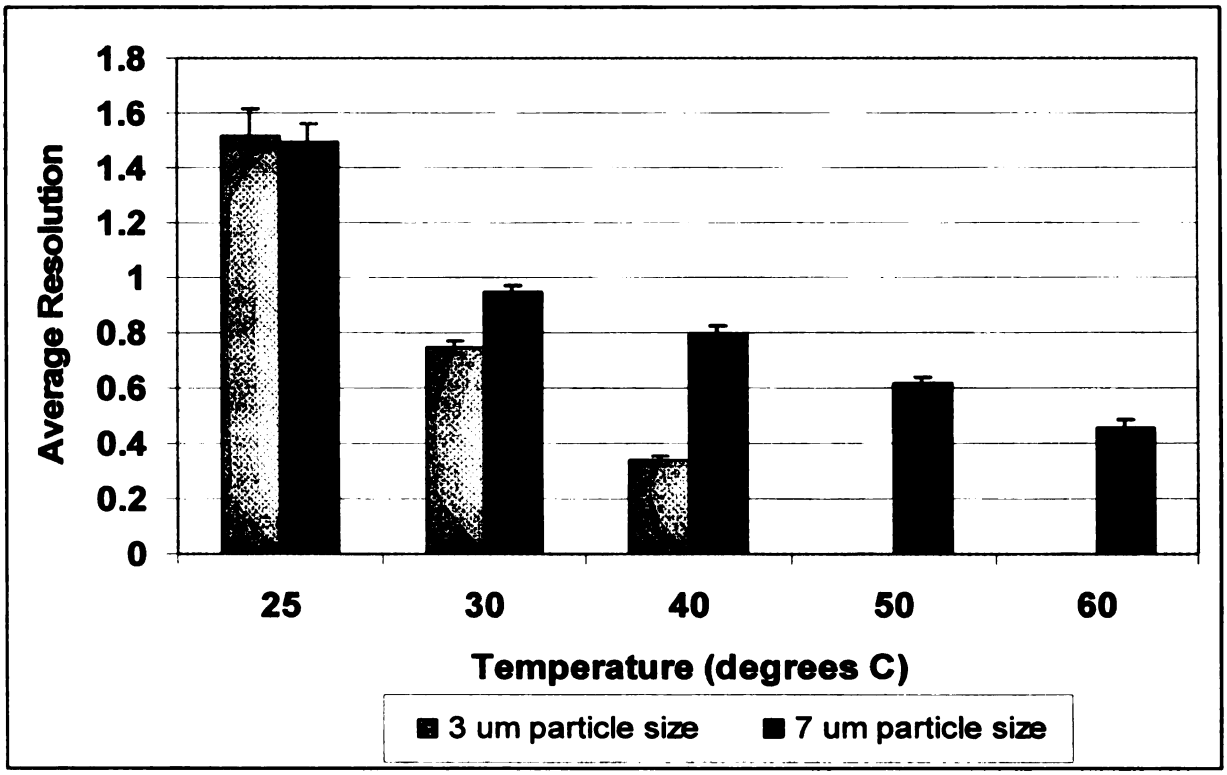

Figure 4.5. Temperature effect on average resolution for 3 and 7 µm particle size resin.

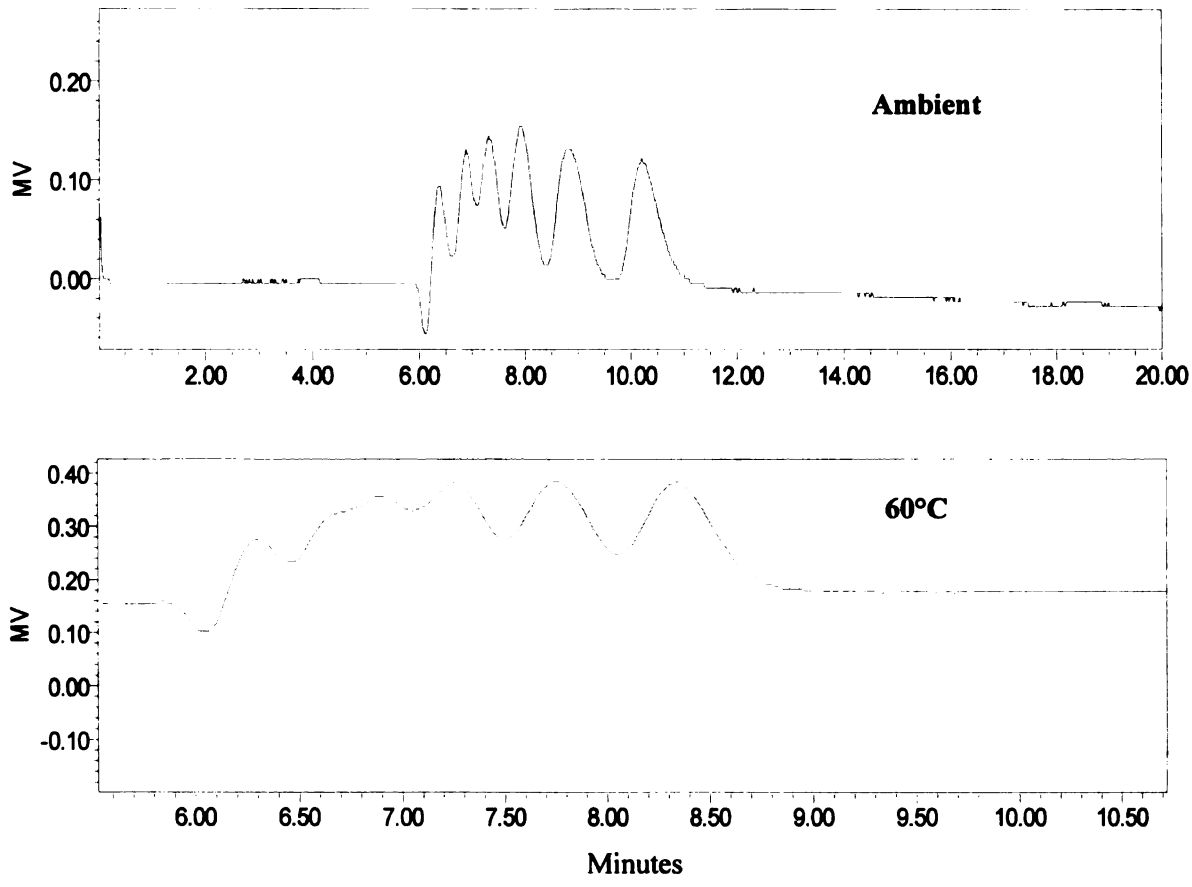

Figure 4.6. HPLC chromatograms of 0.02 mM sugar mixture (lactose and malto-oligosaccharides (DP3-7)) at ambient and 60°C column temperature, with 7µm particle size resin and 0.4 mL/min flow rate of HPLC-grade water.

4.3.3 Particle Size

Overall, the 3-µm particle size did not improve resolution and separation of oligosaccharides when compared to a 7-µm particle size resin. This was unexpected, since it is well-known that smaller particle sizes would create more theoretical plates which would increase resin-chemical interaction thereby refining separating capabilities (74, 76, 77). However, the 3-µm particle size column length was shorter (150 mm) than the 7-µm particle size column (250 mm). This could have certainly diminished theoretical plate count. (Note: Longer

3-μ-m particle size column lengths could not be used since they would cause high backpressures.) Furthermore, higher backpressures within the smaller particle size resin affected resolution capabilities. Thus, the better particle size would be 7 μm because of less backpressure and better separation.

4.3.4 Separation of GOS Mixture

Separation of the GOS mixture containing lactose, GOSs and residual monosaccharides proved to be very difficult under optimized conditions (ambient column temperature, 0.4 mL/min flow rate and 7 μm particle size). The likely reason was the high concentration of lactose in the mixture at ~50% of total sugars which overpowered the GOS peaks which combined to be ~45% of total sugars. Resolution was improved when the solution was diluted 100-fold; however, the separation would not be useful on a process scale due to persistent co-elution as well as the dilution.

