University of Alberta

BIOCONVERSION AND SEPARATION OF MILK CARBOHYDRATES ON NANOMEMBRANES

by

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Cogito, ergo sum …..(I think, therefore I am ..…)

René Descartes

ABSTRACT

Cost-effective processing of dairy whey permeates is important to the environment and economics of the agriculture industry in Canada. Bioconversion of whey permeates is an attractive means of obtaining value-added adjuncts with improved nutritional and functional properties. In the past, cost-effective technologies to recover additional value from whey permeates at a low cost were lacking. Currently, such a technological platform is now feasible with the introduction of new modern bioconversion technologies that incorporate batch or continuous bioreactors, and use ultra- and nano-filtration membranes for the separation of whey permeate components.

In this dissertation, a novel processing methodology is described. This methodology, which is a desirable configuration for food manufacturers includes a stirred batch nanomembrane bioreactor equipped with a crossflow nanomembrane and offers lactose bioconversion with an immobilized biocatalyst, product separation, and biocatalyst recovery in a batch operation.

The major focus of this research was on: a) the development of a new analytical methodology for carbohydrate measurement during the lactose bioconversion process, b) the selection, testing and integration of highly selective nanomembranes to separate the desired substrates, whey permeate carbohydrates, from the reaction mixture, and c) the production of a stable and highly active and specific immobilized biocatalyst. Noticeably, this methodology was designed, developed and tested for the bioconversion of lactose, but could also be used for the bioconversion of other carbohydrate feedstocks.

The food industry in Canada needs an integrated approach to achieve complete lactose reclamation and use. This research project offers such a solution. The research described in this dissertation presents an integrated model of a stirred batch bioreactor that may support not only current, but also future research, and may economically impact the development and bioconversion of whey permeates containing lactose. This may lead to the development of a continuous processing methodology for low cost recovery of lactose from whey permeates and simultaneous conversion to value-added products.

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ABBREVIATIONS

CAGR - compounded annual growth rate

CDC - Canadian Dairy Commission

EL ALR - external loop air lift reactor

GOS - galacto-oligosaccharides

IMO - isomalto-oligosaccharides

GRAS - Generally Recognized As Safe

IMO - isomalto-oligosaccharides

IUPAC - International Union of Pure and Applied Chemistry IUPAC

IUBMB -International Union of Biochemistry and Molecular Biology

IL ALR - Internal Loop Air Lift Reactor

ONPG - *o*-nitrophenyl galactopyranoside

ONP - ortho-nitrophenol

MWCO - Molecular weight cut off

NAD^+ - nicotinamide adenine-dinucleotide (oxidized form)

NADH - nicotinamide adenine-dinucleotide (reduced form)

PVA - polyvinyl alcohol

PEG - polyethylene glycol

Physical constants and units

Bar - pressure units

D - diffusion coefficient of solute ($m^2\ h^{-1}$).

kg/m^3 - kilogram per cubic meter (mass per volume units)

J	- volumetric flux (L m^{-2} h^{-1})
MPa	- megapascal (pressure units)
psi	- pounds per square inch (pressure units)
δ	- solute boundary layer (expressed in length units)

1. INTRODUCTION

1.1 CARBOHYDRATES

Carbohydrates, are defined as polyhydroxy aldehyde or ketone compounds or their polymeric complexes. In chemical terminology, a carbohydrate molecule which cannot be hydrolyzed into smaller carbohydrate complexes is recognized as a monosaccharide (e.g. xylose, fructose, glucose, and galactose). The chemical formula for monosaccharides is described as $(CH_2O)_n$ for $n \geq 3$. Monosaccharides can be classified as aldoses or ketoses. The suffix -ose indicates a specific carbohydrate, the prefix aldo- is a prefix that indicates an aldehyde carbonyl group and the prefix keto- indicates a ketone carbonyl group. Finally, the numerical prefixes tri-, tetra-, pent-, and hex- identify the number of carbon atoms in a carbohydrate molecule. A carbohydrate which can be hydrolyzed into two monosaccharides is called a disaccharide (e.g. sucrose, lactose, maltose and cellobiose), whereas chains of three to ten, monosaccharides are known as oligosaccharides (e.g. fructo-oligosaccharides or galacto-oligosaccharides). Larger complexes or chains of monosaccharides are known as polysaccharides. They can be made up of many hundreds or even thousands of monosaccharide molecules connected together (e.g. starch and cellulose).

Carbohydrates can be found in the natural environment and are important and necessary to all species' survival. The molecular formula for both of the carbohydrates, glucose and galactose is $C_6H_{12}O_6$ and their molecular weight is 180.16 g mol^{-1}. During milk biosynthesis these two monosaccharides are

combined together into a disaccharide known as lactose with a molecular weight of 342.24 g mol^{-1}.

The global demand for carbohydrates increases annually (Urilch, 2009). In addition to being the principle component of most of the major staple food crops carbohydrates are essential ingredients used by the food, biotechnology and fermentation industries. Urilch (2009), in his review of economic trends, reported that the global market for fermentation products, derived from carbohydrates is expected to increase from $15.9 billion in 2008 to $22.4 billion by the end of 2013, for a compounded annual growth rate of 7.0%.

Lactose, a major milk carbohydrate, accounts for 50% of the total dry solids in milk but has a relatively low marketing value for several reasons. One important reason is that approximately 37% of people around the world are lactose intolerant. Whey permeate, a byproduct of cheese manufacturing, contains a high concentration of lactose and is frequently disposed of after processing. The percentage of lactose concentration in whey permeates, calculated as a proportion of total dry solids, is approximately 75% (Mahoney 1997). Overall, approximately 1.2 million tons of lactose is produced annually worldwide without many profitable routes for its direct utilization (Urilch 2009). Therefore, the development of profitable methods for reclamation and use of whey permeate or lactose offers potential economic opportunity to find a use for an otherwise under-utilized commodity. As a waste product, excess lactose is currently an economical burden on the dairy industry.

1.2 LACTOSE

1.2.1 Milk and whey as sources of lactose

Lactose occurs naturally in the milk of mammals. The average lactose content in milk of different mammals is summarized in **Table 1.1**.

Table 1.1 Average concentration of lactose (g/100 g) in milk from various mammals, adapted from Scrimshaw and Murray (1988).

Human	Hog	Cow	Buffalo	Cat	Goat	Dog	Rat
6.9	5.5	4.9	4.8	4.8	4.7	3.1	2.6

When cow's milk is used for cheese production, for approximately every 1 kg of cheddar cheese, approximately 9 kg of sweet whey known also as whey permeate is generated, which on average contains 4.5 to 5.0% (w/v) lactose (Lindmark-Mansson *et al.* 2003). Whey also contains proteins, approximately 6.8% of total solids (Sheth *et al*. 1988; Mawson 1994). Although whey is rich in lactose and proteins, it is frequently disposed of after processing.

Overall, approximately 1.2 million tons of lactose are produced annually worldwide as a by-product of the cheese industry and most of that lactose is disposed of due to a lack of profitable routes for its utilization (Urilch 2009). As a waste product, excess lactose is currently a burden on the dairy industry. Therefore, the development of profitable methods for reclamation and use of whey permeate or lactose offers potential economic opportunity to find a use for an otherwise under-utilized commodity.

Mawson (1994) noted that most whey permeate is disposed of simply by sending it to effluent streams (to water treatment plants). Whey has a high

biological oxygen demand value (35-45 kg/m^3), and is subject to high levies issued by governments for waste treatment. One alternative to disposal is to use the whey as a supplement in pig feed. However, this alternative is not often implemented because it is usually uneconomical (Mawson 1994). Therefore, alternative profitable solutions for whey permeate disposal need to be considered and explored.

In Canada, the total whey generated from cheese production in 2005 was about 270 million kg (Canadian Dairy Commission 2005). The Canadian Dairy Commission (CDC) estimates this amount will continue to increase with an increase in the Canadian population. Over the past several decades, the price of industrial lactose in the world markets was below $1000 per metric ton; however, between 2006 and 2007 it started to increase due to a strong demand from the food and biotechnological industries and its current price is estimated between $2,000-2,500 per metric ton (Williams 2007). Due to the increasing value of lactose on the world markets, novel processing methods (e.g. the cross-flow ultrafiltration and nanofiltration membrane technologies) for the extraction of lactose from whey permeates will be important for capturing the value of this currently under-utilized commodity. In addition, new trends that lead to the production of the value-added products from lactose are also being recognized as a profitable commodity by the dairy industry (Mahoney 1997; Williams 2007). However, novel methods for their profitable production need yet to be developed, evaluated and adopted.

1.2.2 Lactose properties

Lactose is a disaccharide, with a molecular weight of 342.24, made of galactose (β-galactopyranoside) and glucose (β-glucopyranose) monosaccharides joined together with β-(1-4) glycosidic link. **Figure 1.1 s**hows the configuration of the β-lactose molecule composed of its two subunits and drawn as a chair conformation (Holsinger 1997).

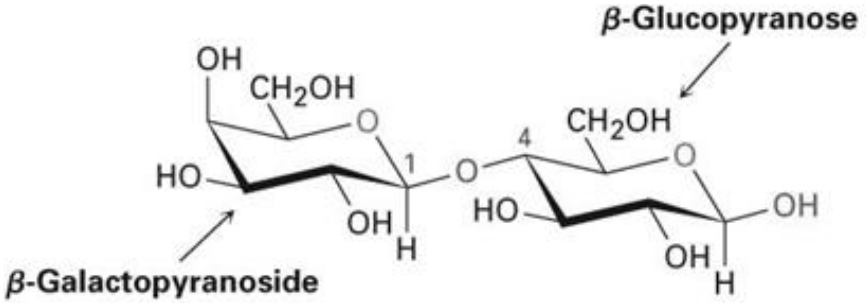

Figure 1.1 β-lactose, [4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranose].

In water solutions, lactose exists in two anomeric forms: α- and β-. When lactose is solubilized in water at 20^{o}C these two forms establish an equilibrium mixture comprised of β- (62.7%) and α- (37.3%) anomers. O'Brien (1997) reported that the ratio of the β/α form is equal to 1.68 at 20 ^{o}C (**Figure 1.2**). However, the process of transfer from one form to another is affected by temperature. The solubility of lactose in water at 20°C is 18.2 g per 100 g of water. The rate of dissolution of lactose in water is affected by the ratio of these two α- and β- forms. The monohydrate α-lactose, with a molecular weight of 360.3 g mol^{-1}, contains 5% water and may be prepared by concentrating a lactose solution to a super saturation state and precipitating it by inducing crystallization below a temperature of 93.5°C. This precipitated lactose, at ambient

temperatures, when in a stable form produces solid crystals of which the most common shape is the “tomahawk-like” shape (Mahoney 1997).

Kearsley (1985) reported that in freshly prepared water solutions of some pure carbohydrate standards, including lactose, glucose and galactose, there was a simultaneous change occurring in the measured optical rotation value. This effect, known as mutarotation, is due to the adjustment in the proportion of α- and β- forms. It was also observed that after some time had passed an equilibrium between the anomeric α- and β- forms of the carbohydrates was established and a constant optical rotation value was measured (**Figure 1.2**). The mutarotation of anomeric forms of sugars becomes relevant to the discussion of the polarimetric method used for quantifications of carbohydrates.

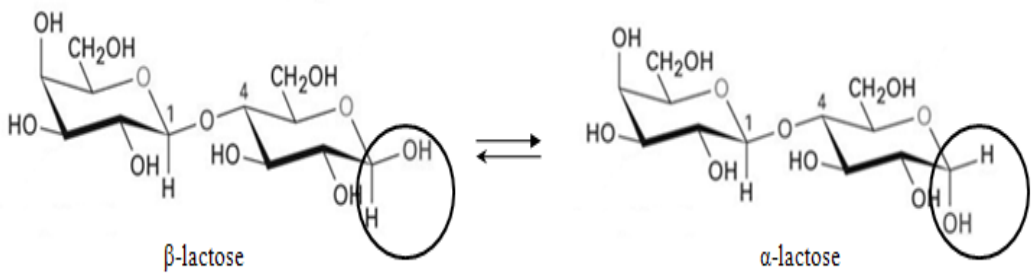

Figure 1.2 Mutarotation of lactose. In water solution the α- and the β- forms of lactose reach an equilibrium.

1.2.3 Lactose as an adjunct

To date, there has been very little application research, technology transfer, and new product commercialization efforts to exploit whey as a source of value-added products. The use of whey increases manufacturer processing costs. For whey to be economical, as a commodity, the value of the products must

exceed the additional production costs. One example of using whey permeates for value-added products is the use of lactose as a sweetener supplement for certain food products. This strategy, has some limitations, which are mainly due to the chemical properties of lactose. For example, with added whey permeate, the quality of ice cream or baked goods, particularly their sensory properties, may be compromised because of the occurrence of lactose crystallization after processing (Mahoney 1997).

There are also some beneficial characteristics of lactose. This carbohydrate is used as an ingradient in food formulations and as a modifier of color, flavor and sweetness. **Table 1.2** compares the relative sweetness of some common carbohydrates showing that lactose is lower in sweetness than that of other sugars.

Table 1.2 Comparison of the relative sweetness level of some carbohydrates,data for relative sweetness were taken from Pazur (1970).

Carbohydrate	Relative Sweetness
fructose	173
sucrose	100
glucose	74
galactose	32
lactose	16

In some categories of food products, this property is used to adjust the sweetness level while maintaining a similar level of carbohydrates necessary to

maintain a balance between food rheological and nutritional properties. In the past, sweet milk stouts traditionally contained 7-12% lactose (Lewis and Young 2001) and were highly recommended for mothers who breast-fed their babies.

1.2.4 Utilization of hydrolyzed lactose

Low "lactase" activity in newborn babies is a known cause of lactose intolerance (maldigestion) in their early childhood (Dahlqvist 1983). "Lactase" is an enzyme which hydrolyzes lactose and in the human body it is expressed by the brush border cells of the small intestine. Many humans, world-wide, are unable to digest (metabolize) lactose and suffer from lactose intolerance. The major cause for the inability to metabolize lactose is the lack of a sufficient amount of lactase (Holsinger 1997). The maintenance of the lactase activity at the right level in the human body after the consumption of foods containing lactose is of significance. Large variations in lactase activity in humans are observed between different racial groups around the world. Simoons (1978) described that the most affected population groups, globally, are those of the American Indian, African, Indian or South East Asian ancestry. Therefore, the use of lactose as a major food additive in food production is limited. Currently, supplements containing the lactase enzyme are available in the market (pharmacies and drug stores) to help those who suffer lactose indigestion problems and to ameliorate lactose intolerance in humans (Mahoney 1997; Elliot *et al.* 2001). In addition, food manufacturers target these affected groups by offering new categories of foods with a reduced content of lactose (Mahoney 1997; Elliot *et al.* 2001).

Market trends in the food production business are consumer-driven and demand that the food industry focus on supplying the market with "health conscious" food products. Currently, food researchers are focusing on the effective bioconversion of lactose into products, which are recognized as value-added. Some lactose-reduced food products have been manufactured successfully (Elliot *et al*. 2001; IDF 2005). **Figure 1.3** shows possible pathways for a generation of value-added products when hydrolyzing lactose with various enzymes (biocatalysts).

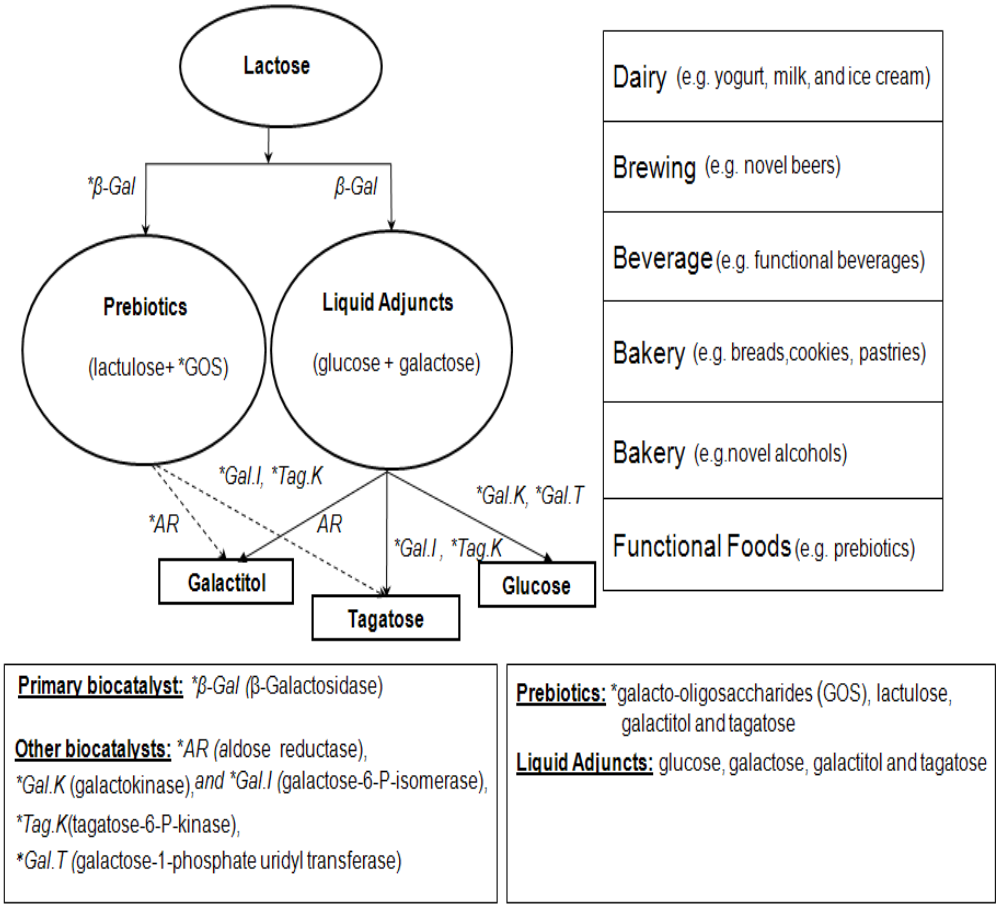

Figure 1.3 Novel value-added products from the bioconverted lactose.

These are classified within two major categories known as a prebiotics and as liquid adjuncts. A description of the two follows. Prebiotics are defined as non-digestible food ingredients that are characterized by health benefits. They affect the host (consumer) beneficially by selectively stimulating the growth and/or activity of one or more beneficial types of bacteria in the colon, and this improves host health (Gibson 2004). Vulevic *et al.* (2004) demonstarted that the prebiotic compounds obtained during the hydrolysis of lactose include galacto-oligosaccharides (GOS). The authors reported that these are generated by the polymerization of galactose residues released during the hydrolysis of lactose. They showed in their *in vitro* studies that GOS are desired in some types of food products because of their "prebiotic effect", which benefits the human gastrointestinal tract. Rycroft *et al.* (2001) concluded that galactose containing oligosaccharides are more effective as a prebiotic than other oligosaccharides such as fructo-oligosaccharides or the inulin type. Palfaram *et al*. (2003) reported that besides GOS, also lactulose and isomalto-oligosaccharides (IMO), all showed their optimum prebiotic effects at pH 6.0 at 2% (w/v).