4.3.5 Other Chromatographic Procedures and Future Directions

The IEC chromatograms in general showed an elution order of ascending DP or molecular weight. This suggests that the main separating mechanism is reverse-phase or non-polar adsorption chromatography. This is characteristic of ion exclusion resins, as the neutral charged compounds adsorb to the membrane layer of the resin and migrate down the column as a result of steric properties. Relative retention times were also fairly close to reversed phase (C18) analysis of malto-oligosaccharides (DP3-7) (81).

Other types of chromatography could be used to separate oligosaccharides. Better resolutions have been achieved by using ion exchange columns (Rs≅2) with an anion exchanger (Ca^{+2}). However, the use of harsh solvents preclude their use for a food application (81). We were able to achieve better resolutions (Rs≅1.5-2) using IMPC. In IMPC, porous beads of a sulfonated polystyrene DVB matrix are coated with a cation, Na^{+} Ca^{+2} or Ag^{+}. This coating allows smaller sugars (i.e. lactose) to adsorb within the pores first due to their steric and relatively more reactive properties, while less reactive and larger sugars (i.e. DP>3) adsorb less readily. This would cause sugars to elute in the order of decreasing molecular weight (i.e. higher DP elute first, lower DP elute last).

Figure 4.7 shows the IMPC and IEC chromatograms under their optimum conditions. The chromatograms in Figure 4.7 show that IMPC is superior to IEC in separating lactose from the oligosaccharides. However, IEC proved to be superior in resolving between oligosaccharides from 3 to 7 DP. The results suggest that a hybrid of the two types of chromatography would be appropriate. Future directions of this research should include an investigation of new resins that combine the adsorption properties of IEC with the pore and counter-ion properties of the IMPC. Also, combining these two column types to operate in series may be appropriate to separate lactose from the oligosaccharides using IMPC, and then separating individual oligosaccharides using IEC. Further investigation in this area is also required.

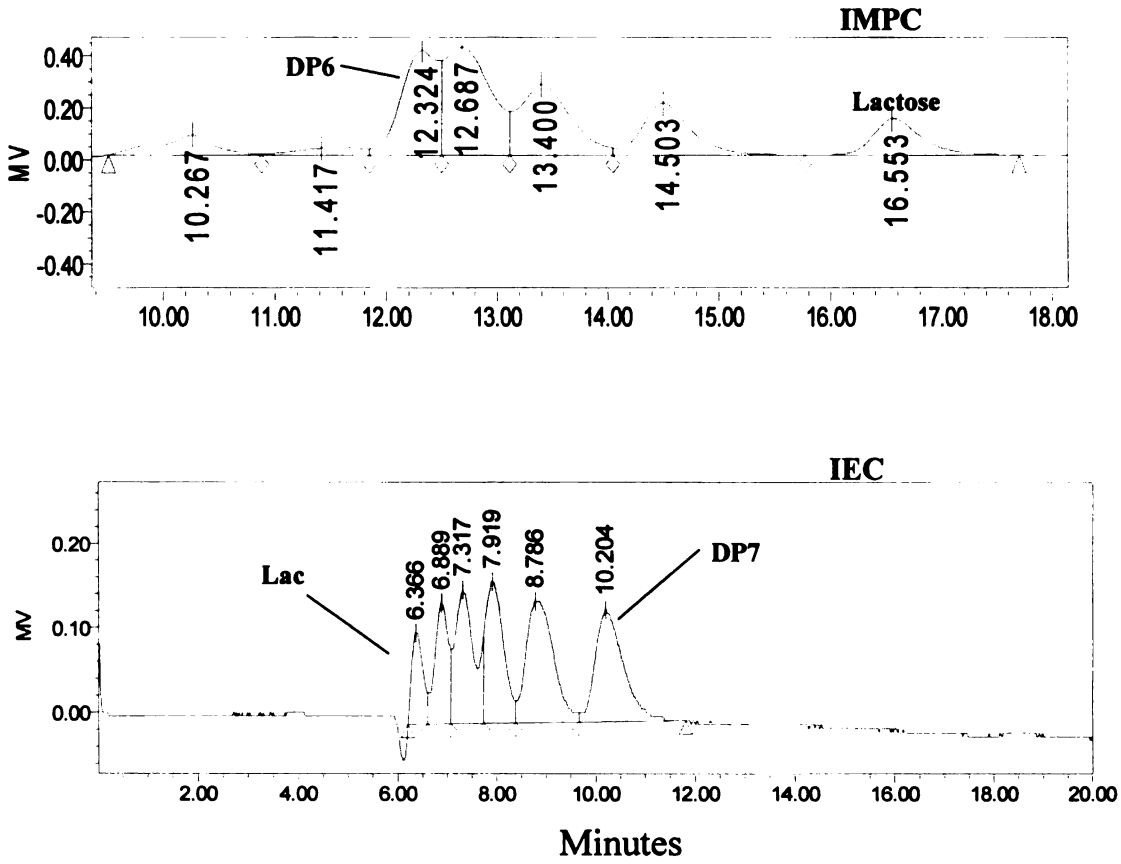

Figure 4.7. Chromatograms of sugar solutions containing 0.02 mM of lactose and malto-oligosaccharides run with HPLC-grade water at 0.4mL/min for ion moderated partition and ion exclusion chromatography.

4.4 Conclusions

The study showed that higher flow rate and higher temperature cause poor resolution in IEC systems. Furthermore, smaller particles size did not effectively enhance resolution and separation of oligosaccharides due to increased backpressures. GOS mixtures could not be resolved because of the high concentration of lactose; however, IMPC was found to effectively separate

lactose from the GOSs. Future work that uses both technologies would be invaluable to the sugar, chemical and pharmaceutical industries.

4.4.1 Novel Contributions

This study has three worthy contributions to science and technology:

1. A new and relatively fast analytical method to analyze oligosaccharides using IEC.
2. A better understanding of the mechanism that underlies oligosaccharide separation within IEC and IMPC systems
3. Preliminary data for further work within research of a combined IEC and IMPC system.

CHAPTER 5

MARKET ANALYSIS

5.1 INTRODUCTION

5.1.1 Prebiotic Market

Functional Foods. A functional food is defined as a food providing health benefits beyond basic nutrition. The functional food market has expanded globally in recent years due to an increasingly large aging and more educated population as well as the increased incidence of obesity and cancer. The estimated sizes of the functional food markets are $18.25 billion in the U.S., $15.4 billion in Europe, and $11.8 billion in Japan (2). The U.S. functional food market grew 8.5% in 2001 and is expected to grow 7.5 % for the next few years (2). Furthermore, nearly two-thirds of grocery shoppers report that their purchase decisions are driven by their desire to either reduce the risk of, or manage, a specific health condition. As a result, experts believe that the functional food market will comprise 10% of all food choices in 10 years (2).

Prebiotic Market. Prebiotics are considered a segment of the functional food market. The products within this market include mostly fructo-oliogosaccharides (FOSs) and inulin, with some production of malto-oligosaccharides (MOSs) and galacto-oligosaccharides (GOSs) (5, 82). These products ordinarily serve as ingredients for food and health novelties (i.e. nutritional beverages, yogurts, nutritional bars). However, the nutraceutical market has adopted inulin and FOSs in supplement form (1-4, 7, 18, 67).

The three largest prebiotic markets in the world are in Japan, Europe and the U.S. Of these, the Japanese market is the most mature with a market size of approximately $800 million in 2003, with a 10% annual growth rate (19). The European prebiotic market is still in the embryonic stages at $103 million but is expected to reach $216 million by 2010 (19). The U.S. market is still in its fledging stages at $15 million in 2003 but is expected to increase markedly to $103.2 million by 2010 (19). Currently, U.S. consumer knowledge of prebiotics is low; however, experts believe that with new research, the rising market success of probiotics and better consumer education through marketing and advertisement, the U.S. prebiotic market will grow quickly. Furthermore, global companies, such as Danone® and Nestle® are targeting the U.S. with their products (1).

There are very few companies that solely manufacture prebiotics. Most of these firms are located in Japan and Europe and they include Nihon Shokuhin Kako Co., Orafti Co., and Sensus Co. (19). The price of prebiotics varies with its application. Prebiotic ingredients range from $2 to $8 per pound solid (83). Currently, prebiotics are manufactured either through hydrolysis (inulin hydrolyzed to FOSs), extraction (i.e. inulin from chicory root) or through an enzymatic process (isomalto-oligosaccharides and GOSs). These prebiotics are offered as mixtures with few functional properties other than as a nutritive additive and bulking agent.

The prebiotic market is mostly segmented to the specialized food/beverage and nutritional supplement markets. Yogurts, certain baked goods and nutritional

bars have been the major foods that contain prebiotic ingredients. Dairy-based drinks and nutritional beverages within Europe and Japan have shown strong market success. Dietary supplements of mostly inulin and FOSs have been marketed, but have shown less success than food-based products. Greater market success and implementation is expected with increase in research and consumer education (5, 82).

5.1.2 Whey and Lactose Markets

Whey. In 2004, 8.8 billion pounds of cheese (excluding cottage cheese) was produced in the U.S. This was a 3.7% increase from 2003 and has been steadily increasing (27). World cheese production in 2004 was estimated at ~40 billion pounds of cheese (27, 28). Market experts predict that cheese production will show an overall increasing trend within the U.S. and the world as the demand for more ethnic cuisines, especially Italian and Mexican, increases (27, 28).

Whey accounts for 90% of the weight during the cheese-making process and presents a serious economic and environmental concern to dairy processors. Though many large cheese manufacturers have found ways to process and market whey, the world's liquid whey surplus reached ~200 billion pounds in 2004 (27). Total U.S. dry whey production in 1999 was 1.18 billion pounds and has seen an increase of ~3 billion since then (27, 84, 85). This is a result of the marked increase in whey protein concentrate (WPC) as an ingredient. This increased demand has raised the market price of dry whey from $0.09 to $0.27 per pound. WPC has a much higher market price of $0.88 to

$0.90 per pound since whey protein has been fractionated from the whey and concentrated (26, 27, 84-86). Experts have pointed that this market price will continue to rise (84-86).

Lactose. Although whey has increased in demand and price, the lactose fraction has had low market value and will continue to lose value as the WPC fraction increases in demand. Currently, market price for dry powder lactose ($0.10 – $0.15 per pound) is about half of dry whey (27). Liquid lactose syrups usually require less processing and can cost about $0.05 per pound solids (27). Low lactose market value is due to a number of reasons. First, lactose is non-digestible by a large number of the population. Second, lactose is 60% less sweet than regular table sugar. Third, lactose has poor solubility and may affect texture and mouthfeel of foods (87, 88).

Lactose has mostly been sold as a fermented food substrate, pharmaceutical caking agent, confectionary ingredient and an animal feed (85, 87-89). It is also exported cheaply to markets in Asia and Europe. These markets often use the whey lactose in their food and pharmaceutical products which they in turn sell to the U.S. (86, 89). The market demand is very low and not expected to increase, thereby driving the market value lower (28).

Adding value to lactose has been investigated by a number of companies and research groups. Some have hydrolyzed lactose to glucose and galactose, others have derivativized lactose to lactitol, lactulose and lactobionic acid (87,

88). Despite these efforts, the value of lactose has still remained low and the surplus of whey lactose still remains high.

We propose that a typical mid-size dairy facility could produce GOS prebiotic syrups using their whey lactose surplus through an enzyme process and subsequent GOS fractionation nanofiltration (NF) process developed in our laboratory. In this paper, we will determine a cost-effective enzyme process by comparing two viable enzyme methods (free- vs. immobilized-enzyme processes). Also, we will conduct yearly cost calculations to produce GOSs within a typical mid-size dairy facility. Additionally, we will conduct a SWOT analysis on the product. Furthermore, we will discuss the importance of purification of individual oligosaccharides and recommended future research directions. Finally, we will provide the main market drivers for the continued and future success of these GOS products.

5.2 Estimated GOS Production Economics

Selection of Enzyme Process. Two types of enzyme processes to produce GOS syrups exist. One process is a free-enzyme system that uses ultrafiltration (UF) technology, while the other process is an immobilized-enzyme system, where cloth is the immobilizing medium. The costs of the free- and immobilized-enzyme systems were determined in Table 5.1. Both systems were calculated to operate at 9600-L/h capacity on an annual basis.

In the free-enzyme system, the reactor volume is typically about 4000 L (90). Free enzyme cost was calculated as follows:

$$4000\,L \;\; x \;\; \frac{4.5\,g\,of\,enzyme}{1\,L} \;\; x \;\; \frac{\$0.063}{1\,g\,of\,enzyme} \;\; \approx \;\; \$1134\;per\;0.5\;months$$

$$\$1134 \;\; x \;\; 24 \;\; \approx \;\; \$27{,}216\;per\;year$$

The immobilized-enzyme system was calculated on the basis of initial cloth weight and the amount of enzyme needed. The amount of initial cloth weight needed for 9600-L/h capacity was determined using **Eq. 5.1** (90).

$$W_2 = \frac{(W_1)(v_2)}{v_1} \qquad \textbf{Eq. 5.1}$$

W_1: Experimental-Scale Cloth Weight (2 g)

W_2: Industrial-Scale Reactor Cloth Weight (g)

v_1: Experimental Flow Rate (5.5 mL/min or 0.33 L/h)

v_2: Industrial-Scale Flow Rate (9,600 L/h)

$$\frac{(2\,g)(9600\,L/h)}{0.33\,L/h} = 58{,}181.8\,g\;of\;initial\;cloth$$

In our experiments, we found that 2 g of initial cloth weight would require 0.5 g of pure enzyme. So that the final cost calculation was:

$$58{,}181.8\,g\;of\;cloth \;\; x \;\; \frac{0.5\,g\,of\,enzyme}{2\,g\,of\,cloth} \;\; x \;\; \frac{\$0.61}{1\,g\,of\,enzyme} \;\; \approx \;\; \$8{,}872.72\;per\;4\;months$$

$$\$8{,}872.72 \;\; x \;\; 3 \;\; = \;\; \$26{,}618.18 \;\; + \;\; \$480(reagent\;cost) \;\; \approx \;\; \$27{,}098\;per\;year$$

From our calculations, the total annual enzyme cost would be slightly lower with an immobilized-enzyme system at 9600-L/h capacity. However, since a membrane filtration system typically exists in a mid-size dairy facility, the capital cost of $500,000 is not included (91). Typically, reactors for immobilized-enzyme systems are not housed within dairy facilities. Thus, an immobilized system would increase capital expenditure by about $250,000, while annual maintenance cost would be about the same as an UF membrane system at $1,000 per year (91, 92). Thus, the free-enzyme system would be the best option for a mid-size dairy facility.

Table 5.1 – Enzyme process costs for UF free-enzyme and immobilized-enzyme systems at 9600-L/h capacity.