Lactose is not readily metabolized by brewer's yeast and therefore must be pre-hydrolyzed prior to it being used as a fermentable adjunct feedstock. Sviridenko *et al.* (1994) reported that it was possible to combine whey lactose hydrolysis in the production of alcoholic beverages by using an enzymatic preparation in a fermentation process together with yeast (*Saccharomyces cerevisiae)*. The authors optimized the parameters of the enzymatic hydrolysis of lactose and the alcoholic fermentation of glucose and obtained higher yields of

ethyl alcohol in the final product. This study should be regarded as providing evidence of an opportunity to use bioconverted lactose syrup as an alternative adjunct in the brewing process. Coton *et al.* (1981), Harju *et al.* (1978) and Poznanski *et al.* (1978) all sought to identify or develop a method for total or partial replacement of the corn syrup adjuncts with hydrolyzed lactose. They suggested that such an alternative could supplement the brewing fermentation process, and provide a new base for alternative beer product development.

The pathways shown in **Figure 1.3** highlight only a few of the many possible applications of bioconverted lactose. The economics of manufacturing prebiotics or liquid adjunct syrups from hydrolyzed lactose, and their successful marketing, depend not only on the processing technology but also on such factors as the availability and cost of whey substrate vs. the availability and cost of alternative sources of carbohydrates.

Recent developments in batch and continuous enzyme reactors for the hydrolysis of lactose using immobilized enzyme systems may contribute to significant cost cutting measures in manufacturing food products derived from bioconverted lactose (Mahoney 1997). Advancements in bioreactor technology generate opportunities for producing a variety of novel liquid adjuncts (e.g. galactitol and tagatose), which can be used as sweeteners (Kim *et al.* 2001; Kuusisto *et al.* 2007). But, all aforementioned issues, including economic as well as processing costs and market demand for hydrolyzed lactose, have to be considered before the initiation of capital investment.

1.3 BIOCONVERSION OF LACTOSE

1.3.1 Biocatalyst sources and characteristics

Enzymes, are proteins that selectively and effectively catalyze specific chemical reactions without being consumed. Like all catalysts, they work by decreasing the amount of activation energy required for a given chemical reaction (Anonymous 1984; NC-IUBMB 2009). Enzyme classification is based on the type of chemical reaction catalyzed. The commonly used system of classification is defined by the International Union of Biochemistry and Molecular Biology (IUBMB). Besides classification of enzymes, enzyme activity and kinetic models are used to compare and evaluate enzyme bio-catalytic activity.

According to the IUBMB enzyme biocatalytic activity is defined as follows: “one international unit (IU) is the amount of an enzyme that catalyzes the transformation of 1 μmol of substrate per minute under standard conditions of optimal temperature, pH, and substrate concentration” (Anonymous 1984). In addition, the definition of enzyme specific activity is the number of enzyme international units per mg of protein.

β-galactosidase, commonly known as a "lactase" (also known as β-D-galactoside galactohydrolase), is classified as a hydrolysis type of enzyme by the International Union of Pure and Applied Chemistry (IUPAC) with the code: EC 3.2.1.23 (NC-IUBMB 2009). The first number of this code describes the type of reaction that is catalyzed by the enzyme (for β-galactosidase it is a hydrolysis

reaction (number 3)), the next two numbers refer to the sub-class of the reaction and the fourth number identifies the specific enzyme.

The β-galactosidase extracted from *Kluyveromyces lactis* is the most common enzyme used to hydrolyze lactose in research laboratories and in manufacturing processes (Mahoney 1997; Zhou and Chen 2001; Juardo *et al.* 2006; Neri *et al.* 2008). It was also the enzyme of choice used in this research project. **Table 1.3** lists several sources from which commercially available β-galactosidase is commonly extracted.

The average molecular weight of β-galactosidase extracted from *Kluyveromyces lactis* is estimated approximately at 120,000 Da. Reports indicate that the enzyme naturally occurs in the dimeric or trimeric form (Cabaille and Combes 1995; Becerra *et al.* 1998). Voget e*t al.* (1994) reported that the activity of the enzyme was enhanced during the hydrolysis of lactose at 45^{o}C in a phosphate buffer (pH 6.6) with the addition of divalent cations like Mn^{2+} in concentrations from 0.1 to 0.2 mmol L^{-1}, and Mg^{2+}, in concentrations from 2.5 to 5.0 mmol L^{-1}. Furthermore, they noted that cations like Zn^{2+} (10^{-3} mmol L^{-1}) and Cu^{2+} (10^{-4} mmol L^{-1}) strongly inhibited enzyme activity. Mahoney *et al.* (1997) found the optimum activity of β-galactosidase to be at a temperature range of 35-40^{o}C and at a pH range of 6.5-7.3. For comparison, Roy and Gupta (2003) evaluated the enzyme activity extracted from the *Kluyveromyces fragilis* during lactose hydrolysis in whey and found that its optimum pH was in the range from 6.0-6.5 at 50^{o}C temperature.

Table 1.3 Examples of different extraction sources of the β-galactosidase.

Type of Source	Specific plant or organism	Reference
Plants	Peach	Lee *et al.* (2003)
	Gram chicken bean	Sun *et al*. (1999)
Yeast	*Kluyveromyces lactis*	Cabaille and Combes (1995)
	Kluyveromyces marxianus	Becerra *et al.* (1998)
	Kluyveromyces fragilis	Roy and Gupta (2003)
Bacteria	*Escherichia coli*	Ayyildiz (1999)
	Lactobacillus acidophilus	Akolkar *et al.* (2005)
	Lactobacillus helveticus	Callanan *et al.* (2005)
	Streptococcus lactis	Rymaszewski *et al.* (1985)
	Streptococcus thermophilus	Drouault *et al.* (2002)
Fungi	*Aspergillus niger*	Pereira Rodríguez *et al*. (2007)
	Aspergillus oryzae	Albayrak and Shang-Tian (2002)
	Aspergillus flavus	Rao *et al.* (1995)
	Penicillium canescens	Sviridenko *et al.* (1994)

The application of β-galactosidase as a catalyst, used to speed up hydrolysis of lactose, is well researched (Cabaille and Combes 1995; Mahoney 1997; Zhou and Chen 2001; Juardo *et al.* 2006; Neri *et al.* 2008). Recently, new methods for improving the extraction of β-galactosidase from new sources are developed and published (Illanes 2000; Bury and Jelen 2000; Grosová *et al.* 2008). However, more work must be done in order to develop economical applications using this enzyme. Therefore, its use in manufacturing foods or

fermented products, is being evaluated and still under extensive review (Bury and Jelen 2000; Grosová *et al.* 2008).

1.3.2 Lactose bioconversion with soluble biocatalysts (mechanism and kinetic model)

There are two principal methods used to hydrolyze lactose; the first involves an application of concentrated acids and the second is the use of β-galactosidase (Mahoney 1997). The latter one is more frequently applied by food manufacturers because of the lack of reaction products that affect the food quality. In addition, the reaction temperature for β-galactosidase in this method is usually between 25 and 55^{o}C, which when compared to the acid method is relatively low. Plus, with the use of β-galactosidase, there is no need for additional purification steps, which are required for acid hydrolysis (Lin and Nickerson 1976; Bury and Jelen 2000; Grosová *et al.* 2008).

The β-galactosidase specifically hydrolyzes the disaccharide α-lactose into glucose and galactose according to the reaction shown in **Figure 1.4**.

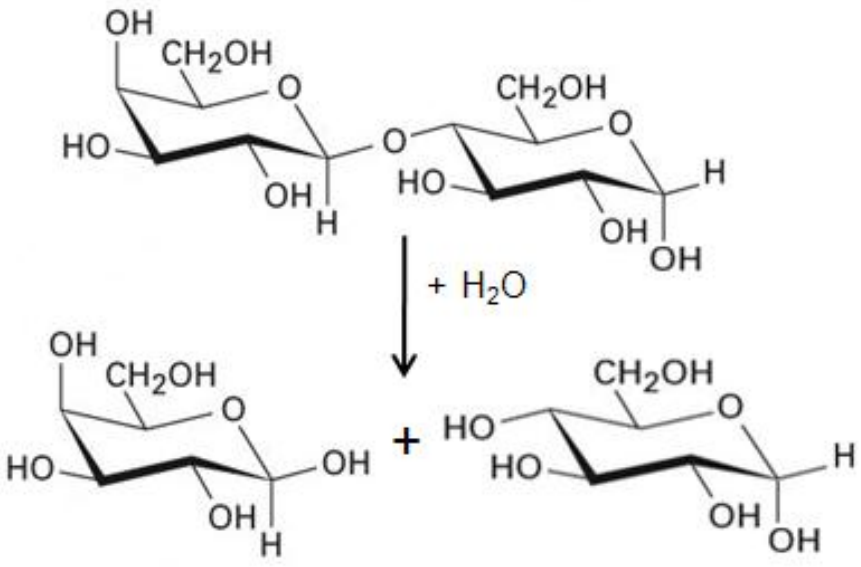

Figure 1.4 Hydrolysis of α-lactose to β-galactose and α-glucose.

Figure 1.5a and **Figure 1.5b** show the mechanism of bioconversion of lactose by β-galactosidase according to a model proposed by Wallenfels and Weil (1972). This model reveals that the β-galactosidase active center is characterized by the presence of active –SH (sulfhydryl) and imidazol groups. In the first step, a lactose molecule reacts with these active groups *via* a bimolecular nucleophilic S_N2 substitution. In greater detail, the sulfhydryl group acting as an acid, donates a proton to the oxygen atom in the lactose β-(1→4) glycosidic linkage. Simultaneously, the imidazol group, a nucleophile, attaches to the C_1 atom in the galactose molecule. At that point, a temporary complex between the biocatalyst and the carbohydrate molecule is generated. Next, the α-glucose molecule is released into the solution. In the second step, release of the β-galactose molecule occurs with the simultaneous attachment of a water molecule or another

electrophilie (e.g. another carbohydrate molecule) and the protonation of the sulfur anion.

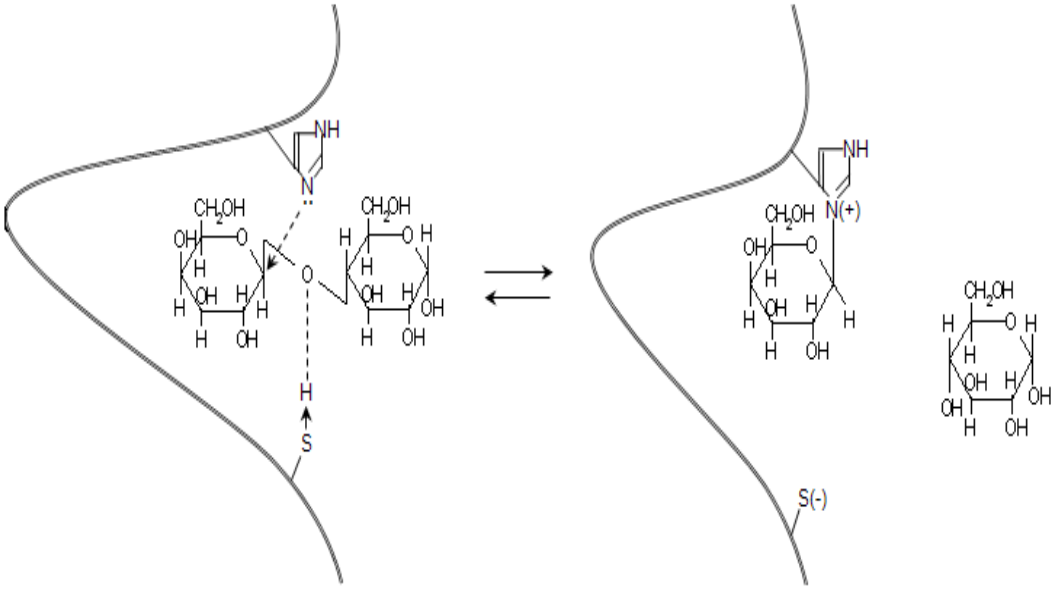

Figure 1.5a Bioconversion of α-lactose by β-galactosidase (step 1) as suggested by Wallenfels and Weil (1972) and as cited in Richmond *et al.* (1981).

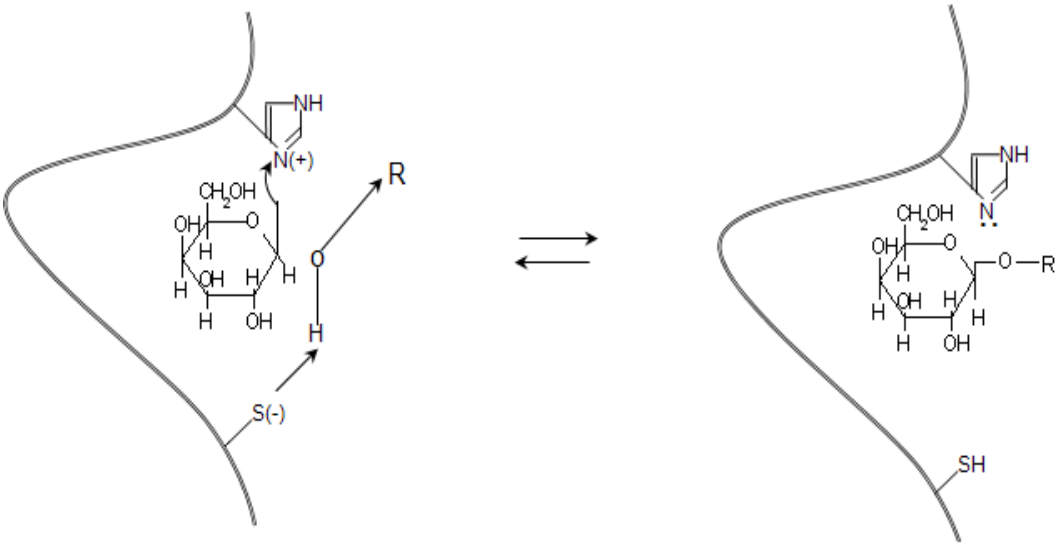

Figure 1.5b Bioconversion of α-lactose by β-galactosidase (step 2); "R" is a nucleophilic acceptor containing OH group (water or carbohydrate) as suggested by Wallenfels and Weil (1972) and as cited Richmond *et al.* (1981).

It is important to note that during the enzymatic hydrolysis of α-lactose in water solutions, only β-galactose and α-glucose forms are released (Shukla 1975; Huber *et al*. 1976; and Huber *et al.* 1981). Both glucose and galactose, similar to lactose, exist in the α- and β- anomeric forms and after lactose hydrolysis undergo mutarotation to reach equilibrium in the aqueous solution. It is reported that β-galactose may act as an inhibitor of the enzyme where its inhibition is 12 times stronger than α-galactose.

Wallenfells and Malhorta (1961) and later Mahoney (1997) suggested that if a water molecule is not readily available in solution as an acceptor during galactose release, then the lactase enzyme enables the attachment of additional galactose or other mono-, di- or tri-saccharides, and results in the formation of the GOS (**Figure 1. 5 c)**.

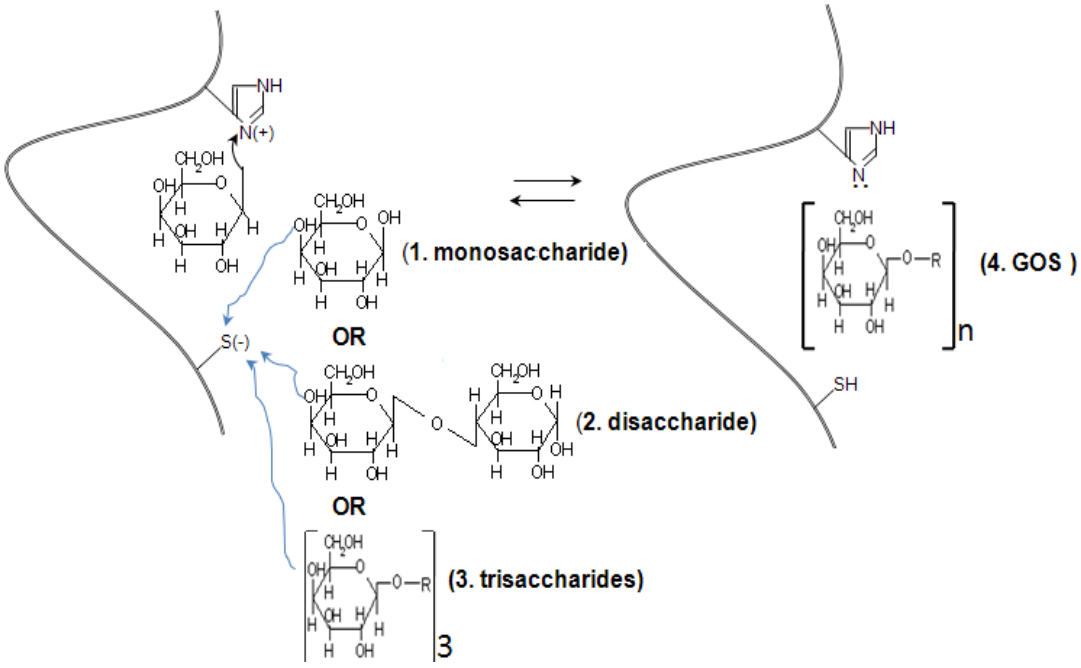

Figure 1.5c Bioconversion of α-lactose by β-galactosidase into mono-, di-, or GOS, as suggested by Mahoney (1997).

Goulas *et al.* (2003) noted that GOS are generated in milk or whey only in small amounts and during the initial phase of the lactose degradation process. Recently, the newly reported bio-technological advances have demonstrated new methods for the production of GOS (Gänzle *et al.* 2008). Cupples *et al.* (1990) evaluated β-galactosidase extracted from *Escherichia coli* during lactose degradation and proposed that the enzyme acts as a general acid by donating a proton to the glycosidic oxygen. The authors elaborated that, as a consequence, galactose molecules were stabilized by the formation of galactosyl transition state intermediates with the enzyme active group, which then react with a water molecule. In addition, they reported that the carboxyl group of the Glu-461 (glutamic acid residue), present in the active center of the enzyme, electrostatically interacted with a positively charged galactosyl transition state intermediate (the galactose carbonium ion). Also, it was suggested that the "degalactosylation" process was accompanied by another side step: activation of a water molecule for reaction with the intermediate by Tyr-503 (tyrosine residue).