	Enzyme Process Cost	
	Free-Enzyme	**Immobilized-Enzyme**
Enzyme	$0.063 per g	$0.61 per g
Reagents:	N/A	$378 per yr (PEI) $102 (GA)
Reactor Volume/cloth weight	4000 L	58,181.8 g
Enzyme Lifetime:	0.5 months	4 months
Estimated Annual Cost:	$27,216	$27,098

	Enzyme Reactor Equipment Cost:	
Equipment Cost: (9600L/h capacity)	No Capital Cost ($500,000)	$250,000
Annual Maintenance:	$5,000	$5,000

Whey Lactose Cost Calculations. The following cost projections were estimated for a typical mid-size dairy facility that generates approximately 4,000,000 pounds of cheese per year, with about 40,000,000 pounds of liquid whey surplus available for GOS production (93). This liquid whey (containing 5% (w/w) lactose) must be further processed with NF and reverse osmosis (RO) to produce liquid lactose at 27% lactose concentration. The following calculations show that the approximate amount of 27% lactose syrup produced from 40,000,000 pounds of whey is 3.36 million liters per year:

$$40{,}000{,}000\ pounds \quad x \quad \frac{0.4536\ kg}{1\ pound} \quad x \quad \frac{\sim 1L}{1\,kg} \quad \approx \quad 18{,}144{,}000\ L\ of\ whey$$

$$\frac{(5\%\ Lactose)(18{,}144{,}000\ L)}{(27\%\ Lactose)} \quad \approx \quad 3{,}360{,}000\ L\ of\ 27\%\ lactose\ per\ year$$

Table 5.2 shows the cost calculations for a dairy facility that produces GOS syrups using the 27% lactose in a UF membrane system at 9600-L/h capacity. Lactose syrup can be purchased from a dairy facility at about $0.05 per pound solids or $0.000110 per g of soluble solids (26-28). This would equate to about $0.0298 per liter of 27% lactose syrup. To be conservative in our calculations, we used this market value for the cost calculation in Table 5.2, though in-house production cost should be considerably less. Our estimated production cost for 27% lactose syrup is $100,128 (Table 5.2).

Table 5.2 – Yearly cost of producing GOS mixture syrup from 27% lactose syrup and UF free-enzyme system at 9600-L/h capacity in a mid-size dairy facility.

	Calculation	Amount
Cost to produce 27% lactose syrup	$0.0298/L x 3.36 million L	$100,128
Free enzyme cost	4000 L x 4.5 g enzyme/L x $0.063/g	$1,134
UF enzyme system maintenance cost		$5,000
Nanofiltration maintenance cost		$5,000
Total Annual Cost		$111,262

GOS Production Cost. The length of production time to process 3.36 million liters of 27% lactose syrup was approximated to be 15 days of 24 h production using a UF membrane system at 9600-L/h capacity. Since the free-enzyme is stable for 2 weeks, yearly enzyme costs are estimated to be about $1,134. UF maintenance costs were approximated to be about $1,000 for this two-week period. The NF cost to fractionate GOSs was also approximated to be about $5,000 for this two-week period (91, 92). These membrane filtration cost projections total to approximately $11,134 per year and are very conservative. Along with lactose syrup costs, the total estimated cost of GOS production is $111,262 per year (Table 5.2).

Annual Amount of GOS produced. Amount of GOS produced can be estimated with a simple series of calculations. First, mass of initial lactose substrate within 3.36 million liters of 27% lactose syrup is determined to be 907.2 million grams of solid lactose. Due to the laws of mass conservation, this mass also represents the mass of total sugars after the enzyme reaction. Thus, to

determine total GOS amount, this figure is then multiplied by 22% (the maximum GOS yield). Total annual GOS amount was calculated to be about 200 million grams or about 440,000 pounds of solid GOSs. The GOS cost per pound solid was figured by dividing the total annual cost in Table 5.2 ($111,262) by 440,000 pounds of solid GOSs. With this calculation, GOS cost was approximated to be about $0.25 per pound solid. This processing cost is low enough to be competitive within the prebiotic market even with 200% mark up (about $0.75 per pound solid), since other prebiotic ingredients range in market price between $2 and $8 per pound solid (83).

5.3 SWOT Analysis

Strengths. In general, GOS syrups have a number of strengths and advantages over other bulk or added ingredients. First, these products have associated prebiotic and health benefits, which can easily be marketed on product labels. GOS mixtures are also "natural" ingredients and as a result will likely be accepted by consumers. Furthermore, these products have some sweetening capability and are lower in calories than conventional sugars. They are also considered soluble fibers and can be used as fat substitutes. These products are very heat stable and can enhance texture, mouth feel and flavor of food/beverage products.

In comparison with other prebiotic ingredients currently available on the market (i.e. FOSs and inulin), these products are less costly at $0.23 to $1 per pound solid of GOSs rather than between $2 to $8 per pound solid of FOSs or

inulin. GOS mixtures also can be easily manufactured within a dairy facility that also produces the raw substrate (lactose). Furthermore, technology and equipment already exists in the U.S. and within these dairy facilities to produce large volumes of these products. FOSs and inulin are often manufactured in facilities that house specialized equipment, and the raw ingredients must be purchased from other facilities. Finally, these facilities are often in foreign countries and are subject to high taxes and tariffs when imported to the U.S.

Weakness. Despite the number of strengths, GOSs have some important limitations. In general, the GOS syrups with a mixture of DPs and lactose may have limited functional properties. For example, the GOS mixture has limited sweetness compared to sucrose, mostly due to the longer chain-lengths. As a result, the GOS mixture can only be used as a complementary sweetener and bulking agent with more intense sweeteners. Also, GOS fat-mimicking capabilities are limited because of the degree of shorter chained GOSs (i.e. DP3) and the limited amphoteric capabilities of the GOSs, in general. Finally, the amount of lactose and the general limited solubility of these products are other weaknesses. Despite these limitations, GOS marketability as a functional food along with their ability to improve mouth feel and flavor may offset these limitations. Furthermore, these products can be marketed and labeled as "natural ingredients" with added health benefits.

GOS prebiotics have the same general weaknesses as other prebiotics currently on the market. However, one important weakness to consider is the

popularity of and established research on the FOSs and inulin. However, there has been much research done showing that GOSs provide the same health and functional benefits as FOSs and inulin. Another weakness is that GOSs are produced from a variable source (whey), which may decline in production or availability. However, with surging cheese and WPC production, whey lactose surplus shows no signs of declining.

Opportunities. The production of GOS mixture syrups has a number of opportunities. Most of these opportunities are in the ingredient, sweetener and fat substitute markets. Another major opportunity is the utilization of the inexpensive and abundant whey lactose surplus, which can be profitable and cost-effective for a dairy processor. Also, GOS syrups can be made very easily within U.S. dairy facilities, since they already contain much of the necessary technology (i.e. membrane filtration). This is another important opportunity for U.S. companies to produce prebiotics domestically, since many of the foreign companies (i.e. Europe and Japan) that produce FOSs and inulin must use specialized equipment and must pay exorbitant taxes and tariffs to import to the U.S. Finally, the GOS process proposed in our report provides glucose and galactose as co-products. These products can be used as fermentable sugars for fuel production, providing yet another opportunity for a processor.

More potential opportunities exist if a process is developed to separate and purify individual chain-lengths. With pure standards of individual OS chain-lengths, more mechanistic research in medicine and nutrition can be done. This

could lead to great strides in medicine and pharmaceutics, which could also open greater market opportunities for the chemical, pharmaceutical and medical industries. Finally, the technology to separate and purify individual GOS chain-lengths could be used with other oligosaccharides for prebiotic, vaccine, biotechnology, medical and pharmaceutical applications.

Threats. There are a number of important threats to the market success of these products. One major threat is the increasing expense and maintenance of the process due to increasing fuel and utility costs. However, continuing research on efficiency and utilization of co-products (i.e. glucose and galactose) could offset this threat. Another threat could be declining cheese/whey/lactose production. However, trends do not seem to suggest this would happen, in fact, cheese production and demand is expected to continue to increase strongly. Another threat may be more cost-effective alternative uses for lactose, thereby decreasing lactose supply and increasing lactose market price. Thus, research in process efficiency and versatility with other products (i.e. FOSs) may be important. Finally, current prebiotic mixtures (FOS or inulin) may eventually be produced more inexpensively than the GOS mixtures. However, FOS and inulin market price is far greater than the GOS production cost, and continuing GOS process-efficiency research will assure continued competitiveness.