Mahoney (1997) validated the transition-state theoretical model and added that Glu-537, instead of Glu-461, acts as a nucleophile residue and that the His-418 residue is also important and acts as a catalytic residue, which enhances activity of the enzyme. Benković and Hammes-Shiffer (2003) showed that the enzyme catalytic reaction follows the transition-state theoretical model. They noted that the "preferential binding of the transition state complementariness" is of more critical importance than the binding of the substrate or reaction products.

Several kinetic models have been proposed describing a type of

Michaelis-Menten kinetics used in evaluating β-galactosidase during lactose hydrolysis (Woychik and Wandolski 1972; Bisswanger 2004). **Figure 1.6** outlines a Michaelis-Menten-type kinetic model for lactose hydrolysis by β-galactosidase with competitive inhibition caused by the reaction product galactose described earlier by Mateo *et al.* **(**2004). In this model, galactose competes for the enzyme active site with lactose.

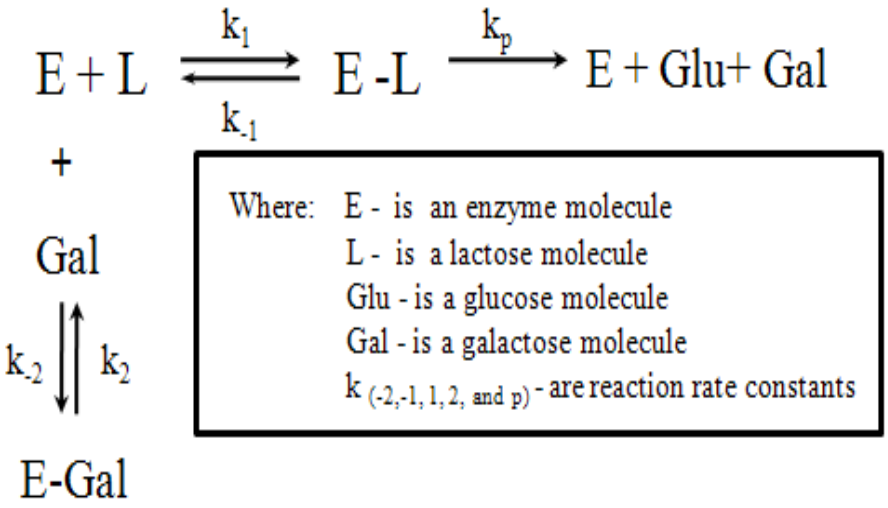

Figure 1.6 Michaelis-Menten kinetics model for lactose hydrolysis by β-galactosidase with competitive product inhibition, adapted from Mateo *et al.* (2004).

Figure 1.7 shows mathematical equations 1, 2 and 3, which describe this model. The model makes the assumption that this reaction occurs under steady-state conditions during the formation of the enzyme/lactose complex.

Báleš (2006) described several mathematical equations that were selectively used for the evaluation of the enzyme kinetics. The author applied these also to evaluate the internal rate of substrate diffusion in immobilized enzymes or cells.

$$\frac{d\,(E\text{-}L)}{dt} = 0 \quad \text{(for steady state conditions)} \qquad (1)$$

$$v = \frac{d\,Glu}{dt} = \frac{k_p\,(E\text{-}L)_o}{L + K_m(\,1 + Gal/K_i)} \qquad (2)$$

or

$$v = \frac{d\,Glu}{dt} = \frac{V_{max}\,L}{L + K_m\,(1 + (L_o - L)/K_i)} \qquad (3)$$

Where: v - is velocity of reaction (mmol min^{-1})
v_{max} - is maximum velocity of reaction (mmol min^{-1})
$v = v_{max}$ for $L \rightarrow \infty$
K_m = Michaelis-Menten constant which is $= (k_{-1} + k_p) / k_1$
K_i = inhibition reaction constant $= (k_2/k_{-2})$

Figure 1.7 Mathematical equations 1, 2, and 3 describe lactose hydrolysis by β-galactosidase with galactose as a competitive inhibitor according to a Michaelis-Menten kinetic model, adapted from Mateo *et al.* (2004).

1.3.3 Immobilization methods

The high cost associated with the use of β-galactosidase in a so-called "soluble" or "free" form during bioconversion of lactose contributes significantly to the overall cost of "lactose-reduced" food products (Mahoney 1997). This cost could be partly mitigated through enzyme reuse. Immobilization of enzymes is one strategy that may allow for higher enzyme stability for reuse, and in many cases can provide for easier recovery of the enzyme for further reuse. Therefore, the development of reliable and robust methods that focus on the immobilization of soluble enzymes on stable supports may be a solution. As a consequence, some

of the currently developed methods and food processing technological platforms aim at efficient use of immobilized enzymes and at the development of an integrated batch, semi-batch, and continuous bioreactors equipped with membranes (Jurado e*t al*. 2006; Grosová *et al*. 2008). Before some of these methods can be used by the food industry in Canada they are required by law to be approved by the Canadian Food Inspection Agency (CFIA). Similarly, in the United States of America these methods need to achieve "Generally Recognized as Safe" (GRAS) status which is granted by the Food and Drug Administration (FDA).

According to the definition published by IUPAC, "immobilized enzymes are enzymes, which are physically confined or localized, with retention of their catalytic activity, and which can be used repeatedly or continuously" (Worsfold 1995).

IUPAC recognizes four categories for the classification of immobilized enzymes by the immobilization method:

1) covalent bonding,
2) intermolecular cross-linking with the help of reagents,
3) adsorption on a wate-soluble matrix, and
4) entrapment inside a water insoluble polymer or semi-permeable membrane.

Furthermore, IUPAC recognizes three categories of support that are used to immobilize enzymes. They are: 1) hydrophilic biopolymers based on natural polysaccharides (e.g. agarose, dextran, chitosan, and cellulose), 2)

lipophilic synthetic organic polymers (e.g. polyacrylamide, polystyrene, nylon, and polyvinyl alcohol), and 3) inorganic materials (e.g. aluminum, glass wool, pore glass and iron oxide)

Important attributes that should be considered when selecting a support material for the immobilization process are as follows: 1) high binding affinity to the enzyme, 2) resistance to chemical solvents and changes in different pH values and temperatures, 3) inertness, 4) mechanical and chemical stability, and 5) lack of solubility in water (Illanes 2000). The material used to entrap an enzyme molecule has a significant effect on the enzyme activity and needs to be tested and verified prior to its use. **Table 1.4** shows advantages and disadvantages of using immobilized enzymes over free enzymes (Grosová *et al*. 2008). Noticeably, there are many more advantages than disadvantages when using immobilized enzymes.

Table 1.4 Advantages and disadvantages of enzyme immobilization.

Advantages	**Disadvantages**
enzyme reuse is possible	additional cost of the entrapment material
small risk of product contamination	cost of the used immobilization method
high stability of the entrapment material	lower specific activity
high enzyme concentration is achievable	delayed substrate diffusion
use in different processing methods	
improved control of enzymatic reactions	
use in continuous applications is possible	

A detailed review of immobilization methods commonly used to immobilize β-galactosidase has been recently published (Grosová *et al*. 2008). The most common methods focus either on covalent binding of β-galactosidase covalent binding to activated supports or immobilization through entrapment into a gel matrix. Covalent binding is a result of amide, ether, thio-ether, or carbamate linkages. On the other hand, entrapment methods are based on mixing of the enzyme with a soluble polymer solution and subsequent solidification of the mixture leading to entrapment of enzyme inside the gel structure.

All immobilization methods have some effect on enzyme stability and catalytic activity (Illanes 2000). Haider and Husain (2009) noted that enzymes immobilized on membranes were often characterized by a lower activity in comparison to the soluble enzymes. They found that the entrapment of the β-galactosidase, extracted from the *Aspergillus oryzae*, on a packed bioreactor column filled with calcium alginate cross-linked with the concanavalin A (con A) was a more efficient alternative than one with an entrapped enzyme on a membrane. They also reported that lactose was effectively reduced in milk (77% (w/w)) and whey (86% (w/w)) during the batch process.

Recently, Brena and Batista-Viera (2006) reviewed enzyme stabilization methods, and found that methods that use the multipoint covalent binding of enzymes to the immobilization matrices improve their stability. Báleš (2006) reviewed methods used for evaluation of kinetics and immobilized cells and enzymes. He indicated that the catalytic reaction, due to the heterogeneous nature of immobilized enzymes, is one of several reaction steps, which leads to the

biotransformation of a substrate into a product. It was noted that the substrate needs to overcome the resistance of the external fluid layer (external diffusion) and the internal structure of the immobilizing porous polymer (internal diffusion) in order for it to enter into the immobilized active site of the enzyme for catalysis.

In this research project, β-galactosidase was entrapped in a polymer matrix using one of nonionic hydrophilic polymers; polyvinyl alcohol (PVA) or polyethylene glycol (PEG). The methodology was adopted from the LentiKat's® a. s. company in the Czech Republic. Rebroš *et al.* (2006) reported that the immobilization matrix composed of PVA and PEG offered several advantages such as a high stability of the immobilizing hydrogel during storage, a fast diffusion rate and a high stability at different pH and temperature values. In addition, the biocatalyst retained a high activity before and after processing and there was a lack of β-galactosidase leak when used (Rebroš *et al.* 2006; Grosová *et al*. 2009). Although β-galactosidase on PVA/PEG supports has been studied in some detail, its application and use in a membrane bioreactor have not yet been tested.

1.3.4 Assays used for the evaluation of the β-galactosidase activity

The experimental measurement of β-galactosidase activity is based on the evaluation of initial reaction rates of the enzyme. Several methods that are used to evaluate the activity of β-galactosidase have been reported (Kim *et al.* 1997; Illanes 2000; Acevedo *et al.* 2009; Gong *et al.* 2009). There are two basic categories of assays used for measurement of β-galactosidase activity. The first

category of β-galactosidase assays is based on the measurement of the accumulation of reaction products (i.e. glucose, and galactose) according to predetermined time intervals, and stopping the enzyme, by denaturation, with a concentrated salt or acid solution. The second one uses analytical instrumentation to accurately and continuously record concentrations of reactants and products during the course of the reaction (Illanes *et al.* 1990; Illanes 2000). Frequently, the selection of a method is subject to the availability of equipment or reagents in the laboratory.

Currently, the most common method used to evaluate β-galactosidase activity to degrade lactose is a spectrophotometric assay that measures degradation of the *o*-nitrophenyl galactopyranoside (ONPG) (Shah and Jelen 1990; Illanes 2000). In this method, the colourless ONPG, the substrate, is degraded by β-galactosidase into *o*-nitrophenol (ONP), a yellow compound that absorbs light at 420 nm. The change in absorbance at 420 nm is monitored with a spectrophotometer and the reaction velocity (reaction rate) is plotted as a function of the concentration of ONP *vs.* time. One unit of β-galactosidase activity is defined as the amount of enzyme, which liberates one μmole of *o*-nitrophenol from ONPG per minute at 37^{o}C.

Kleyn (1985) described another enzymatic method that could be used for determining β-galactosidase activity to degrade lactose. In the first step of this assay, lactose is hydrolyzed by β-galactosidase to glucose and β-galactose in the presence of water. In the second step, the β-galactose is oxidized by added nicotinamide adenine-dinucleotide (NAD^{+}), to galactonic acid in the presence of

β-galactose dehydrogenase enzyme and forms the reduced compound, NADH. The amount of formed NADH is stoichiometric with the amount of lactose present in solution and measured by the spectrophotometer set up at 340 nm and having a slit width of 10 nm. One unit of β-galactosidase activity is defined as the amount of enzyme, which liberates one μmole of NADH per minute at 20 to 25^{o}C. These two enzymatic assays have a few drawbacks such as their complicated procedure and their failure to differentiate between individual carbohydrates.

Kim *et al.* (1997) evaluated the ONPG method and noted that a small change in the composition of the buffer reaction system may affect β-galactosidase's ability to hydrolyze the ONPG. In addition, they observed that pH and the concentration of metal ions significantly influences the ONPG type assays. Consequently, they questioned the use of this method to evaluate β-galactosidase activity in the enzyme preparation.

An alternative to elaborated ONPG and NAD^{+} methods is to use high performance liquid chromatography method (HPLC) to measure the concentration of substrates and products during the lactose hydrolysis process (Jeon *et al*. 1984). The use of HPLC for carbohydrate concentration determination, including lactose, glucose, galactose and other carbohydrates, has been tested, and a certified HPLC method exists for the quantitative measurement of lactose (AOAC 1995). The HPLC method offers accurate and fast measurement of individual compounds in a complex carbohydrate mixture and it is an effective tool for measurement of β-galactosidase activity. Pirisino (1983)

and Clement *et al.* (1992) reported that the HPLC method has several advantages over the ONPG method, including: 1) a lack of interaction between reactants, 2) a high accuracy, and 3) a high reproducibility.

1.3.5 Analytical methods used for monitoring lactose bioconversion

Evaluating lactose decomposition in reduced milks or dairy products is a challenge because of the complexity of the product matrix and analytical methods (Pirisino 1983; Jeon *et al.* 1984; Mahoney 1997; Bury and Jelen 2000).

Several authors have proposed methods for assaying the concentration of carbohydrates in dairy products (**Table 1.5)**. The most commonly used methods for the evaluation of lactose and its degradation products in dairy substrates (e.g. milk or whey) are spectroscopic and analytical methods such as: UV/Vis, NIR, FTIR, HPLC, and GC.

All of these methods are labour-intensive and require expensive equipment and highly trained personnel. Therefore, new low-cost, simple and robust methods, are in demand for improving monitoring degradation of lactose in dairy feedstocks.

Table 1.5 Analytical methods used to measure carbohydrate concentration in dairy products.

Method	Carbohydrates	Reference
UV/VIS	glucose	Zaitoun (2006)
HPLC	glucose, galactose, lactose	Jeon *et al.* (1984); Anonymous (2007)
GC	galactose, α-lactose, β-lactose, α-epilactose	Olano *et al.* (1986)
NIR	fructose, glucose, sucrose	Lanza and Li (1984)
FTIR	fructose, glucose	Sivakesava and Irudayaraj (2001)
μFIAS	glucose, galactose, lactose	Rajendran and Irudayaraj (2002)

1.3.6 Polarimetry as a method of choice

Lactose, glucose and galactose are optically active carbohydrates and, in their simple solutions, rotate the plane of linearly polarized light clockwise. This property is called dextrorotatory and the magnitude of this rotation is specific for each of these carbohydrates. This property is used by polarimetric reference methods to measure lactose, method #16.055, and glucose concentrations in food products, method #31.032 (AOAC 1984a; AOAC 1984b). Measurement is rapid and the degree and direction of the optical rotation of carbohydrates are determined by the concentration and molecular structure of the chiral molecules (Kearsley 1985).

The standard measure of the degree of optical rotation was first described in Biot's law by the following equation (Lesney 2004):

$$[\alpha]_{\lambda}^{T} = \frac{\alpha}{c \cdot l} \qquad (4)$$

where: $[\alpha]_{\lambda}^{T}$ is a specific rotation, T is the temperature, λ is a wavelength, α is an optical rotation, l is an optical path length in dm and c is a concentration of solute expressed as $g \cdot mL^{-1}$. The major disadvantage of this method is that it measures only one carbohydrate and requires time-consuming preparatory analytical steps.

During this research project, using the above mentioned optical rotation principles, a new polarimetric method was developed to monitor the bioconversion of lactose into glucose and galactose. This new method is robust, simple to perform and through subsequent use of a predictive mathematical model, offers the possibility for significant cost and time savings. Hence, it enables a rapid, non-destructive and quick evaluation of carbohydrate concentrations during the production of lactose-reduced milk products. Moreover, it is conceptualized that using a multi-wavelength digital polarimeter would result in further optimization of this new method.

1.4 BIOREACTOR TECHNOLOGY FOR BIOCONVERSION OF LACTOSE

1.4.1 Bioreactors in general

In general, "bioreactor" refers to any device or system that supports a biologically active environment. A typical bioreactor is a cylindrical tank in

which a biochemical process is carried out; this process typically involves organisms or biochemically active enzymes, and it can either be aerobic or anaerobic (Decker and Reski 2008). Commonly, bioreactors are manufactured in different volumes, as small bench scale and as large as industrial tanks (e.g. liters, hectoliters) and are often made from non-corrosive stainless steel. Types of bioreactors are divided into three main categories (according to the mode of operation and the feedstock flow pattern): 1) batch bioreactors, 2) fed-batch bioreactors, and 3) continuous bioreactors. Furthermore, continuous bioreactors can be divided into two sub-categories: plug-flow reactors (PFR) and continuous flow-stirred tank reactors (CSTR).

In a batch bioreactor, the initial concentration of a substrate(s) declines during the reaction process. Once the substrate is depleted, the process is stopped and the reacting batch is removed from the bioreactor tank (Harrison *et al.* 2003). On the other hand, in the fed-batch bioreactor the concentration of the feed is readjusted to the desired value during the reaction process and the end reaction products are removed at the end of the fed-batch process.

The characteristic feature of a continuous bioreactor, or flow bioreactor, is a perpetual flow of feedstock within the bioreactor. Such bioreactors are generally smaller than a batch reactor (Williams 2002). They handle the substrates as a flowing stream, and it may be designed as a pipe or as a tank, with or without baffles (device required to arrest the swirl in a tank), in a stand-alone configuration or as a series of interconnected stages. Furthermore, for the reactor

to work in the continuous mode, it is necessary to balance the incoming feedstock flow rate needs with the outgoing product.

Villadsen and Reuss (2006) noted that the residence time for the substrate in a continuous bioreactor needs to be standardized in order to meet the requirements for thermodynamic steady-state conditions and to guarantee the continuous substrate processing. The authors indicated several factors that may limit the final performance of the bioconversion process: 1) the reaction stoichiometry, 2) the reaction rate, 3) the kinetics of reaction and 4) the physico-chemical parameters (e.g. mixing or mass and heat transfer). In addition, they elaborated that detailed knowledge about these parameters is required to successfully design, develop and control a continuous bioreactor. It was also noted, that the CSTR type of bioreactor offers a complete mixing of feedstock *vs.* lack of mixing in the PFR type. As a consequence, the reaction conditions within the CSTR bioreactor are uniform whereas within the PFR the reaction conditions change from inlet to outlet of the bioreactor tank and lead to high substrate and low product concentrations.

Jørgensen *et al.* (2006) offered a detailed review of multivariate modeling principles used for the monitoring and control of bioreactors. The authors offered detailed explanation of advanced mathematical methods such as: 1) a principal component analysis, 2) a fault diagnosis, and 3) a feedback control. Moreover, the authors suggested that their methods are needed to ensure a high bioconversion yield in a batch or continuous bioreactor. Nienow (2006) suggested that knowledge about the different types of mixing processes (e.g. suspension,

dispersion, blending, pumping) is necessary to ascertain fluid flow patterns and to meet the mass transfer criteria in the bioreactor. In addition, they noted that well mixed conditions were obtained experimentally in stirred tank bioreactors, where highly turbulent flow conditions were maintained through high mechanical power inputs per unit volume.

Figure 1.8 shows the bioreactor classification based on the type of the mixing system used. Sajc *et al.* (2000) noted that in all of these cases, a suitable mass transfer driving force was necessary to ascertain mixing of reactants subject to their rheological properties.

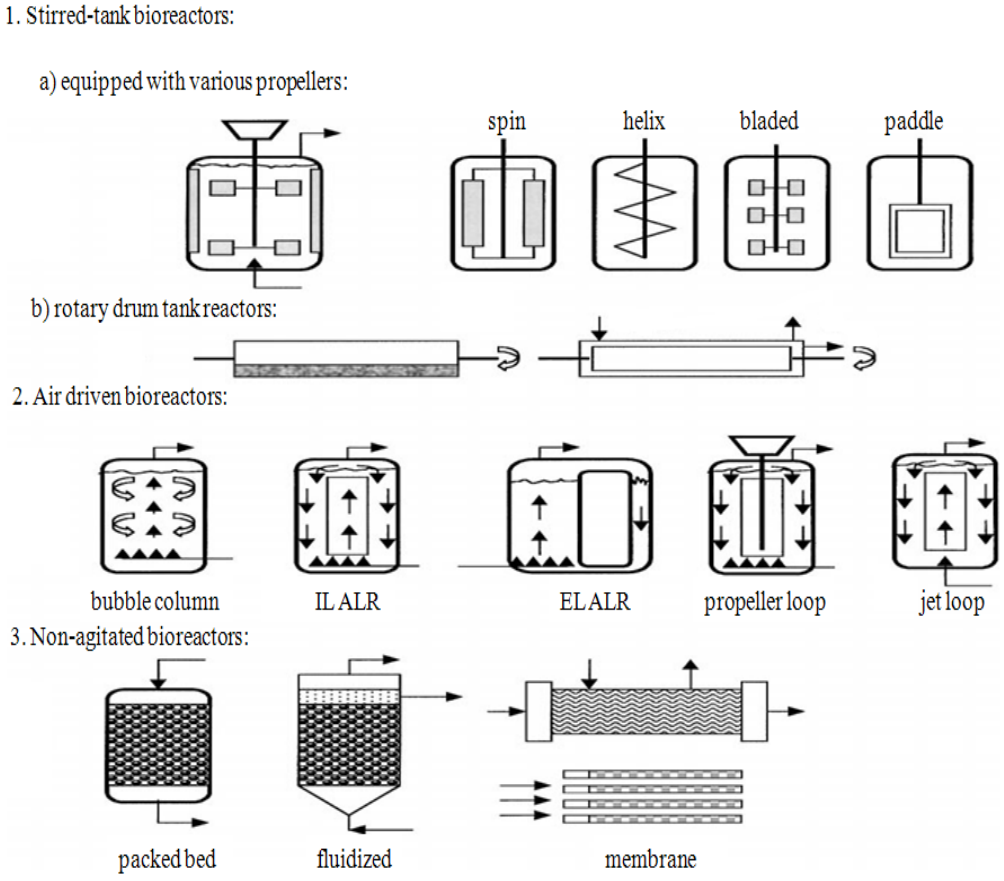

Figure 1.8 Different types of bioreactors classified based on the mixing system, where IL ALR and EL ALR is the Internal Loop Air Lift Reactor and the External Loop Air Lift Reactor, adapted from Sajc *et al.* (2000).

Further to this, Nienow (2006) indicated that the proper selection of propeller was required to avoid excessive levels of mixing and shear, which could result in damage to the immobilized biocatalyst. Villadsen and Reuss (2006) noted that the first issue that needs to be addressed before commencement of the design of a membrane bioreactor is making the appropriate selection of the required category (e.g. batch reactor, fed-batch reactor, or continuous reactor). They indicated that the desired choice by all processors is the continuous bioreactor type, which demands in addition to high capital costs a high level of controls. The authors observed that the batch reactor was a more suitable alternative if there was a need for 1) the pH level adjustment in the bioconverted feed or 2) the additional mixing of the feedstock.

Villadsen and Reuss (2006), also elaborated that the type of the kinetics of the reaction occurring in the bioreactor also needs to be considered. They indicated that the packed-bed bioreactors had an intrinsic reaction kinetic advantage over the continuous stirred tank bioreactors. In the continuous stirred tank bioreactor, the average reaction rate was lower than that in the packed-bed bioreactor. According to their report, this was because of the change in concentration of available reactants during the bioconversion process. Moreover, they suggested that the type of the immobilized biocatalyst (enzyme) needs to be also considered and indicated that the risk of damaging the biocatalyst through mechanical stirring was higher in the CSTR bioreactor type than in the PBR bioreactor type. Finally, the authors recommended using only stable forms of the immobilized biocatalyst in the CSTR bioreactor.

1.4.2 Bioreactors used for lactose degradation

Currently, several commercial plants use bioreactors equipped with free or immobilized enzymes to hydrolyze lactose (Mahoney 1997; Petzelbauer *et al.* 2003; Li *et al.* 2006). The authors reported that the most common systems used by the industry were stirred-tank bioreactors, operating in the batch mode after a free β-galactosidase enzyme was added at the start of the lactose reduction process. Alternatively, packed-bed bioreactors were used by some researches to hydrolyze lactose. But they described that only a few of these were tested in an industrial setting.

Mahoney (1997) reviewed the use of bioreactor technology containing immobilized enzymes for lactose hydrolysis (**Table 1.6)**. Some of these bioreactors were used to produce low lactose milk (Pastore and Morsi 1976) and others to process whey into hydrolyzed syrups (Moore 1980; Harju 1987). According to these authors, some of these systems were successfully introduced in the food industry. But, the common problem in all of these applications was a loss in enzyme activity during extended processing; therefore, there was a need for enzyme replacement. In addition, the kinetic characteristics of the immobilized enzyme were changed. The major issue was a decrease in the diffusion mass transfer rate of lactose due to the type of immobilization method used. This resulted in different amounts of intermediates and final products produced.

Table 1.6 Commercial bioreactors with the immobilized β-galactosidase for lactose hydrolysis.

Process Name (Country)	**Product ($ cost/ kg)**	**Immobilization of β-galactosidase (type)**	**Reference**
SNAM Progettti (Italy)	lactose reduced milk (no data)	cellulose triacetate fiber (entrapment)	Pastore and Morsi (1976)
Corning Inc. (Europe; US)	whey hydrolyzed to lactose syrup- (~0.46)	on porous glass beads (covalent binding)	Moore (1980)
Rohm Gmbh (Germany)	whey hydrolyzed to lactose syrup- (~0.06)	acrylic beads (covalent binding)	Sprossler and Plainer (1983)
Valio (Finland)	whey hydrolyzed to lactose syrup- (~0.13)	phenol-formaldehyde resin (covalent binding)	Harju (1987)
Sumitomo Chemicals (Japan)	lactose reduced milk and whey (no data)	"Sumylact" phenol-formaldehyde resin (covalent binding)	Mahoney (1997)

1.4.3 Membrane filters

Currently, there are two different membrane filtration methods, which are available for separation of liquid and solid materials and can be used in the design of membrane bioreactor systems, Anonymous (2003). The first one is called a Normal Flow Filtration method, commonly recognized as a "dead-end" filtration method, and the second one is called a Tangential Flow Filtration method and is commonly known as the "crossflow" filtration method (**Figure 1.9**). Both methods are pressure driven methods. In the "dead-end" filtration method, a feed

flow is directed perpendicular to a membrane surface and small solute molecules are filtered across the membrane pores. The large solute molecules that are too large to pass with applied pressure are retained and accumulated on the membrane surface. This accumulation over time (called caking) is a cause for the filter capacity to be exhausted and the filtration process to stop.

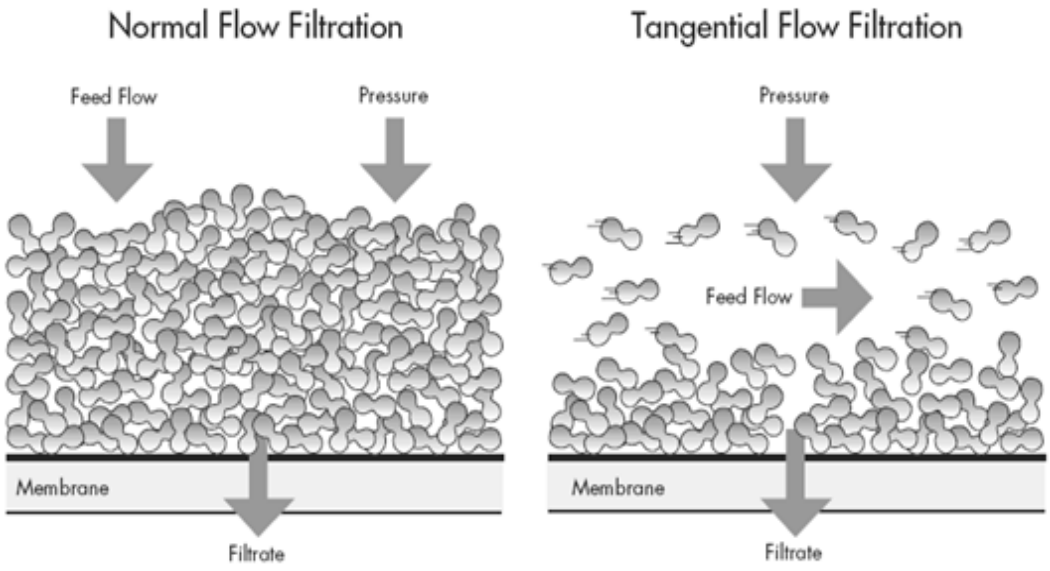

Figure 1.9 Comparison of filtration methods, the courtesy of Millipore Corporation, Anonymous (2003).

In the "crossflow" method, the liquid flow direction in the feed channel present in the crossflow membrane unit is parallel or at a tangent to the membrane surface. Therefore, the caking effect is minimal and the crossflow filter capacity and the filtrate volume are high. The crossflow of solute molecules across the membrane, which is known as flux (*J*), is subject to membrane resistance, which will be different for compounds with a different molecular weight (e.g. mono or disaccharides). In addition, factors such as carbohydrate concentration, feed ionic strength and the fluid external hydrodynamic

characteristics (e.g. feed flow rate, pH, and temperature) will influence their flux and their retention/separation characteristics.

Figure 1.10 shows the effect of solute feed flow along the length of the feed channel in the crossflow filter on the feed pressure in the inlet ($P_{F)}$ and in the outlet (P_R). The low flow rate on the filtrate pressure side is a cause for the low filtrate pressure (P_f).

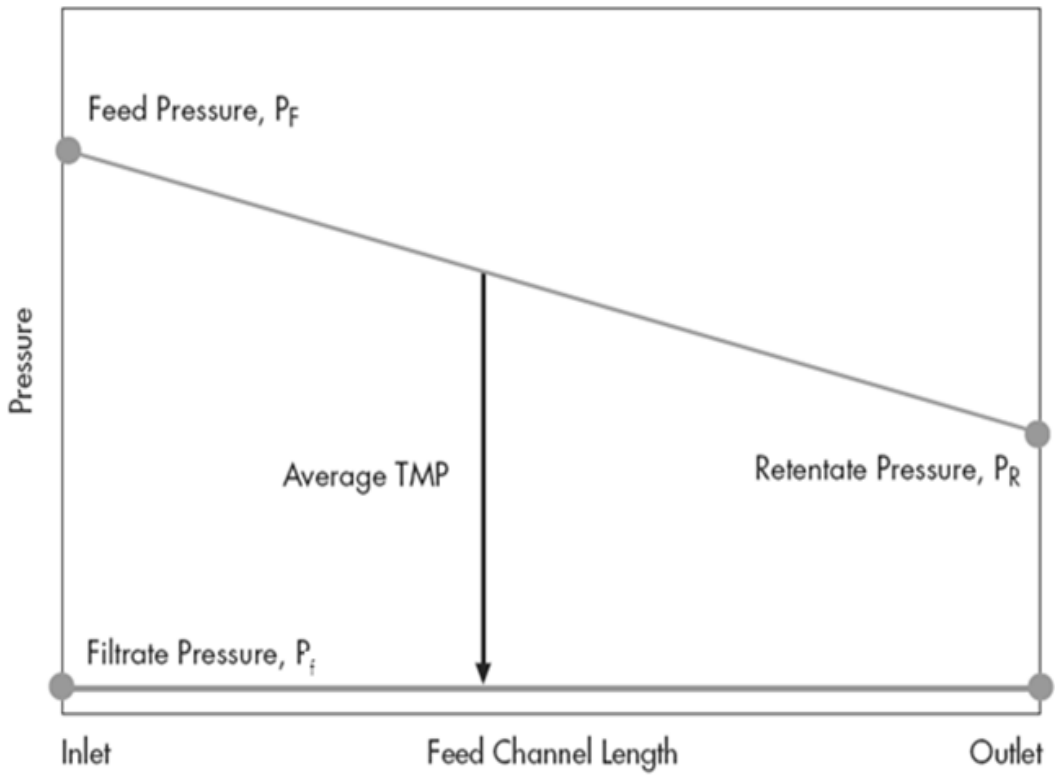

Figure 1.10 Pressure distribution in the crossflow membrane feed channel. The trans-membrane-pressure (TMP) is the average applied pressure value from the feed to the filtrate side of the membrane, the courtesy of Millipore Corporation, Anonymous (2003).

The separation of solute molecules on crossflow nanomembranes is described by the solution-diffusion model (**Figure 1.11)**. The pump pressure forces a portion of the feed (Feed Flow), flowing parallel to the membrane active surface, to pass across the membrane into the filtrate stream (permeate). The

outcome of this process is an increase in a concentration gradient of the feedstock, passing over the nanomembrane surface at a specific feed flux (*J*), and at the length of the flowing feed channel.

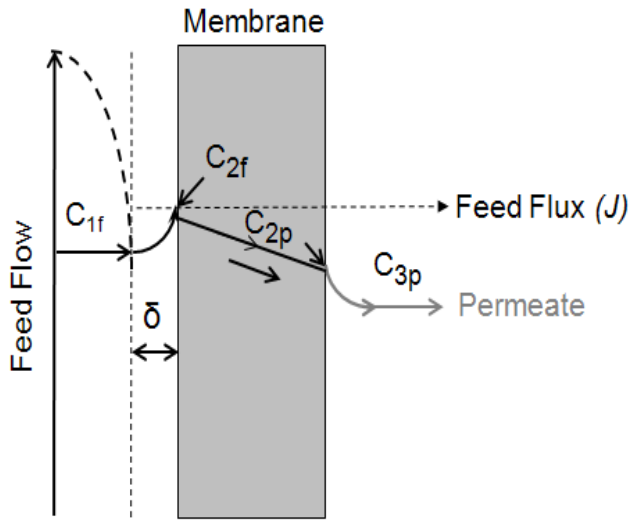

Figure 1.11 Schematic model of transport of a solute in the nanomembrane during the crossflow process. Where: $\mathbf{c_{1f}}$ is the concentration of carbohydratesolution in the feed stream that flows parallel to the membrane surface,**δ** is a solute boundary layer, c_{2f} is the concentration of solute at the membrane surface, and $\mathbf{c_{2p}}$ and $\mathbf{c_{3p}}$ refer to the concentration of solute in the permeate stream within and after the membrane respectively, adapted from Harrison *et al*. (2003).

Steinle-Darling *et al*. (2007) indicated that the feed (water and solute molecules), when diffusing through the crossflow membrane, must overcome the membrane boundary layer and membrane before passing through it. They reported that an increase in a solute concentration boundary layer (ratio c_{2f}/c_{1f}), known as a "concentration polarization phenomenon", affected the performance of crossflow membranes.

Harrison *et al.* (2003) elaborated further that at steady state conditions the rate of convective mass transfer of solute toward the membrane's active surface

was equal to the rate of mass transfer of solute diffusion away from the membrane surface and offered the following equation:

$$J = \left(\frac{D}{\delta}\right) ln\left(\frac{c_{1f}}{c_{2f}}\right) \qquad (5)$$

where: J is a transmembrane feed flux, D is a diffusion coefficient of the solute, and the δ, the c_{1f}, and the c_{2f} are parameters which are described earlier in **Figure 1.11.**

Charcosset (2006) described "dead-end" and "crossflow" filtration methods, which were available for separation of liquid and solid materials. Also, he indicated that the crossflow system offered several advantages as compared to "dead-end" method: 1) a high efficiency of separation, 2) a high retention of immobilized enzymes and biomass, 3) a high throughput of bioconverted feeds, 4) the opportunity to improve operating controls, and 5) a high membrane bioreactor compactness. During the crossflow filtration process, part of the feed is rejected or retained by the membrane (the concentrate or retentate), whereas the other part flows through the membrane (permeate).

Harrison *et al.* (2003) provided a general classification for the crossflow systems and membranes and indicated that membranes are commonly produced from semi-permeable polymeric or ceramic porous materials. Furthermore, they explained that crossflow membranes are packed in module assemblies and are available in several configurations such as: flat sheet, spiral wound, tubular, hollow fiber, and a rotating cylinder. Examples of those commonly used filter modules are demonstrated below in **Figure 1.12**.