5.4 Pure Chain-Lengths and Target Markets.

As suggested above, the technology to separate individual GOSs and perhaps other oligosaccharides provides a number of other target markets. This technology could diversify and widen prebiotic market success. However, we expect that these prebiotics will only capture incremental shares of each of these markets, as many of them are well established and quite broad.

Low Calorie Sweeteners. The low calorie sweetener market comprises of artificial and natural sweeteners with a caloric value less than conventional table sugar (sucrose). This market was estimated to be around $11 billion worldwide in 2002 (94). This market is dominated by saccharin, aspartame and sucralose (Table 5.3). From 2002 to 2004, saccharin and aspartame sales had decreased by ~10%, while sucralose sales rose by 136% (94). This remarkable increase was due mostly to the intense and extensive marketing and advertising of sucralose as a natural sweetener made from sugar. Since the general public regards natural products as more safe and beneficial, the Splenda™ brand has reaped the benefits.

Table 5.3 - Major sweeteners in the low calorie sweetener market compared to DP3 GOS.

Sweetener	Commercial Name	Calories (cal/g)	Type	Sweetening Power to Sucrose	Market Price ($/pound)
Saccharin	Sweet N' Low Sugar Twin	0	Artificial	500x	9.07
Aspartame	Nutrasweet Equal	0	Artificial	200x	13.61
Sucralose	Splenda	0	Natural	600x	15.88
GOS (DP3)	None	1.7	Natural	0.6 to 0.8x	$0.50*

*Cost of GOS production and purification. Does not include mark up.

The DP3 GOSs, if separated and purified, would be a natural sweetener with prebiotic benefits. This ingredient may be marketable as not only low calorie (<1.7 cal/g) compared to sucrose (4 cal/g) but also as a fiber and prebiotic (7, 70). This could be an excellent market opportunity as prebiotic awareness increases and soluble fiber consumption increases. However, since DP3 has limited sweetening power (20-30% less than sucrose), its marketability is limited as a complementary bulking agent to be used along with more intense sweeteners (7, 70). As a result, DP3 could only capture a small percent of the market. Also, DP3 production costs are uncertain, as this depends on the purification process. As a result, it cannot be appropriately compared with other sweeteners in Table 5.3. However, since GOS mixtures were estimated to be about $0.25 per pound solid, the final cost after purification of DP3 is not

expected to be above $0.50 per pound solid. Which even after mark up would still be far less than other low calorie sweeteners (Table 5.3).

Fat Substitutes. The fat substitute market comprises of fat-, protein- and carbohydrate-based low caloric compounds that replace fat. Examples of these products include Olestra™, Simplesse™ and gums, respectively. These products help mimic textural, mouth feel and flavor properties of fats (95). The fat substitute ingredient market is expected to reach $1.015 billion by the end of 2006 and is expected to grow annually at 15% (95). Most fat substitutes are natural with the exception of a few (i.e. Olestra™, Methocel™ gums). This makes their marketability to the public much easier thereby increasing their implementation in food products. However, each type of fat substitute has a specific set of limitations and applications. Thus, there is a growing market for other fat substitutes with new applications.

The DP6 or DP7 GOSs could offer some textural and flavor enhancements in certain foods and beverages that would ordinarily use gums or Simplesse™ (7, 70). Advantages of the GOSs are their obvious prebiotic, "natural" and fiber benefits but also their excellent heat stability, since certain gums and protein-based fat substitutes lose functionality with high temperatures or shearing (95). The market price of gums or Simplesse™ is about $2.40 to $12 per pound (96). We estimate that DP6 or DP7 purification would yield about $0.50 per pound solid production cost. Though this is speculative and does not include mark up (roughly 200%), we suspect that these GOSs would be

competitive. However, DP6 and DP7 compounds may be limited to beverages and semi-solid foods (i.e. yogurt) (7, 70). Thus, we expect that only a small fraction of this market would be captured with the DP6-7 GOS product.

Nutritional Supplements. This market includes over-the-counter vitamin, mineral and health supplements. The estimated size within the U.S. is around $17 billion (97). Immune-enhancing supplements are currently at $2.12 billion and seem to be growing the fastest among nutritional supplements (97). Currently, prebiotic supplements, which also can be categorized as immune-enhancing, only comprise a small percentage of this market in the U.S. However, with continued research, consumer education, and the U.S. population's continuing appeal for supplements, it is expected that prebiotic supplements will capture more of the nutritional supplement market in coming years. Currently, only inulin and FOSs are offered in supplement form in the U.S. (19, 98). Europe and Japan have had more diverse product lines that also include GOSs, MOSs and soybean oligosaccharides (19). Most of these products are not refined and contain mixtures of different chain-lengths as well as other components. The market price depending on the supplement can range from $15 to $40 for a bottle of a hundred 250 mg prebiotic supplements (19). GOSs, either in pure chain-length or mixture, could be offered as a nutritional supplement at a competitive market price.

Chemical Industry. Purifying oligosaccharides will allow companies to select and choose an appropriate chain-length for a particular application while maintaining prebiotic and health properties. Purifying oligosaccharides is a very costly and/or secretive process. Japanese firms have dominated the pure oligosaccharide market, supplying mostly to chemical companies at exorbitant prices. The Japanese produced $46 million worth of nine different types of oligosaccharides in 1990 (18). Pure oligosaccharide standards can cost anywhere from $100 to $600 per mg (99). Currently, pure GOS standards are not readily available on the market (99). This demonstrates huge market opportunities for processors that can cost-effectively manufacture pure forms of individual GOS or oligosaccharide chain-lengths.

Emerging Markets. Production of individual GOS chain-lengths may propel more mechanistic research of prebiotics in the areas of nutrition and medicine. As more research communities demand pure standards of individual DPs, this could translate to market opportunities in the chemical industry (7, 22-25, 67). Among the known benefits of reduced colon cancer risk and enhanced immunity, recent research has shown that prebiotics may help individuals afflicted with Chrone's Disease and irritable bowel syndrome (IBS) (100). Recent research has also suggested that oligosaccharides of certain chain-lengths could serve as effective vaccines against pathogenic microbes (21-24, 66, 101-103). This could open up market demand for these pure DPs in the pharmaceutical industry.

5.5 MARKET DRIVERS

Consumer Interest and Functional Foods. GOS consumer interest is very high in Japan and Europe. These prebiotics are added to a variety of products including breads, fermented dairy foods and beverages (yogurts, kefir, LAC milk, etc.), jams, confectionery foods, baby foods, infant formulas and specialized foods for the elderly and infirmed (7). Recent advances in sweetening capability, flavor modification and health benefits of GOSs have sparked more interest in the U.S. market as well as in the Japanese and European markets (7, 9, 70, 71). Furthermore, U.S. consumer interest in nutraceuticals or functional foods is exponentially rising (1, 2). The Institute of Medicine's Food and Nutrition Board has defined functional foods as "any food or ingredient that may provide a health benefit beyond the traditional nutrients it contains" (3). The nutraceutical market is projected to grow to $60 billion by 2010 totaling to 10% of the U.S. food market with a 12% annual growth rate (4). The high market interest in functional foods is directly related to the increasing incidence of various health problems. Oligosaccharides directly address important health issues, including obesity, colon cancer, and immunology.

The following discussion is about the main drivers of the prebiotic market. Our discussions focus on prebiotic oligosaccharide mixtures, but we also include discussions on how prebiotic success may be improved if individual oligosaccharide chain-lengths are purified.