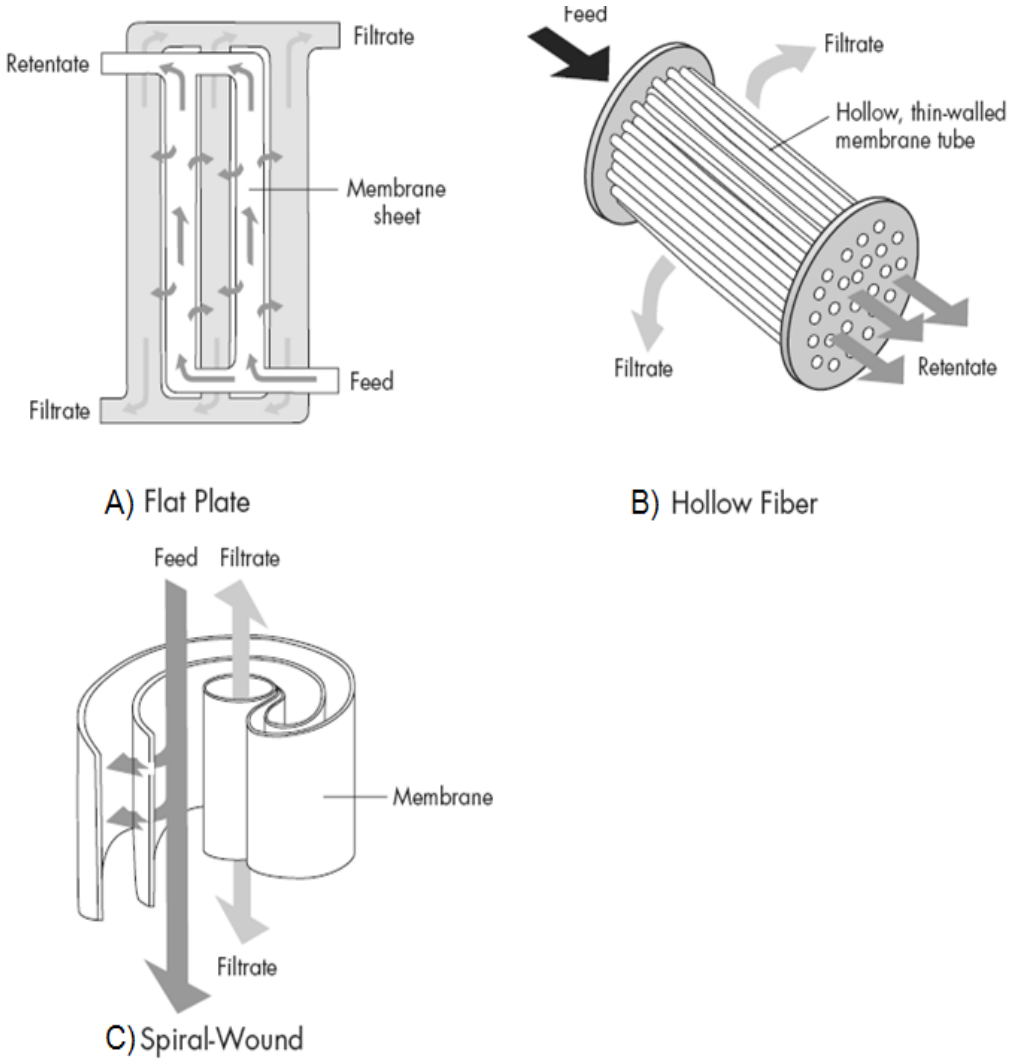

Figure 1.12 A) Flat plate, B) Hollow fiber and C) Spiral-wound crossflow filtration modules, the courtesy of Millipore Corporation, Anonymous (2003).

In addition, Harrison *et al.* (2003) indicated that the selection of the correct membrane pore size and module configuration is always subject to several technological process requirements (e.g. mechanical, hydrodynamic and cost). They elaborated that the structural characteristics and thickness of those materials impact solute transport. The term Molecular Weight Cut Off (MWCO), is

frequently used to describe the nominal size of molecules that diffuse across the membrane where they are rejected at a 90% level.

They noted that the MWCO and the membrane structure may be affected during the separation process due to applied pressure, temperature, feed flow rate, feed concentration and the type of carbohydrate used. Moreover, the crossflow filtration systems could be equipped with membranes with different pore sizes and could be used in several industrial applications (e.g. water purification, extraction or recovery of value-added components, solids concentration, and liquid phase mixing) (**Figure 1.13).**

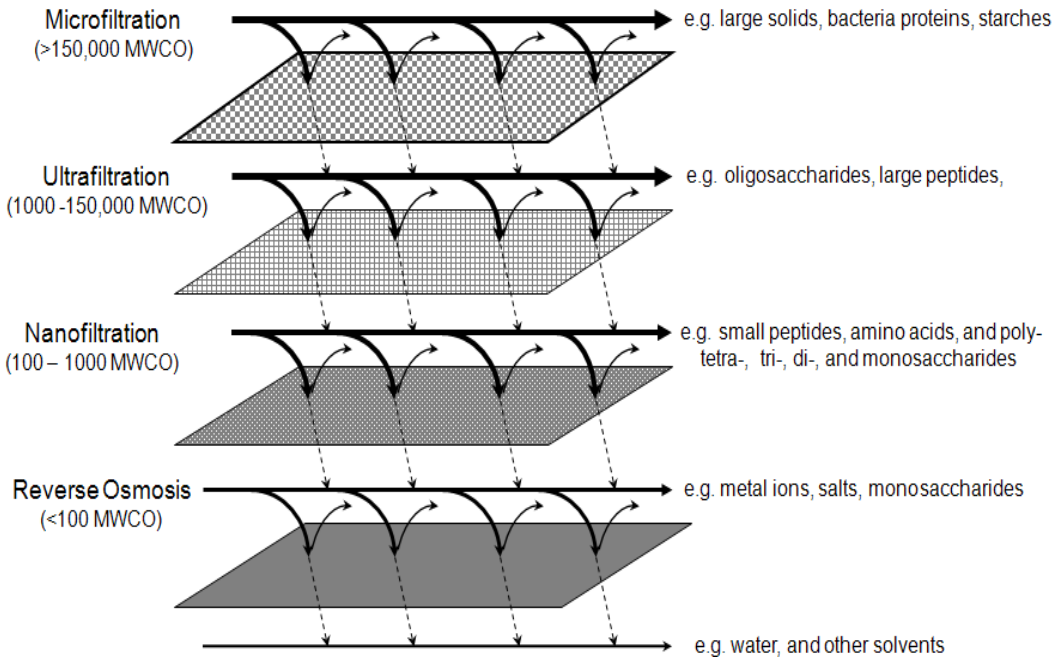

Figure 1.13 Crossflow membranes classifications based on the MWCO.

1.4.4 Characterization of nanomembranes

Nanomembranes, which are used in the food industry are membranes with pore sizes from approximately 100 to 1000 MWCO. Currently, in the dairy

industry nanomembranes are employed in the concentration and demineralization of sweet whey (Jeantet *et al.* 2000). A nanomembrane is made of a very thin laminate, which is composed of an active surface layer of porous material (e.g. polymer) and a support layer (made of more porous polymer compound), approximately 100 to 400 nm thick. The nanomembrane active surface is commonly made of a polyamide (PA) or a polyethersulfone (PES) polymer. The ultra thin composite materials are generated by the interfacial polymerization process and laminated on the surface of the porous support materials, commonly a polysulfone. Overall, nanomembranes are characterized by high mechanical strength and flexibility, and are resilient to corrosive chemicals and temperature, and offer a high biological stability (Cricenti *et al.* 2006; Tang *et al.* 2007).

Jeantet *et al.* (2000) evaluated spiral wound nanofiltration membranes used in the fractionation of sweet whey and attributed a significant nanomembrane "fouling" to the "concentration polarization phenomena". Bellona *et al*. (2004) reported that the concentration and size of solute molecules in the solvent were critical for their permeation or rejection by the membrane. It was noted that the transport of feed across the membrane is affected by a few gradients: 1) a solute concentration gradient, 2) the pressure and 3) the temperature gradient. Goulas *et al.* (2003) offered their own evidence of carbohydrate separation of oligosaccharides on nanomembranes. They indicated that most carbohydrates are neutral molecules in water solutions and their mass transport across nanomembranes is controlled by convection and diffusion processes. They added that the convection process improves with an increase in

the applied nanomembrane pressure that the diffusion process is dependent on the carbohydrate concentration gradient. Goulas *et al*. (2003) proposed several mass transfer criteria that could be used to evaluate, compare and characterize nanomembranes for their ability to separate feed components. These evaluation criteria are described by the following equations:

$$1)\quad J_v = V_p / A\,T \qquad (6)$$

where J_v *is* the volumetric flux of permeate, V_p is the permeate volume, A is the membrane effective area, and T (h) is the time required for the production of V_p liters of permeate, respectively.

$$2)\quad VCR = V_f / V_r \qquad (7)$$

where VCR is the volume concentration ratio and V_f and V_r are the volumes of the initial feed solution and the retentate, respectively.

$$3)\quad R_i = ln(C_r/C_f)/ ln(VCR) \qquad (8)$$

where R_i is the rejection value specific for a given solute *"i"*, C_r and C_f are the concentrations of that solute in the retentate and the initial feed respectively.

$$4)\quad Y = C_r V_r / C_f V_f \qquad (9)$$

where Y, the yield, is the ratio of retentate components ($C_r V_r$) to the initial feed components ($C_f V_f$). In addition, Jeantet *et al.* (2000 evaluated solute etention of different components including lactose with the help of the following equation:

$$R_{\%} = (C_r - C_f)/ C_f * 100 \qquad (10)$$

where $R_{\%}$ is the solute retention, C_r and C_f are the concentration of that solute in the retentate and feed, respectively. Furthermore, they found that the whey nanofiltration process could be optimized and whey permeate was characterized by a lower chemical oxygen demand and lower mineral content.

Cricenti *et al.* (2006) reported that the nanomembrane filtration system offers some advantages over the reverse osmosis filtration (lower applied pressure) or the ultrafiltration system (a high rejection of low molecular weight compounds). They suggested that the passing of carbohydrate across the membrane was a function of solute molecules size, polarity, as well as membrane material and charge. They used the horizontal attenuated total reflectance-fourier transform infrared spectroscopy (HATR-FTIR) method to investigate nanomembrane composition before and after processing of salt solutions. They indicated that this method revealed an interesting modification to the membrane surface, which helped them to confirm that the nanomembrane surface was composed of the polysulphone material.

Tang *et al.* (2007) offered a proof of the comprehensive characterization of the physico-chemical properties of uncoated and coated nanomembranes using analytical methods that included transmission electron microscopy (TEM) and attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR). They elaborated that it is necessary to evaluate the physico-chemical properties of the membranes to determine and optimize their selection before their application at the CFF process. Bowen *et al.* (1999) demonstrated an application of the Atomic force microscopy (AFM) for the analysis of nanomembranes' surfaces.

They suggested that the knowledge of nanomembranes' surface composition is important for understanding their performance.

1.4.5 Separation of carbohydrates on nanomembranes

Recently, there has been several publications which have used different carbohydrates feeds and demonstrated that nanofiltration of carbohydrates on membranes was possible (Table 1.7).

Table 1.7 Nanomembranes used in the separation of carbohydrates from their feeds.

Nanomembranes (type/supplier)	**Feedstock/ Carbohydrates (concentration %)**	**Reference**
DS-5 (DK 3840 C) (GE Osmonics, Minneapolis, MN, USA)	Whey lactose and minerals (~5%)	Jeantet *et al.* (2000)
DS-5DK and DS-5HL (GE Osmonics)	grape juice sugar (~ 3%)	Ferrarini *et al.* (2001)
NF-CA-50 and NF-TFC-50 (Intersep Ltd., UK)	mixture of lactose and glucose and oligosaccharides (~ 20-30%)	Goulas *et al.* (2003)
DS-5DK, DS-5 DL, and NF270 (GE Osmonics; Dow Filmtec)	mixture of xylose, glucose, mannose and chemicellulose (~ 20-59%)	Sjöman *et al.* (2006)
DS-5DK, DS-5 DL, and NF270 (GE Osmonics; Dow Filmtec)	mixture of xylose, glucose (~ 2- 30%)	Sjöman *et al.* (2007)
DS-5DK and DS-5HL (GE Osmonics)	mixture of lactose and lactic acid (~ 4.9 - 5.2%)	Li *et al.* (2008)

In addition, Sjöman *et al.* (2007) found that the xylose retention value was lower than the glucose retention value. They concluded that the best separation

conditions between glucose and xylose carbohydrates were achieved from their concentrated solutions (10 and 30%), and at high pressure conditions (30 bar). Lopez-Leiva and Guzman (1995), and later Goulas *et al.* (2003), used nanofiltration membranes for the purification and separation of carbohydrate mixtures composed of oligosaccharides, lactose, glucose and galactose. The authors reported that the concentration and some purification of oligosaccharides is possible on nanomembranes.

1.4.6 Membrane Bioreactors

The bioreactors integrated with membranes are used to achieve two objectives: feedstock bioconversion and its simultaneous separation on membranes. Currently, membranes, which have unique separation characteristics, are integrated into the membrane bioreactor systems. The membrane bioreactor technology is a desired configuration and it is adopted for the development of bioconversion methods (Sajc *et al.* 2000; Harrison *et al.* 2003; Charcosset 2006). The membrane bioreactor consists of two major elements: the bioreactor tank and the semi-permeable membrane filter.

Harrison *et al.* (2003) offered their own examples of crossflow membrane filtration units coupled to a bioreactor. They indicated that there were four basic bioreactor-membrane configurations: 1) batch concentration, 2) diafiltration, 3) purification, and 4) complete feed recycle. In the batch concentration configuration, soluble or suspended solids are concentrated as a retentate in the tank. This is achieved by the removal of the liquid permeate,

which is filtered across the crossflow membrane. In the diafiltration configuration, the liquid containing small soluble molecules flows across the crossflow membrane and the volume of disposed permeate is adjusted with added water or buffer. In the purification configuration, the small molecules pass across the membrane, but the larger molecules are retained in the bioreactor tank. Finally, in the complete feed recycle configuration, both retentate and permeate are recycled back to the bioreactor tank.

Sajc *et al.* (2000) compared membrane bioreactors to non-membrane bioreactors and indicated that the application of membranes in the bioreactor system offers several advantages: 1) a higher selectivity towards bio-converted substrate, 2) a higher surface-area-per-unit-volume, 3) an improved level of control for contacting and mixing two or more phases, 4) an efficient use of biological cells or enzymes (biocatalysts), and 5) an opportunity for the immobilization of soluble enzymes or biological cells within or outside of the bioreactor membrane.

Charcosset (2006) reviewed some operating principles of bioreactors equipped with membrane filters and their application in the industry. The author described two types of membrane bioreactors: A) a batch stirred bioreactor equipped with an external membrane unit and B) a continuous bioreactor. The characteristic feature of this second system was that the membrane filter was installed inside of the bioreactor tank together with an enzyme attached to its surface (**Figure 1.14)**.

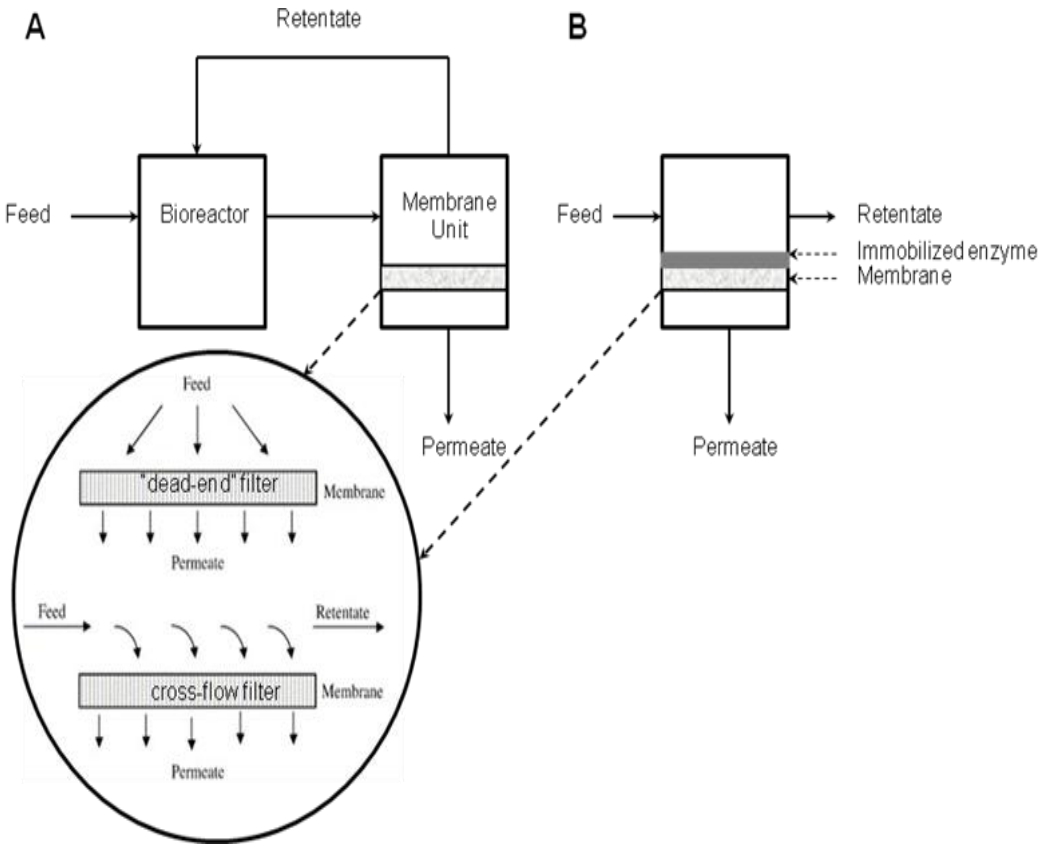

Figure 1.14 Two types of the bioreactor systems (A and B) equipped with membranes, adapted from Charcosset (2006).

According to Charcosset (2006), in the first type of membrane bioreactor, the feed is pumped into the stirred bioreactor tank and it is then bioconverted using soluble enzymes with simultaneous filtration across the membrane unit. The unfiltered feed, as retentate, is concentrated and re-circulated back to the stirred bioreactor tank and the filtrate, as permeate, is separated and pumped to the storage tank. The use of soluble enzymes in batch stirred bioreactor is limited to the following issues: 1) a low productivity of bioreactor, 2) a high cost of added enzymes, 3) a high cost and difficult recovery of enzyme,

4) a possible product and effluent contamination, and 5) a high variability in final product composition.

In the second type of membrane bioreactor, the feed is pumped continuously into the bioreactor tank and bioconverted on contact with membranes containing the attached enzyme, and then subsequently separated into retentate and permeate streams. A membrane bioreactor that uses the membrane as a separation media for retention of an enzyme and larger molecules in the bioreactor tank is recognized as enzyme membrane reactor (EMR).

Huffman-Reichenbach and Harper (1981) noted earlier that permanent immobilization of the enzyme to the membrane may be difficult. The authors failed in their attempt to immobilize the β-galactosidase enzyme on a polysulphone type membrane surface with a molecular weight cut off from 10,000 to 50,000. They found that the retention of the enzyme was very low and indicated that the enzyme leaked through the membrane into the permeate stream. But according to the authors, the molecular weight (MW) of the β-galactosidase used in their research was approximately 100,000. Therefore, they concluded that membranes have a dynamic porous structure which can be affected by many factors such as solubility of filtered compound, flux rate, solution pH, and concentration of salts.

Haider and Husain (2009) reported that enzyme membrane reactors are characterized by a lower enzymatic activity when compared to batch bioreactors with soluble enzymes. They observed that enzymes entrapped by membranes suffered conformational changes which resulted in their lower binding capacity.

Rios *et al.* (2004) reviewed the function and use of enzyme membrane reactors. They observed that the major advantage of such a technological platform was that it enabled the selective separation of enzymes from the processed feeds. In addition, the authors noted several other benefits of this relatively new technology: 1) improved control of the bioconversion process, 2) lower variability in the finished product content, 3) reusability of the biocatalyst and 4) opportunity for continuous processing of used feeds and 5) lower total cost of operation.

Several membrane bioreactor systems with immobilized enzymes were used for hydrolysis and separation of lactose (López Leiva and Guzman 1995; Chockchaisawasdee *et al*. 2005; Shahbazi *et al*. 2005, Grosová *et al*. 2008). These authors indicated that enzymatic membrane bioreactors simplify technological steps and offer material and cost savings in the carbohydrate bioconversion processes. Therefore, they described that there is an ongoing search for new improved methods with a focus on immobilized enzymes and improvements to the EMR bioreactor design for bioconversion of lactose. Nevertheless, there have not been many studies published that could show results for the bioconversion of lactose with its simultaneous CFF nanofiltration in the batch stirred reactor.

This dissertation project, utilizing the above mentioned research information, describes the bioconversion of lactose with the β-galactosidase immobilized on polyvinyl alcohol (PVA) in the bioreactor and its simultaneous crossflow filtration on nanomembranes.

1.5 RESEARCH OVERVIEW AND OBJECTIVES

The research focus of this dissertation project was on the design, development and testing of a measurement methodology and a lactose bioconversion platform that incorporated the stirred bioreactor system equipped with a nanomembrane. The proposed hypothesis was that this conceptual design would lead to the development of the effective bioconversion of lactose or other similar carbohydrates.

At the start of this project, not much was known about fast and rapid analytical assays that would permit the evaluation of lactose concentration and its parent compounds, glucose and galactose, during the lactose bioconversion process. Identification of these compounds frequently requires the use of costly and complicated sample pretreatment procedures that may affect the sample matrix and lead to poor repeatability in results. Generally, most studies describing lactose bioconversion in the bioreactor system, equipped with the ultra-filtration membranes or nanomembranes, were conducted with a small volume reactor system (Iorio *et al*. 2006). The efficiency of the immobilized biocatalysts used in the lactose bioconversion process, although well researched, is continuously updated with new innovative solutions, which focus on the biocatalyst's increased efficiency and stability and on its possible reuse (Grosová *et al*. 2008). This is because there is an ongoing need to lower costs of soluble biocatalysts and where possible replace them with reusable immobilized forms (Mahoney 1997; Illanes 2000).

The specific objectives and summary of the research contained in this dissertation are as follows:

Chapter 2. In this chapter, the major goal was to develop a fast, rugged, and simple method for measuring the concentration of individual carbohydrates during the lactose hydrolysis process in milk. The digital polarimeter equipped with a flow-through cell and a prediction model, developed with the commercially available commercial Design-Expert® software program, to evaluate the concentration of carbohydrates in permeate from the UF treated milk on the Koch HFK 131 ultrafiltration membrane, nominal molecular weight cut-off of 10,000.

Chapter 3. In this chapter, the major goal was to investigate the separation of lactose from glucose and galactose on two commercially available nanomembranes, the Filmtec NF270-400 and the Koch TFC-SR3 with a nominal molecular weight cut-off of 200-400.

Chapter 4. In this chapter, the major objective was to evaluate the activity of a novel biocatalyst during the bioconversion of lactose. We immobilized β-galactosidase of *Kluyveromyces lactis*, a biocatalyst, in LentiKat's® polyvinyl alcohol (PVA) spherical beads, and compared its activity to a commercially available soluble enzyme when hydrolyzing lactose in the stirred membrane bioreactor equipped with the nanomembrane.

Chapter 5. In this chapter, the major objective was to develop and apply the batch-stirred membrane bioreactor for the whey lactose bioconversion. Lactose was bioconverted by a novel immobilized biocatalyst and an enzyme was entrapped on the PVA LentiKat's® matrix which was retained in the bioreactor tank with the stainless steel screen. Simultaneously, bioconverted lactose was crossflow filtered on the Koch TFC-SR3 nanomembrane at a constant feedstock pressure that was controlled with the pressure and mass flow controller unit.

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2. RAPID MEASUREMENT OF CARBOHYDRATES IN MILK WITH A MODIFIED POLARIMETRIC METHOD [a]

2.1 INTRODUCTION

Lactose, glucose and galactose are optically active carbohydrates and in their simple solutions rotate the plane of linearly polarized light clockwise. This property has its specificity for each of these carbohydrates, and is used by polarimetric reference methods to measure lactose and glucose concentrations in food products (AOAC 1984a, 1984b). The principle of the degree of optical rotation was first described by Biot's law (Lloyd and Goodall 1989; Rudolph Research Analytical 2006). This measurement is rapid and non-destructive. The specific optical rotation value for individual carbohydrates is determined by the carbohydrate concentration and molecular structure (Kearsley 1985; Lesney 2004). The enzymatic hydrolysis of lactose by the enzyme lactase results in the direct conversion of this unique disaccharide into two monosaccharides, glucose and galactose (Gänzle *et al.* 2008). The partial or complete conversion of lactose renders it useful in numerous applications. Applications include the production of lactose-free milk that retains usable carbohydrates and a new source of glucose and galactose that can be used in food products and/or for food and industrial fermentations (Bury and Jelen 2000; Elliot *et al.* 2001; IDF 2005).

Various researches have proposed several alternative methods to evaluate the

[a]A portion of this chapter was presented orally at the 2007 IFT Annual Meeting & Food Expo in Chicago. Pikus, W., McCaffrey, W.C., Bressler, D., and L. Ozimek. 2007. Measurement of milk sugars with digital polarimeter at low pH. Book of Abstracts IFT 2007 (46-04):63

concentrations of these carbohydrates in solution. Although many of these methods could arguably be more accurate than the polarimetric method, they are substantially more costly and labor-intensive (Pirisino 1983; Lanza and Li 1984; Ona *et al.* 1998; Sivakesava and Irudayaraj 2001). Southgate (1991) reviewed the application of the polarimetric method to measure the concentration of carbohydrates in dairy foods and described the major advantages of this method over other analytical methods with regard to its robustness, non destructiveness, low cost and very short measurement time. Schallenberger (1985) reported that a variation in the optical rotation of individual carbohydrates in their mixed solutions containing two carbohydrates could be used to calculate the concentration of individual components.

The development of new prediction models based on an estimates of the concentration of individual carbohydrates and other chemicals in a mixture was reported by Scheffe (1958), Myers (1964), Contreras *et al.* (1992), Obermiller (2000), and Bro (2003). An important advantage of these models is that data can be evaluated quickly and accurately by use of mathematical equations. Therefore, it was envisaged that successfully integrating one of these prediction models, as suggested above, with an on-line digital polarimeter should lead to the successful development of a new fast method, which would cheaply and accurately evaluate lactose, glucose and galactose in their mixture during the lactose hydrolysis process. However, the direct polarimetric on-line measurement of carbohydrates in milk or lactose-reduced milk is not possible without the prior removal of interfering components such as protein and fat. To eliminate this obstacle, an

ultrafiltration (UF) treatment can be used to separate major milk components effectively (Lopez-Leiva and Guzman 1995).

The major objectives of this study which were: 1) to develop a fast, robust, and simple method to measure the concentration of carbohydrates in a ternary mixture of lactose, glucose, and galactose, with the help of a digital polarimeter equipped with a flow-through cell and a commercially available Design-Expert® prediction program, and 2) to explore its suitability for measuring the concentration of individual carbohydrates "on-line" during the lactose hydrolysis process in milk permeate from the UF treated milk in a stirred batch reactor equipped with UF membrane.

2.2 MATERIALS AND EQUIPMENT

2.2.1 Materials

Skim milk powder (Safeway Inc. brand, Edmonton, Alberta, Canada) was used as received. The α-lactose monohydrate, D-glucose and D-galactose standards, each 99.8% pure, were purchased from Sigma-Aldrich Canada Ltd. (Toronto, Ontario, Canada). Citric acid, 99.9% pure, was acquired from VWR Inc. (Toronto, Ontario, Canada) (purity data supplied by the manufacturers). The potassium phosphate buffer system components, 99.9 % pure, and the pH 4.0 and 7.0 buffers and hydrogen peroxide 30% (w/v) were purchased from Fisher Scientific (Edmonton, Alberta, Canada). Solutions of 1 N NaOH and 1 N HCl used for the pH adjustment were acquired from VWR Inc. The Validase®

biocatalyst (Yeast Lactase, β-galactosidase, EC 3.2.1.23, derived from *Kluyveromyces lactis,* activity 50,000 ortho-nitro-phenyl-β-D-galactopyranside g^{-1} units (ONPGU g^{-1} units), with a protein content 30g L^{-1} , was donated by Valley Research Inc. (South Bend, Indiana, USA). Doubly de-ionized water was produced by the filtration of tap water through the membrane/cation exchange assembly obtained from Millipore Inc., Jaffrey (New Hampshire, USA). The lactose, glucose and galactose standard solutions were prepared in a 0.1 M potassium phosphate buffer containing 2 mM $MgCl_2 \cdot 6\ H_2O$ and pH 6.9 adjusted with citric acid. The α-lactose monohydrate, D-glucose and D-galactose will be referred to in this paper as lactose, glucose and galactose.

2.2.2 Membrane

The Koch HFK 131 ultrafiltration membrane, nominal molecular weight cut-off (MWCO) of 10,000 (Koch Membrane Systems Inc., Wilmington, Massachusetts, USA), was purchased from Sterlitech Co., (Kent, Washington, USA), in a flat sheet configuration. Before use, a new membrane was preconditioned by immersing in fresh doubly deionized water and holding it at room temperature for 24 h. Double deionized water was recycled through the membrane system after installation of the preconditioned membrane at a flow rate set at 72 L h^{-1} with a membrane back pressure set at 9.7 bar. The protein in milk retentate and permeate was measured with the Coomasie Plus® Bradford Assay and using the BSA protein standard (Pierce Biotechnology, Inc., Rockford,

Illinois, USA). The protein rejection value of the Koch membrane was evaluated according to Goulas *et al.* (2003).

2.2.3 The stirred membrane batch reactor system

Experiments concerning lactose hydrolysis in milk were carried out in a stirred membrane batch bioreactor (**Figure 2.1)**. The major components of this setup are: a) the digital polarimeter, model Autopol II (Rudolph Research Analytical, Hackettstown, New Jersey, USA), b) the SEPA CFII membrane unit containing one membrane with an effective filtration area of 140 cm^2 (Sterlitech Co., Kent, Washington, USA), c) the 10 L feed vertical stainless-steel flat bottom tank (with overall dimensions - length 46.0 and diameter 20.0 cm) equipped with a double wall, and custom designed at the Department of Agricultural, Food and Nutritional Science (AFNS), the University of Alberta, Edmonton, Canada, and d) the Hydra-Cell pump, model D03 (Wanner Engineering Inc., Minneapolis, Minnesota, USA).

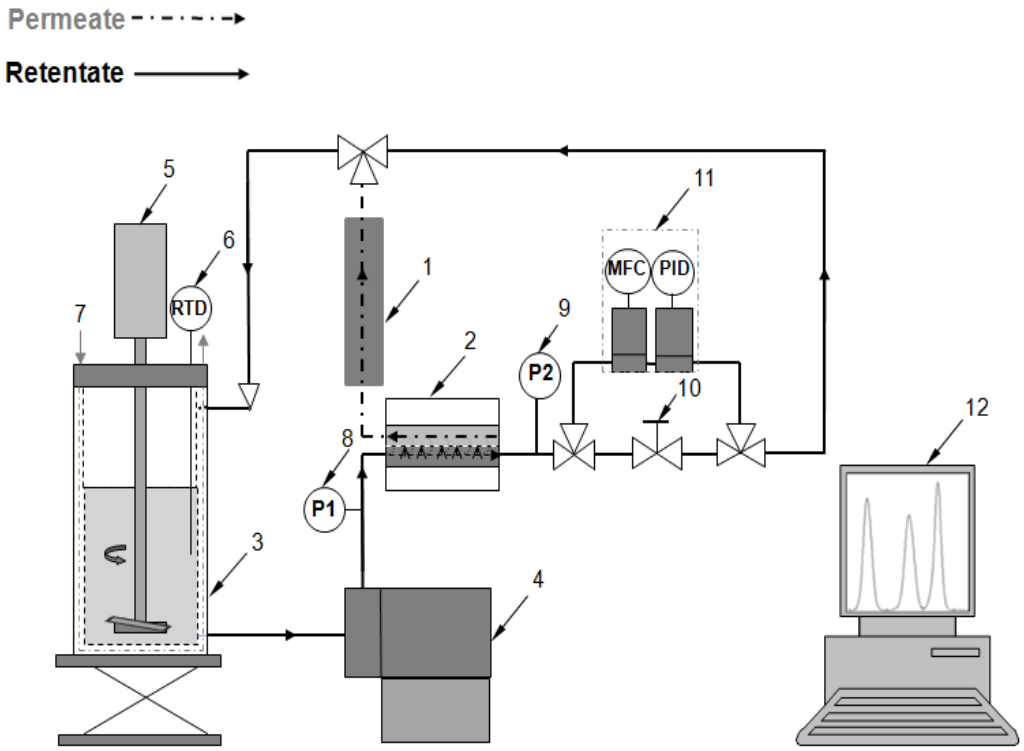

Figure 2.1 The components of the stirred membrane batch bioreactor system with "on-line" polarimeter are 1) the polarimeter, 2) The SEPA CFII membrane CFF unit, 3) the 10 L feed stainless-steel tank, 4) the Hydra-Cell pump, 5) the 50-500 rpm stirrer (Fischer Scientific, Edmonton, Alberta, Canada), 6) the Pt-100 two wire thermocouple probe (Fischer Scientific, Edmonton) connected to the Fluke 701 process calibrator (Fluke Electronics Canada Inc., Mississauga, Ontario, Canada), 7) the water bath, model Haake F3 (Fischer Scientific, Edmonton, Alberta, Canada), 8) and 9) the analog pressure gauges (NoShok Inc., Berea, Ohio, USA), 10) a manual needle pressure regulator valve (Swagelok Inc., Edmonton, Alberta, Canada), 11) the automatic back pressure mass flow controller valve (Bronkhorst High-Tech BV, Nijverheidsstraat, AK Ruurlo, Holland), and 12) a computer equipped with mass flow control software (Bronkhorst High-Tech, BV Nijverheidsstraat).

2.2.4 Polarimeter

The digital polarimeter, (model Autopol II, Rudolph Research Analytical, Hackettstown, New Jersey, USA), was used to measure the carbohydrate optical rotation for both batch and continuous on-line measurements in permeate

samples. The instrument was equipped with a temperature controlled flow-through quartz cell set at 37.0 ± 0.5^{o}C, 10 mL volume and 200 mm long, and a tungsten-halogen lamp set at λ = 589 nm operating wavelength. The accuracy of the instrument's optical rotation set up was verified with the control plate, purchased from an instrument manufacturer (Rudolph Research Analytical, Hackettstown), and calibrated according to the National Institute for Standards and Technology (NIST), Rudolf Research Analytical (2006). The phosphate buffer reference was set at a zero value. The optical rotation was measured continuously during the lactose hydrolysis process in UF milk permeate.

2.3 METHODS

2.3.1 Evaluation of carbohydrate standard solutions with the polarimeter

The individual carbohydrate standard solutions containing lactose, glucose and galactose in concentrations ranging from 0.0 to 4.0% (w/v) were prepared by a proportional dilution of each carbohydrate in a phosphate buffer. In addition, their mixed standard solutions, containing lactose, glucose and galactose, blended in equal amounts, each ranging from 0.0 to 4.0% (w/v), were prepared by thoroughly mixing the concentrated stock solutions, 20% (w/v), with a phosphate buffer. This was followed by an intermittent mixing for 5 min. To standardize measurements and to avoid the influence of mutarotation, all fresh carbohydrate standards were kept for 24 h at room temperature and the pH values for all samples were reconfirmed immediately prior to the polarimetric analysis. Before

taking the polarimetric measurement, samples were mixed for 5 min and allowed to rest for 30 min at 37.0 ±0.5°C in a thermostatically controlled water bath (model 1235, VWR, Toronto, Ontario, Canada).

The response factors (RF_x), specific for glucose, galactose, and lactose, were estimated experimentally from a linear correlation between the measured optical rotation value and the corresponding concentration value for each carbohydrate by applying the following equations 11, 12 and 13:

$$RF_{glu} = [\alpha_{glu}]_{\lambda}^{T} / S_{glu} \quad (11)$$

$$RF_{gal} = [\alpha_{gal}]_{\lambda}^{T} / S_{gal} \quad (12)$$

$$RF_{lac} = [\alpha_{lac}]_{\lambda}^{T} / S_{lac} \quad (13)$$

where S_{glu}, S_{gal}, and S_{lac} are the concentrations of each carbohydrate component, $[\alpha_x]_{\lambda}^{T}$ is the measured optical rotation value at a set measurement temperature (T) for each carbohydrate and instrument wavelength (λ). All analytical results were corrected for the dilution after a pH adjustment.

2.3.2 Determination of carbohydrate concentrations with HPLC

The HPLC method was adopted with a run time for each sample modified to 16 min (Anonymous 2007). The experiments were performed on an Agilent 1200 Series chromatograph equipped with a Model G1329A autosampler, a Model G1311A quaternary pump and a Model G1362A refractive index detector operated at 35°C internal temperature (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). The analytical column was a Nucleogel Sugar

Ca^{2+} column (300 mm x 6.5 mm) proceeded by a guard column (21 mm x. 4.0 mm) (Marcherey-Nagel Gmbh & Co. KG, Düren, Germany), and the column was heated at 87°C with an external column heater (Bio-Rad, Hercules, California, USA). The mobile phase was H_20, filtered by passing through 0.5 µM filters, (Millipore, Bellerica, Illinois, USA), and degassed, while the 20 µL permeate sample was injected on to the column with the flow rate set at 0.5 mL min^{-1}. The HPLC results for all samples were quantified using Agilent "ChemStation", version 2007, software, (Agilent Technologies).