Obesity. Obesity is reaching epidemic proportions, where one in every four American adults is obese (104) About 39.8 million or 57% of American adults are considered overweight (104). Obesity is also a global problem; the World Health Organization has estimated that over 300 million adults worldwide are obese (105). A major reason for these statistics is the widespread prevalence of calorie-dense/low-fiber foods (104).

GOSs tackle the obesity issue in three important ways, either to be used as low-calorie sweeteners, fat-replacers or well-tolerable fibers. Specifically, the trisaccharide (DP3) has the greatest sweetening power of the oligosaccharides and has a caloric value (<1.7 cal/g) of less than half of sucrose (7). Pure forms of this trisaccharide will decrease the need for large amounts of higher calorie sweeteners (i.e. sucrose, high fructose corn syrup). Furthermore, DP3 is very palatable, natural and safe with added health benefits; this would make it more appealing than synthetic low-calorie sweeteners (i.e. saccharin, aspartame) currently viewed as "dangerous" and/or objectionable in taste and mouth feel by many consumers (2). Oligosaccharide mixtures currently available on the market fail to offer adequate sweetness because longer chain oligosaccharides reduce the sweetening power of the mixture. However, these longer chains, if separated and purified, can be used as fat-replacers due to their higher molecular weights and unique chemical interactions with each other as well as with other food components to offer a desirable texture, mouthfeel and flavor, which mimics that of some lipoid ingredients (7). In either the sweetening or fat-replacing application, GOSs serve as soluble fiber that is well tolerated by humans at 15-

20 g GOSs per day without noticeable side effects (6). Currently, the majority of Americans are deficient in daily dietary fiber intake thereby suggesting the need for oligosaccharide ingredients to meet dietary guidelines (7).

Colon Cancer. Colorectal cancer is the third most common form of cancer and the second deadliest in the U.S. (106). In 2005, the U.S. alone had approximately 145,300 new cases of colorectal cancer with about 56,300 deaths (106). GOSs and other prebiotics have been and are being investigated for their role in reducing the incidence of colorectal cancer.

Numerous research studies have associated oligosaccharide consumption with elevated butyrate or other short chain fatty acid (SCFA) levels in the intestinal tract. These SCFAs serve as an important energy source for epithelial cells of the distal colon (102). As a result, epithelial cells are more capable of repairing mutagenesis and thus preventing tumor formation (66, 101). It has been suggested that colonic bacteria, especially *Bifidobacterium* and *Faecaolibacterium* anaerobes, metabolize non-digested oligosaccharides to produce butyrate and other SCFAs as well as other fermentation products (107). Currently, there is a need to understand the exact metabolic pathway, as well as why certain chain lengths seem to increase butyrate better than others (23, 25, 66). Availability of pure chain lengths of oligosaccharides may help elucidate this.

Immunity. Human immunity has been a "hot" area of research because of the high incidence and prevalence of bacterial and viral infections. Oligosaccharides have been shown to enhance immunity in a number of ways. Oligosaccharides can *indirectly* enhance immunity by promoting the growth of beneficial intestinal microflora, which secrete chemicals that enable intestinal cells to combat infection against harmful pathogens (i.e. *Salmonella spp., L. monocytogenes, E. coli*) (22, 67, 108). These same probiotic bacteria may also stimulate immunomodulation by the host (109). Oligosaccharides may also have a *direct* immunological effect. Some studies showed that oligosaccharides of a certain chain-length mimic epithelial cell receptors where pathogens (i.e. *Salmonella* and *E. coli*) would attach, thereby reducing attachment and infection (67). Other studies have identified that certain oligosaccharide chain-lengths strongly stimulate immune response against dangerous strains of *Vibrio spp., Streptococcus spp.* and *Shigella spp.* (21, 24, 103). The mechanism is still unclear, but some have suggested that the oligosaccharide chain-lengths mimic the lipopolysaccharide cell membranes of these pathogens thus stimulating the host to produce antibodies that would combat future infection by these and other pathogens (21, 24, 103). Therefore, pure forms of oligosacharide chain-lengths have potential applications as vaccines and medications against infectious agents.

Aging Population. As the largest cohort in the U.S., the Baby Boomers are aging and have become enamored with staying healthier, living longer and

looking better. The number of Americans over age 55 will increase from 67 to 76 million from 2005 to 2010 accounting for most of the total population growth (97, 110). This has been the cause for the speculatively large growth rate of the functional food and nutraceutical markets. This cohort is educated and generally keen in learning about new ways to improve their health. As a result, they may be eager to learn and experiment into the prebiotic market. This has been one of the main reasons for the expected growth in the prebiotic market.

5.6 Conclusions

Our market analysis has shown that with the surging functional food and prebiotic markets along with the increasing whey lactose surplus, conditions are ideal for GOS syrup production. Furthermore, this assessment showed that mid-size dairy facilities could easily implement a free-enzyme GOS process, since they already house the necessary equipment and technology (i.e. membrane filtration). A SWOT analysis revealed that the strengths and opportunities to produce GOS mixtures far outweigh the weaknesses and risks. Finally, new technology that could separate and purify individual GOS chain-lengths could offer greater opportunities in many other target markets.

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions. In chapters 1 and 2, optimum enzyme conditions were determined for UF free-enzyme and immobilized-enzyme systems. Optimum initial enzyme and lactose concentrations were 42.3 U/mL and 270 g/L, respectively. These conditions yielded relatively high GOS yields (20-22%) in short start-up times (15 min) when compared to the literature. Chapter 2 revealed that the developed continuous UF free-enzyme system may be the most feasible process for a typical GOS manufacturer. This is because of the system's high agitation and possibly higher pressure over batch or immobilized systems, which led to consistently higher GOS yield (~22%), higher GOS throughput and shorter start-up time. In this same system, we showed that residual monosaccharides had no significant inhibitory effect on GOS production, which is an important finding when considering the process in Objective #2. Furthermore, UF systems may be more desirable for processors (especially dairy processors), since enzyme costs and capital expenditure would be lower than immobilized-enzyme systems. Thus, we conclude that the free-enzyme UF system developed in our studies accomplishes Objective #1 as an optimal and commercially-feasible system to produce GOSs.

Chapter 3 showed that a simple and easily scalable NF diafiltration process would achieve over 90% removal of monosaccharides and ~50% removal of lactose from resultant mixtures of the enzyme process, with <19% loss of total GOSs. The novel NF process used in these studies manipulated pH and concentration to optimize the fractionation of GOSs. Though an ideal

fractionation or preconcentration would be 100% retention of GOSs with 100% permeation of lactose and monosaccharides, the GOS fractionation in this study may be adequate for the production of a GOS syrup. Furthermore, the process is commercially feasible since many processors house NF technology and could easily implement this developed process. Thus, we conclude that Objective #2 was accomplished using a novel NF system that manipulates pH and concentration.

In chapter 4 IEC showed to be successful in separating oligosaccharides from DP3 to DP7 with ambient column temperature, 0.4 mL/min HPLC-grade water mobile phase flow rate and 7 μm particle size. Despite the good separation of oligosaccharide standards, the GOS syrup derived from the processes developed in Chapters 1 though 3 did not show good separation using IEC, since the lactose peak overpowered the GOS peaks due to the relatively higher lactose concentration. However, in subsequent studies, we showed that IMPC could very effectively separate lactose from the oligosacchardes, but individual oligosaccharide separations were not resolved well. From these data, a new or combined chromatography technology can be conceived, where IMPC could first separate lactose from the GOSs and then IEC could more refinely separate the individual GOSs. Though our results are preliminary and not yet commercially scalable, we believe that these findings have made important strides towards accomplishing Objective #3 to separate and purify individual GOSs.