2.3.3 Development of the prediction model

The optical rotation data for different lactose, glucose and galactose concentrations related to lactose hydrolysis in milk for an initial lactose concentration set at 4.0% ± 0.1 (w/v), was fitted into the simplex matrix table of the Design Expert®, DOE 7.1.6 modeling software (Stat-Ease Inc., Minneapolis, Minnesota, USA). The data matrix was transformed with the help of the general polynomial quadratic equation (14) which was proposed by Scheffe (1958):

$$E(Y) = \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j \qquad (14)$$

where $E(Y)$ is the response (e.g. optical rotation value) for the dependent variable, $\beta_i X_i$ is the response due to the pure components concentrations (X_i) in the mixture, β_i is the linear coefficient and $\Sigma\beta_{ij} X_i X_j$ is the response due to the interaction between component concentrations in mixture ($X_i X_j$).

2.3.4 Evaluation of lactose hydrolysis in milk with the prediction model

The commercially available biocatalyst Validase®, 4 mL, was added to 4 L of reconstituted skim milk. The lactose concentration was adjusted exactly to 4.0% ± 0.1 (w/v) by mixing with water as indicated by the milk powder manufacturer and verified by HPLC. The hydrolysis reaction was carried out in a stirred membrane batch reactor for 1 h at 37 ± 0.5°C. The permeate, free of fat and milk proteins, was delivered to a flow-through cell in the polarimeter. The milk with the added enzyme was mixed by stirring, set at 50 rpm, and pumped at a constant speed of 40 L h^{-1} in a recirculation mode. The lactose hydrolysis data were evaluated with the prediction model and compared to HPLC. The amount of the galacto-oligosaccharides (GOS) formed during hydrolysis of lactose was estimated by subtracting concentration of lactose, glucose and galactose from the initial concentration of lactose 4% (w/v).

2.3.5 The Effect of pH

The effect of pH on the optical rotation of carbohydrates was evaluated by measuring the optical rotation of appropriate carbohydrate mixtures in a pH range from 6.0 to 7.5. This is related to natural milk pH (Mahoney 1997) and to the enzyme optimum activity (Kim *et al.* 1997). The concentrated standards of lactose, glucose and galactose were diluted exactly to 4.0% ± 0.1 (w/v) by thoroughly mixing their concentrated stock solutions, each 20% (w/v), with a phosphate buffer and adjusting the pH with 1 N NaOH or 1 N HCl for individual samples to 6.0, 6.5, 7.0, and 7.5.

2.3.6 Statistical analysis

All experiments were repeated in triplicate and the data averaged. The data collected from the polarimeter for lactose, glucose and galactose standard solutions were evaluated by linear regression and by the two tailed Student's t-test, Microsoft Excel 2003 SP 2.0, (Microsoft Co., Redmond, Washington, USA) and the Statistica 5.0A software, (Stat Soft Inc., Tulsa, Oklahoma, USA) and P-values of 0.05 or less were considered significant. The modified "on-line" polarimetric method, integrated with the prediction model for the evaluation of lactose hydrolysis process, was developed and validated by the analysis of variance (ANOVA) using Design- Expert® software.

2.4 RESULTS AND DISCUSSION

2.4.1 Evaluation of carbohydrate standards

The response factor estimated for galactose (RF_{gal}) was significantly higher than the response factors for lactose (RF_{lac}) and glucose (RF_{glu}) (**Table 2.1)**. This significant difference in RF was the key to evaluating galactose, glucose and lactose concentrations in the carbohydrate mixture or in permeate. In the current experiment, there was no variability between the specific rotation values and an increase in the concentration of glucose, and galactose in their water solutions after storage for 24 h, which was reported earlier by Schallenberger (1985).

Table 2.1 Response factors (RF_x) and linear regression coefficient (R^2) for carbohydrates and their proportional blend in phosphate buffer at 37.0°C, and at pH 6.9 and λ = 589 nm.

Carbohydrate	(RF_x)	[a]R^2
lactose	0.55	>0.99
glucose	0.56	>0.99
galactose	0.79	>0.99
blend	1.89	>0.99

[a]R^2 = regression coefficient > 0.99.

Figure 2.2 illustrates the results for carbohydrate standards, which indicate that there was an extremely high positive correlation ($R^2 > 0.99$) between the optical rotation value and the known concentration of lactose, glucose or galactose in their individual and standard mixture solutions. The optical rotation was measured in triplicate for each sugar standard and all samples showed a small standard deviation (SD ±0.04). As expected, the measured optical rotation values changed proportionally to the amounts of carbohydrate added to the phosphate buffer. Such results have been noted previously; polarimetry is well known to give a linear response when used to measure the concentrations of pure or mixed sugars (Kearsley 1985).

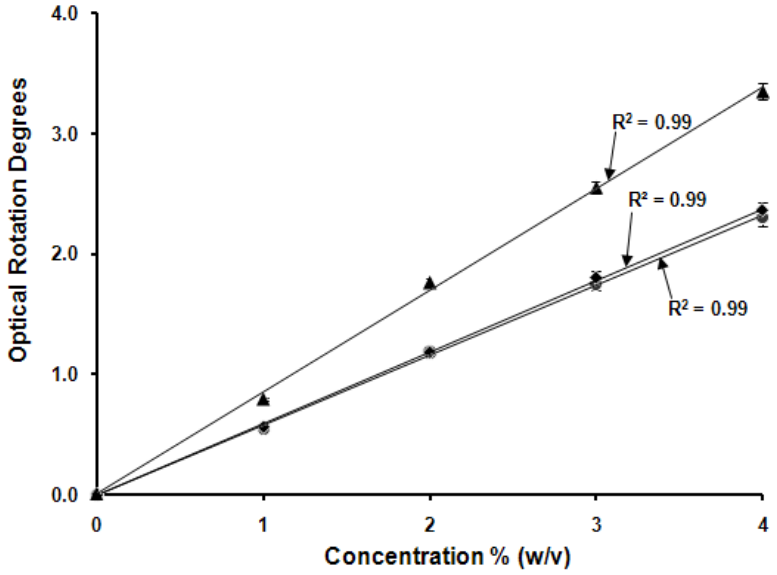

Figure 2.2 Optical rotation standard curves of lactose (●), glucose (◆), galactose (▲), in phosphate buffer at pH 6.9, 37.0°C, and λ = 589 nm. Data are averages from three experiments.

2.4.2 The prediction model

At first, the model was evaluated with mixtures of pure sugars using polynomial quadratic equation (14) which was proposed by Scheffe (1958). In this evaluation, it was observed that the theoretical model development would result in predictions valid only for lactose degraded to glucose and galactose in equimolar proportions. However, because of the production of galacto-oligosaccharides (GOS) during lactose degradation, the new model was adjusted based on actual experiments with pure solutions of lactose containing the enzyme. As an outcome of this adjustment, the model was able to recognize its biological effect.

Figure 2.3 shows the outcome of the data matrix transformation, using the Design Expert® software "Mixture Quadratic Model". The model is displayed as

an equilateral triangle with numerically scaled sides for its carbohydrate mixture components: glucose *(A)*, galactose *(B)*, and lactose *(C)* which are its weighted percent fractions. The triangle contains a model space contour of a parallelogram with an "optimal diagonal line" running across major points in its space which are highlighted as black dots.

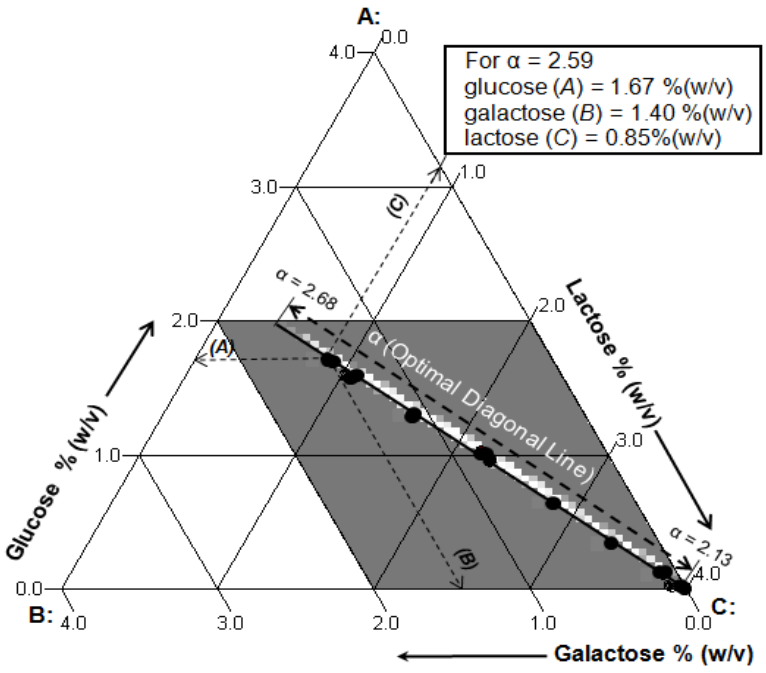

Figure 2.3 Evaluation of carbohydrate concentrations during lactose hydrolysis in bioreactor by input of optical rotation value α (●) to the "Mixture Quadratic Model.

Each point on this diagonal line corresponds to the measured optical rotation value which is in a direct correlation to the concentration of carbohydrate components in the mixture related to hydrolyzed lactose and described by the

model polynomial equation (15) specific for the initial lactose concentration of 4%:

$$Y = -11.74*A -16.37*B + 0.53*C+14.61*A*B+ 3.29*A*C+4.06*B*C \quad (15)$$

By inputting the experimentally derived optical rotation values for pure carbohydrate components to the new model, as described earlier in section 2.3.3, a prediction can be made about the progress of lactose hydrolysis and the concentration of carbohydrates during hydrolysis of lactose in milk. The carbohydrate concentrations can be estimated automatically with the model by entering the optical rotation value into the polynomial equation. To illustrate the model operating principles, the optical rotation value point measured at $\alpha = 2.59$ was highlighted on an diagonal line within a space contour of a parallelogram. Carbohydrates concentration values were found for: *(A)* glucose = 1.67% (w/v), *(B)* galactose = 1.40% (w/v) and *(C)* lactose = 0.85% (w/v), by direct referencing to their corresponding triangle sides.

As indicated above, the initial concentration of lactose in milk before hydrolysis must be known and the numerical values in the model and the polynomial equation (15) are valid only for milk with lactose concentration set at 4% (w/v). However, once a model is developed it can be used for the design of a new model and a new polynomial equation for milk with different lactose concentrations.

2.4.3 Evaluation of lactose hydrolysis with the prediction model

The model was adjusted based on actual experiments with solutions evaluated during hydrolysis of lactose (data not shown). Data acquired "on-line" by polarimetric measurement and by HPLC during the hydrolysis of lactose in skim milk is illustrated in **Table 2.2**.

Table 2.2 Average concentration values of lactose (lac), glucose (glu) and galactose (gal) predicted with the new model (eq. 15), and compared to HPLC during the hydrolysis of lactose in skim milk in the stirred membrane batch reactor.

Bioconversion Time (min)	Measured Optical Rotation	Prediction Model lac + glu + gal % (w/v)	HPLC lac + glu + gal % (w/v)	SEM [a]
0	2.15	3.95 + 0.00 + 0.00	4.00 + 0.00 + 0.00	<0.1
10	2.14	3.91 + 0.02 + 0.01	3.95 + 0.01 + 0.02	<0.1
20	2.13	3.85 + 0.11 + 0.08	3.85 + 0.10 + 0.07	<0.1
30	2.18	3.36 + 0.34 + 0.30	3.20 + 0.34 + 0.30	<0.1
40	2.24	2.83 + 0.64 + 0.53	2.59 + 0.62 + 0.51	<0.1
50	2.43	2.20 + 1.00 + 0.80	1.98 + 0.93 + 0.76	<0.1
60	2.49	1.61 + 1.29 + 1.10	1.50 + 1.29 + 1.08	<0.1
70	2.53	1.06 + 1.50 + 1.31	1.06 + 1.48 + 1.26	<0.1
80	2.56	0.85 + 1.51 + 1.33	0.88 + 1.47 + 1.26	<0.1
90	2.59	0.85 + 1.67 + 1.40	0.85 + 1.64 + 1.35	<0.1

[a]SEM = Standard Error of the Mean. None of the means for each sugar were significantly different ($P > 0.05$).

Figure 2.4 shows that the lactose conversion with the enzyme was rapid and the lactose was reduced on average to 0.81% (w/v) after 90 min. At the end of this process, on average, 1.65% (w/v) of glucose and 1.32% (w/v) of galactose

were formed. There was a significant correlation ($P < 0.05$) between the predicted values and HPLC values found in the permeate carbohydrates. A small difference was observed for the optical rotation versus the HPLC data at the end of the lactose hydrolysis process. It was also noticed that during the first sixty minutes of lactose degradation its concentration value predicted with the model was higher than its concentration value measured by HPLC.

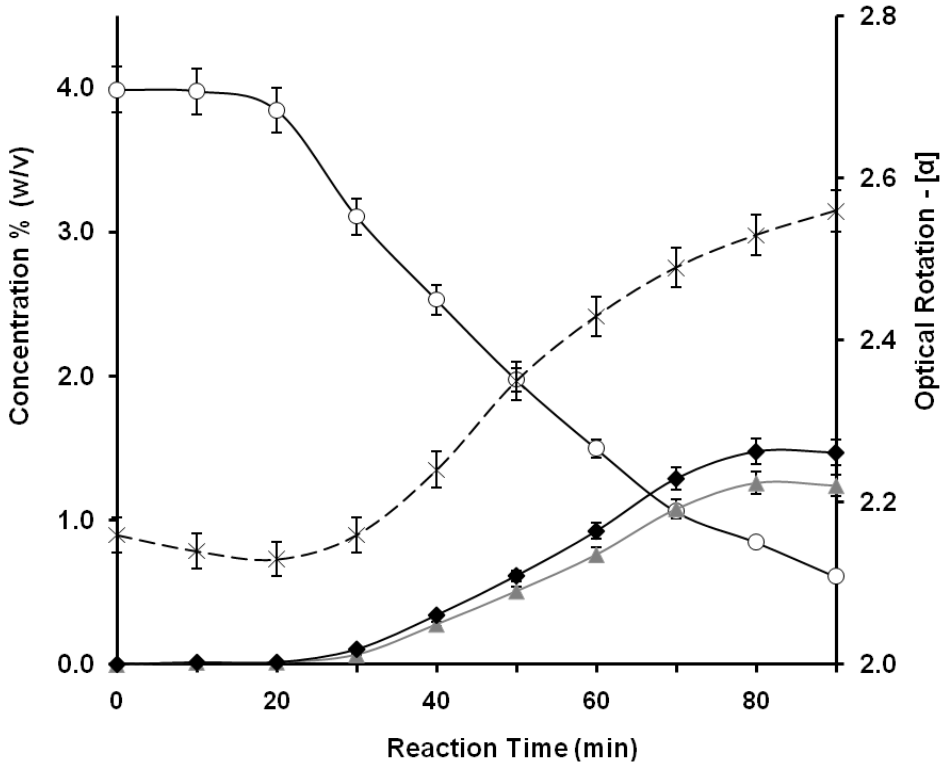

Figure 2.4 Measured optical rotation [α] (-⁎-) in on-line flow-through cell and average concentration of lactose (–○–), glucose (–◆–) and galactose (–▲–) evaluated by HPLC during hydrolysis of lactose with enzyme in skim milk (lactose concentration 4% (w/v), at 37.0°C). Error bars show standard deviation values based on triplicate tests.

This difference could be explained by the formation of optically active galacto-oligosaccharides (GOS) during lactose degradation (**Figure 2.5)**. Their

estimated total concentration varied and increased during the lactose hydrolysis from 0% (w/v) up to maximum 0.39% (w/v), but declined at the end of the process to 0.16% (w/v). However, this trend was not clear.

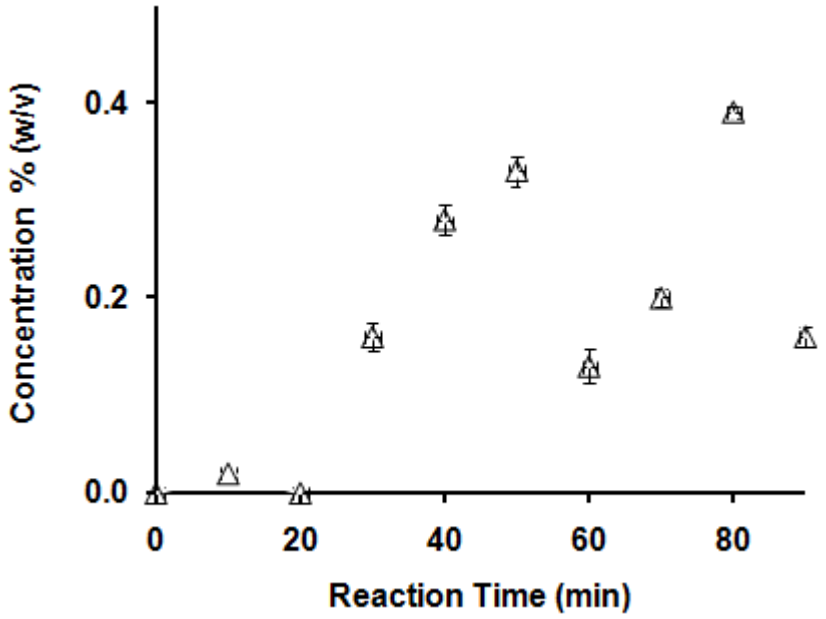

Figure 2.5 The calculated average concentration of the GOS (△)during hydrolysis of lactose 4.0% (w/v) with enzyme in skim milk at 37.0°C. Error bars show standard deviation values based on triplicate tests.

This may have affected the optical activity data and accuracy of the prediction for the lactose concentration with our model. Gänzle *et al.* (2008) reported that during the lactose degradation to glucose and galactose their equimolar balance is shifted towards higher concentrations of glucose because the galactose molecule reacts with lactose and forms the intermediary polymers, the galacto-oligosaccharides (GOS). They explained that the hydrolysis of lactose by β-galactosidase undergoes at least three steps: 1) the formation of an enzyme-

lactose complex, 2) the release of glucose, leaving the galactosyl group joined as an enzyme-galactosyl complex, and 3) the transfer of galactose to nucleophilic acceptors containing a hydroxyl group such as water or acceptor sugars and formation of GOS. However, an earlier investigation indicated that the final outcome of the reaction of lactose with the enzyme in the milk is the equimolar amount of glucose and galactose concentration (Mahoney 1997).

To improve our model prediction for hydrolysis of lactose in skim milk, attempt was made to account for the formation of the GOS by optimizing the model parameters and "training" it with the data acquired by HPLC during hydrolysis of lactose in skim milk. The results of model validation with ANOVA showed that the model terms are valid and the Sequential Model Sum of Squares test suggested the choice of the Quadratic Model (**Table 2.3**).