Since purification could not be fully accomplished in our studies, a cost and market assessment for production of GOS syrup containing a mixture of GOS DPs and lactose was done. The process economic assessment showed that the free-enzyme UF system would be more cost-effective and commercially feasible than an immobilized-enzyme system for a mid-size dairy processor. The cost assessment also showed that GOS syrup production from in-house equipment and substrate (whey lactose) was estimated to be around $0.25 per pound solid for a mid-size dairy processor. This production cost is much lower than the current market price range for prebiotic mixtures ($2 to $8 per pound solid), suggesting that even after mark-up the GOS product will be competitive in the prebiotic market. A SWOT analysis further revealed that the strengths and opportunities of the GOS product outweighed the weaknesses and potential risks. Furthermore, it was concluded that the surging functional food, prebiotic, cheese and WPC markets create ideal conditions for GOS production within U.S. dairy facilities.

Future Directions. The above research has resolved many of the production and fractionation problems in GOS production. Also, the research has shown some great strides in technology that purifies individual oligosaccharides as well as market analysis on the market potential of these products. However, further research is required in a number of areas. Some of these avenues of research are listed in point form below:

- Test enzyme process using whey lactose permeate (with 27% lactose concentration) to identify any enzyme inhibition due to other components within the permeate.
- Develop a generic mathematical model to predict GOS production.
- Investigate the use of genetically enhanced β-galactosidase to improve GOS yield and production.
- Improve immobilization process for less enzyme loss and better GOS yields/throughputs.
- Research the utilization of the glucose and galactose streams (i.e. fuel production, enzyme production from microbe-cultivation, etc.).
- Investigate other membrane types or compositions to enhance GOS fractionation from monosaccharides and lactose. If lactose removal is high enough, then IMPC would not be necessary for DP purification.
- Develop a cost-effective hybrid chromatography that uses IMPC and IEC.
    - Scale-up the chromatography process to test for purity, commercial feasibility and cost-effectiveness.
    - Conduct simulated moving bed chromatography experiments
    - Test individual DPs for nutritional, pharmaceutical and functional effects.
- Conduct extensive market analysis on individual GOS DPs once a purification process is developed.
- Use market analysis and pilot-scale processes as teaching tools for outreach programs directed at the dairy industry.

APPENDIX 1

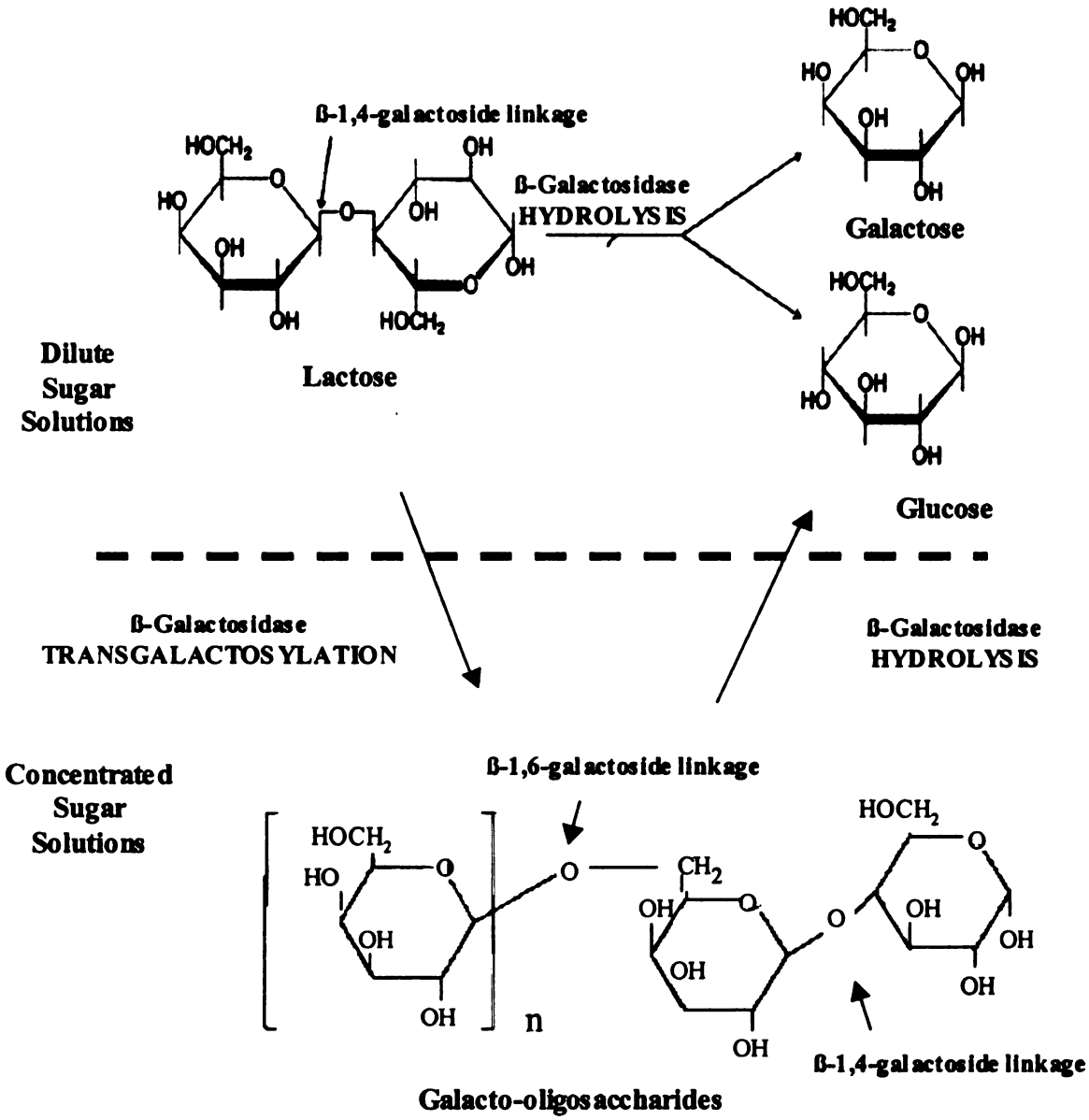

Figure A.1.1. The ß-galactosidase hydrolysis and transgalactosylation mechanism of ß-1,4-lactose.

APPENDIX 2

Table A.2.1 Results of Bradford Assay in absorption (AU) at 595 nm for initial, permeate and retentate fractions of a UF run at 100-400 p.s.i., 18.9L/min and 40°C for 30 min.

	Pressure							
	100 p.s.i.		200 p.s.i.		300 p.s.i.		400 p.s.i.	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Initial (5 L)	259	253	253	256	260	270	263	254
Retentate (2.5 L)	540	531	537	541	540	536	540	544
Permeate (2.5 L)	ND	ND	ND	ND	ND	ND	ND	ND

APPENDIX 3

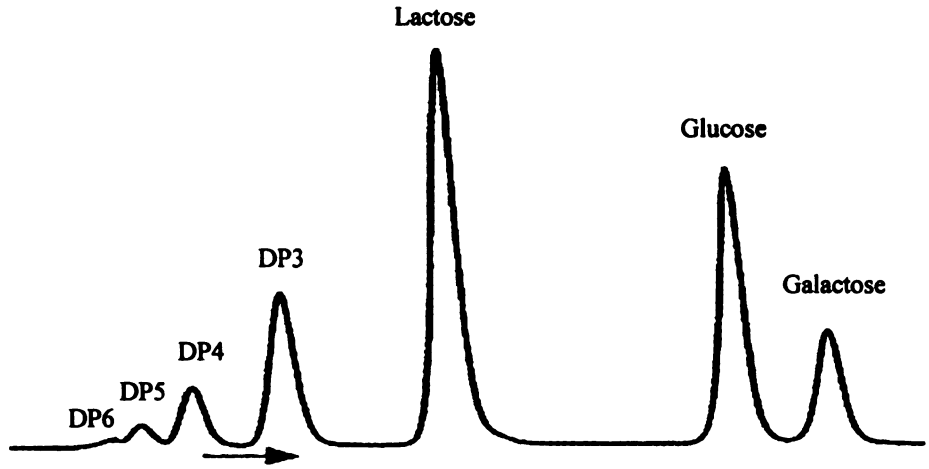

Figure A.3.1. A typical HPLC chromatogram showing various sugars found in lactose hydrolysis. Elution time (min): 10.1 for DP6, 12.2 for DP5, 12.8 for DP4, 13.8 for DP3, 16.2 for lactose, 19.5 for glucose and 20.5 for galactose.

APPENDIX 4

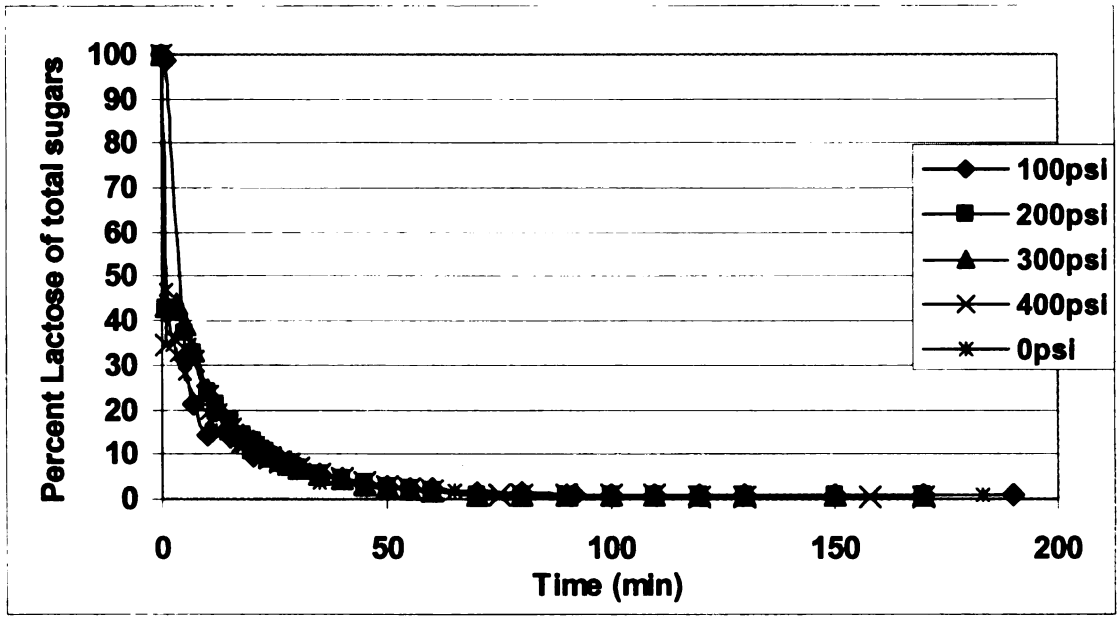

Figure A.4.1. Lactose conversion over time (min) of a 5% (w/v) lactose solution containing 42.3 U/mL of ß-galactosidase at pH 4.5 and 40°C during UF at various fluid pressures and a 0 p.s.i. control batch solution.

APPENDIX 5

Table A.5.1. Effluent, retentate and permeate samples collected from 5 and 24 h immobilized-enzyme and UF free-enzyme runs and plated on Tripticase Soy Agar with 0.6% Yeast Extract and incubated at 22, 35 and 40°C for 48 h. NG = No Growth.

		22°C for 48h		35°C for 48h		40°C for 48h	
		5 hr	24 hr	5 hr	24 hr	5 hr	24 hr
Immobilized enzyme system	Sample 1	NG	NG	NG	NG	NG	NG
	Sample 2	NG	NG	NG	NG	NG	NG
UF Free-Enzyme System	Permeate 1	NG	NG	NG	NG	NG	NG
	Permeate 2	NG	NG	NG	NG	NG	NG
	Retentate 1	NG	NG	NG	NG	NG	NG
	Retentate 2	NG	NG	NG	NG	NG	NG

APPENDIX 6

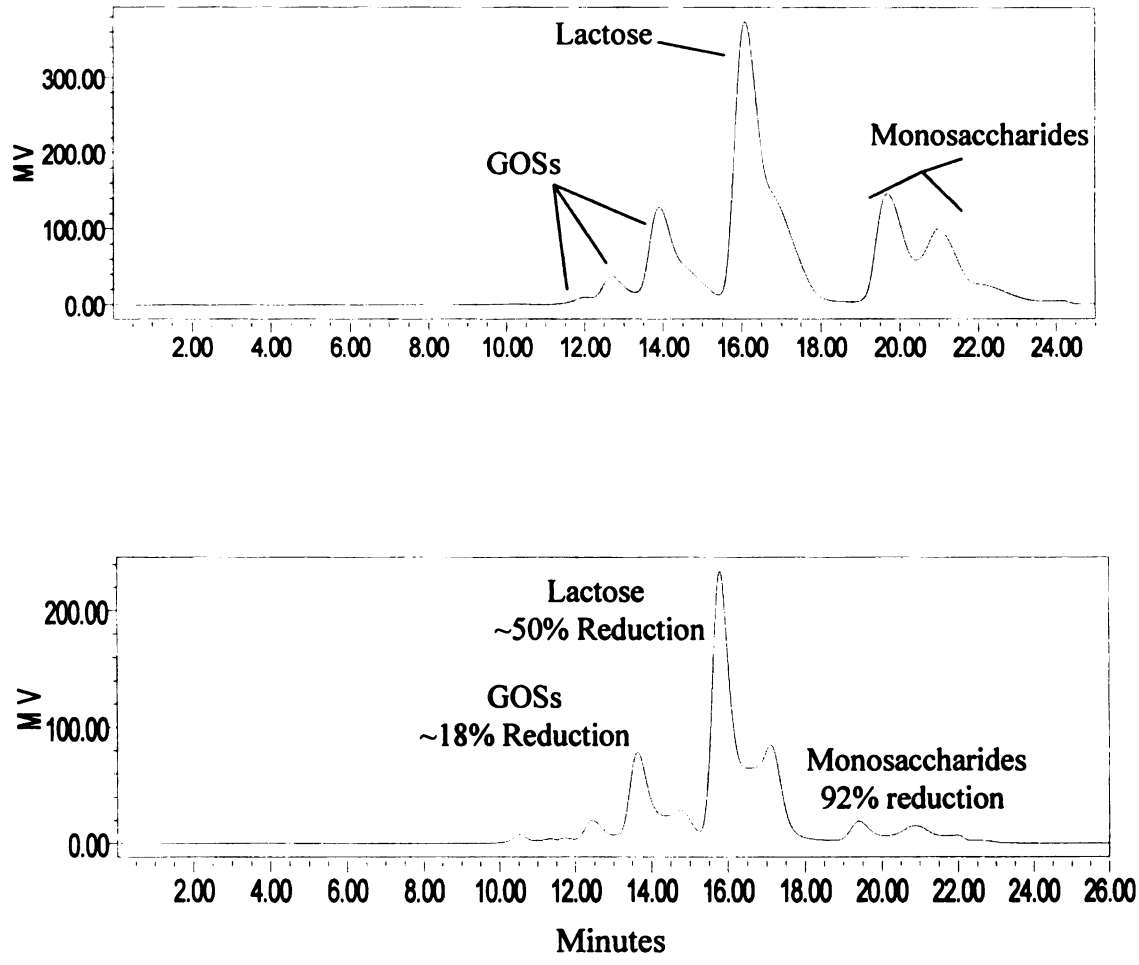

Figure A.6.1. HPLC chromatograms of various sugars present during initial (top) and post-diafiltration (bottom) samples.

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