Table 2.3 Model selection with the "Sequential Model Sum of Squares" test.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F -Value	P-value Prob > F
Means vs. Total	161.70	1	161.70		
Linear vs. Mean	0.72	2	0.31	941.42	< 0.01
Quadratic vs. Linear	7.5E-003	3	2.5E-003	17.1	< 0.01[a]
Special Cubic vs. Quadratic	3.7E-004	1	3.7E-004	2.80	0.10
Cubic vs. Special Cubic	1.1E-003	3	3.9E-004	4.02	0.02[a]

[a]"Sequential Model Sum of Squares" test suggests the choice of the best polynomial equation in the model.

This model validation was confirmed by the Model Summary Statistics that showed maximal values of the "Adjusted R-Squared" and the "Predicted R-Squared" (**Table 2.4**).

Table 2.4 Model summary statistics.

Source	Standard Deviation	R^2	Adjusted R^{2a}	Predicted R^{2a}
Linear	0.02	0.98	0.98	0.98
Quadratic	0.01	0.99	0.99[a]	0.99[a]
Special Cubic	0.012	0.99	0.99	0.99
Cubic	0.01	0.99	0.99	0.98

[a]"Model Summary Statistics" suggests selection of the Quadratic Model for the maximal values of the "Adjusted R-Squared" and the "Predicted R- Squared".

The average permeate flux, it was also noted, declined continuously, and at the end of the lactose hydrolysis process, was reduced from its initial value of 15 mL min^{-1} on average by 80-90% (w/v), which shows that fouling of the membrane by proteins likely occurred (Louie *et al.* 2006). This, although by itself significant ($P < 0.05$), did not affect the permeate delivery to the flow-through cell and the "on-line" polarimetric measurement of optical rotation. Additionally, the filtration of milk on the membrane revealed that all lactose, glucose, and galactose passed through the membrane but all milk proteins were rejected.

2.4.4 Effect of pH

In evaluating the pH effect on the optical rotation value, the data showed that the pH had no significant effect ($P > 0.05$) on the optical rotation for lactose, glucose and galactose in the pH range from 6.0 to 7.5. The selected pH range for this test, from 6.0 to 7.5, was to approximate a natural milk pH at 6.95 (Mahoney 1997) and a lactase optimum activity, from 6.5 to 7.0 (Kim *et al.* 1997). O'Brien (1997) reported that some carbohydrates could react with other milk components at extreme low or high pH levels, and therefore, could affect optical rotation results. However, in his work such reactions would be outside the pH range used for optical rotation tests.

2.5 CONCLUSIONS

The new polarimetric method is robust, simple to perform and, through the subsequent use of a predictive mathematical model, offers the possibility for significant cost and time reduction. Hence, it enables a rapid, non-destructive and quick evaluation of carbohydrate concentrations during the production of lactose hydrolyzed milk products. By using the polarimeter flow through cell in conjunction with the "Mixture Quadratic Model", it is possible to continuously monitor the lactose hydrolysis process in a batch. A minor limitation of this method is that there is a need to adjust the input for initial lactose values in the model matrix table when using milk with different initial lactose concentrations. Even so, the new modified polarimetric method is a good system and offers an

alternative to other more expensive methods for accurate evaluation of carbohydrates in mixtures during the production of lactose reduced milk.

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3. SEPARATION OF LACTOSE, GLUCOSE AND GALACTOSE WITH COMMERCIAL NANOMEMBRANES [a]

3.1 INTRODUCTION

The crossflow filtration process (CFF) using nanomembranes to optimize the separation or fractionation of selected carbohydrates has been under investigation as an alternative to other technologies. New reliable CFF membranes are needed to achieve efficient and effective fractionation, purification and concentration of carbohydrates and feed-stream components (IDF 1987; Cricenti *et al.* 2006). The separation of disaccharides from monosaccharides with nanomembranes requires a high pressure, a highly specific membrane and a thorough control of the feedback pressure, which is important to prevent large fluctuations in the CFF flux (Jeantet *et al.*2000). Selecting the right nanomembrane for this specific application to satisfy the requirements of the end user technological process is difficult. This is due to proprietary materials and technology for which the nanomembranes are produced and applied (Tang *et al.* 2007). The commercially available nanomembranes are usually made up of thin film polyamide polymers (PA), several hundred nanometers thick, and are coated on the polysulfone (PS) support as described by Tang *et al.* (2006). It is necessary to understand their physicochemical properties in connection to their application to achieve improvement in their performance (Louie *et al.* 2006).

[a] A portion of this chapter was presented orally and as a poster at the EFB Workshop in Brac, Croatia. Pikus, W., McCaffrey, W.C., Bressler, D., and L. Ozimek. 2008. Sugars cross-flow Filtration on Nanomembranes.

There is a lack of detailed information in published research literature that demonstrates the effective use of nanomembranes for the separation of carbohydrates such as lactose from its subcomponents, glucose and galactose. Therefore, it is important to investigate the separation, filtration and/or concentration in feed streams containing lactose, glucose and galactose by using the CFF nanomembrane technology. Such a method offers an opportunity: 1) to formulate novel adjuncts used in the production of human foods or animal feeds, (Abril and Stull 1989; Lopez-Leiva and Guzman 1995), 2) to generate lower biological waste loads (Saddoud *et al.* 2007), 3) to enhance their value (Mahoney 1997; Goulas *et al.* 2003), and 4) to lower manufacturer processing costs (Curcio *et al.* 2006).

Lactose, a major carbohydrate present in dairy wastes, is a disaccharide that is not readily utilized by the food industry. In cheese whey, a cheese processing byproduct, lactose accounts for approximately 75% of the total dry solids (Siso 1996). This carbohydrate, if recovered from the waste, has a potential to improve or to generate novel value-added food ingredients (Gänzle *et al.* 2008; IDF, 2005). The partial or complete degradation of lactose into the monosaccharides, glucose and galactose, renders it useful in numerous applications (Mahoney 1997; Petzelbauer *et al.* 2003; Pirisino 1983). Some of these applications include the production of lactose-free milk that still retains usable sugars, a new source of glucose and galactose that can be used in food products (adjuncts), and for food and industrial fermentations (ethanol, buthanol, platform chemicals etc.) (Bury and Jelen 2000; Elliot *et al.* 2001; Goulas *et al.* 2003). It has been demonstrated

that lactose reduced milks are used for the production of frozen dairy desserts or in ice cream products to improve ice cream shelf-life and its sensory properties (Sheth *at al.* 1998). The partially hydrolyzed lactose adjuncts, containing blended amounts of lactose, glucose and galactose, are also desired for the formulation of novel fermented beverages (Crumplen *et al.* 1990).

The solute separation process for all membranes is described by the solution-diffusion model, which shows that both water and solute molecules, when diffusing through the membrane, must overcome the membrane layer to permeate through it (Steinle-Darling *et al.* 2007). The concentration and size of solute molecules in the solvent is critical for their permeation or rejection process by the membrane (Bellona et al., 2004). The Molecular Weight Cut Off (MWCO) is the terminology, which is most often used by manufacturers and researchers to describe the nominal size of molecules after they diffuse across the membrane and are rejected at a 90% level (Harrison *et al*. 2003). The MWCO and the membrane structure may be affected during the separation process due to applied pressure, temperature, feed flow rate, feed concentration and the type of carbohydrate used (Jeantet *et al.* 2000; Shahbazi *et al.* 2005; Jurado *et al.* 2006; Tang *et al.* 2006). The major objectives of this work were: 1) to evaluate two commercial nanomembranes for their ability to separate proportional blends of lactose, glucose and galactose when operating the CFF nanomembrane system in a batch concentration mode, and 2) to evaluate the nanomembranes' structure before and after their use with the help of Transmission electron microscopy and Fourier transform infrared spectroscopy.

3.2 MATERIALS & METHODS

3.2.1 MATERIALS

3.2.1.1 Carbohydrates

The α-lactose monohydrate, D-glucose and D-galactose standards, each with 99.8% purity, were purchased from Sigma-Aldrich Canada Ltd. (Toronto, Ontario, Canada). trichloroacetic and citric acid, both 99.9% pure, were acquired from VWR Inc.(Toronto, Ontario, Canada). The pH 4.0 and 7.0 buffers were purchased from Fisher Scientific (Edmonton, Alberta, Canada). Solutions of 1 N NaOH and 1 N HCl used for the pH adjustment were acquired from VWR Inc.

3.2.1.2 Phosphate buffer

The buffer was prepared by mixing 0.01 M K_2HPO_4, 0.015 M KCl and 0.012 M $MgCl_2 \cdot 6\ H_2O$ into doubly deionized water which was produced with a resistivity of 18.2 MΩ · cm by filtering of tap water through the reverse osmosis membrane/cation exchange system purchased from Millipore (Canada) Ltd. (Etobicoke, Ontario, Canada). The phosphate buffer pH value was adjusted to 6.7 as described in the procedure for carbohydrates. All buffer components were purchased from Fisher Scientific (Edmonton, Alberta, Canada).

3.2.1.3 Whey

The fresh batch of pasteurized skim milk, 5 L, a product of Saputo Inc. (Montreal, Quebec, Canada) was purchased at Safeway Canada Inc. (Edmonton, Alberta, Canada) and aliquotted into 1 L sterile glass bottles. Next, the bottles, were put into a water bath (model Haake F3, Fischer Scientific, Edmonton) and heated up to 32^{o}C for 1 h. One mL of rennet enzyme, strength 580 IMCU$\cdot$ mL $^{-1}$, 92.0% Chymosin pure, acquired from Renco Inc., Eltham, New Zealand, was diluted in 20 mL of fresh deionized cold water. One mL of diluted enzyme was then added to each milk bottle, mixed thoroughly and kept at 32^{o}C for 45 min. The formed cheese curd was stirred thoroughly for 2 min and fresh whey, free of cheese curd particles, was separated with a cheese cloth (Safeway Inc. brand, Edmonton, Alberta, Canada), poured into another set of sterile 1 L glass bottles, and stored at 4^{o}C for 12 h.

3.2.1.4 Validase

The Validase biocatalyst (β-galactosidase (EC 3.2.1.23)), derived from *Kluyveromyces lactis*, activity 50,000 *ortho*-nitro-phenyl-β-D-galactopyranoside g^{-1} units (ONPGU g^{-1} units) with a protein content 30 g L^{-1} , was donated by Valley Research Inc. (South Bend, Indiana, USA).

3.2.1.5 Nanomembranes

Filmtec NF270-400 (nominal pore size 270-300 MWCO) and Koch TFC-SR3 (nominal pore size 200-400 MWCO), in a flat sheet configuration,

were donated by the Dow Chemical Company (Midland, Minnesota, USA) and the Koch Membrane Systems, Inc.(Wilmington, Massachusetts, USA), respectively.

3.2.1.6 CFF membrane system

The schematic of the nanomembrane test setup is shown in **Figure 3.1**.

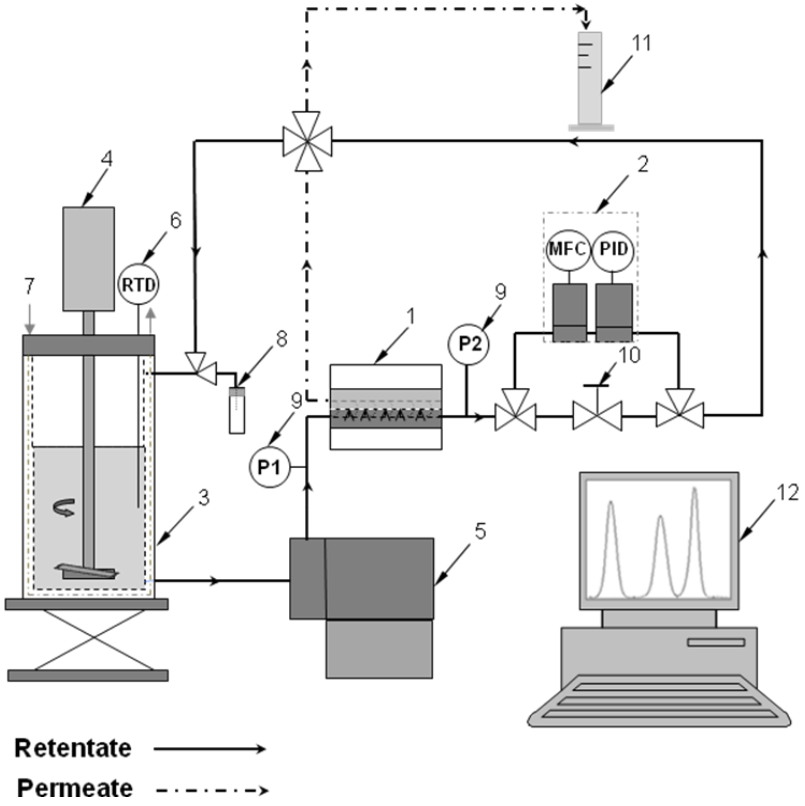

Figure 3.1 The experimental CFF nanomembrane system elements: 1) SEPA CFII membrane CFF unit, 2) PID controller, 3) 10 L stainless-steel feed tank, 4) stirrer, 5) high pressure pump, 6) Pt-100 thermocouple probe, 7) warm water inflow from water bath, 8) retentate sample collection port, 9) analog pressure gauges, 10) automatic back pressure relief valve with the manual needle pressure regulator valve, 11) 2 L graduated cylinder as the permeate volume collector, and 12) computer with installed pressure/mass flow controls.

The major components of this setup are: 1) a SEPA CFII membrane CFF unit with an effective flat membrane filtration area of 140 cm2 (Sterlitech Co., Kent, Washington, USA), 2) a proportional-integral-derivative (PID) mass flow/controller (Bronkhorst High -Tech BV Nijverheidsstraat, AK Ruurlo, Netherlands), 3) a 10 L feed stainless-steel tank, custom designed and equipped with a double wall at the Department of Agricultural, Food and Nutritional Science (AFNS), the University of Alberta, Edmonton, Canada, 4) a 50-500 rpm stirrer (Fischer Scientific, Edmonton), 5) a Hydra-Cell pump, model D03 (Wanner Engineering Inc., Minneapolis, Minnesota, USA), 6) a Pt-100 two wire thermocouple probe (Fischer Scientific, Edmonton) connected to the Fluke 701 process calibrator (Fluke Electronics Canada Inc., Mississauga, Ontario, Canada), and 7) a water bath, model Haake F3 (Fischer Scientific, Edmonton), 8) the retentate sample collection port, 9) an analog pressure gauges (NoShok Inc., Berea, Ohio, USA), 10) an automatic back pressure relief valve with the manual needle pressure regulator valve (Swagelok Inc., Edmonton, Alberta, Canada), 11) a 2 L graduated cylinder as the permeate volume collector, and 12) a computer with installed pressure/mass flow control software (Bronkhorst High -Tech BV Nijverheidsstraat).

3.2.2 METHODS

3.2.2.1 Preparation of carbohydrate standards

Four proportional blends, (A-D), of lactose, glucose and galactose were prepared as a feed solution by proportionally mixing them, from 0.0 to 5.0 %

(w/v) with a 0.01 M phosphate buffer solution for their total amount of 5% (w/v). Their weighted amounts were expressed as a percent value (w/v) of the total for ternary mixture of lactose, glucose and galactose A (80:10:10), B (60:20:20), C (40:30:30), and D (20:40:40). It should be noted that the weighted amount of α-lactose monohydrate was recalculated and reported as lactose. For all samples the pH values for the carbohydrate standards and proportional blends were reconfirmed immediately prior to the nanomembrane filtration process. All analytical results were corrected for the dilution resulting from the pH adjustment. The HPLC value corresponded to the mean of the three replicates and the phosphate buffer solution was used as a blank with a zero value reference.

3.2.2.2 Crossflow filtration process of mixed sugars

At the start of the CFF process, the tank was filled with 3 L of proportionally blended feed solution. Next, the feed solution was recycled through the whole system and back to the feed tank at a constant feed flow rate of 72 L h^{-1} set at the Hydra-Cell pump, the nanomembrane back pressure was set at ambient pressure, and the temperature of the feed solution was adjusted to 37 $\pm 1^{o}$C. After 30 min, the feed solution was directed to the nanomembrane for 5 h with the back pressure set to 1.38 MPa (200 psi). The retentate solution was recycled into the feed tank after its separation from the permeate solution with a constant flow rate of 72 L h^{-1} at 37 $\pm 1^{o}$C. The permeate solution volume was collected in a graduated cylinder, and its volume was noted and the mass was measured on a digital balance during and at the end of the CFF process. The

permeate volume, its weighted mass and the back pressure were all recorded at the start and every 30 min throughout the CFF nanomembrane process.

The cleaning in place process (CIP) of the CFF nanomembrane system was completed after each experiment by rinsing and recirculating with cold double deionized water for 10 min, and then by recirculating with 0.1 N NaOH solution, at 30 ±1^{o}C for 15 min. Finally, the system was rinsed with 3 L of doubly deionized cold water, disinfected with a 0.5% (w/w) solution of hydrogen peroxide, and rinsed with 3 L of double deionized cold water.

3.2.2.3 Bioconversion of whey lactose and crossflow filtration

The feed tank was filled with 3 L of whey lactose, at 5.3 ±0.1% (w/v), and was hydrolyzed with added soluble biocatalyst Validase®, (2 g L^{-1}) for 30 min at whey pH 6.7 and at 37 ±1^{o}C. During the bioconversion process whey lactose was thoroughly mixed with the enzyme using a stirrer with a speed set up manually at 50 rpm, and pumped at a constant flow rate of 72 L h^{-1} with the Hydra-Cell pump, in the recirculation. After 30 min, the feed solution was CFF filtered on the nanomembrane for 5 h with the back pressure set to 2.07 MPa (300 psi).

3.2.2.4 Membrane flux, volume concentration and carbohydrate rejection

The new nanomembrane sheets were preconditioned by immersing in the fresh double-deionized water and by holding them immersed at room temperature for 24 h. After their installation in the nanomembrane unit, water was recycled in

the CFF nanomembrane system and flushed through the membrane at a flow rate set at 72 L h^{-1} and a back pressure set at 1.38 MPa (200 psi), which was controlled by a PID mass/flow controller interfaced via RS 232 with a computer. The temperature of the feed and stirrer speed were adjusted manually with available controls and set up at 37 ±1^{o}C and 50 rpm, respectively. The water volumetric flux for each nanomembrane was measured after 15 min, counted from the start up time of the crossflow filtration process on the membrane, to ensure that the installed nanomembrane coupons were without defects. This procedure was adopted as the standard measure for each experiment. The volumetric fluxes for mixed carbohydrates and whey CFF tests were also evaluated. It should be noted here that the volumetric permeate flux for whey was measured after 30 min, counted from the start up time of the crossflow filtration process on the nanomembrane. The volumetric fluxes of water, carbohydrates and whey, the volume concentration ratios (VCR) and the rejection values (R%) for individual carbohydrates in their proportional blends were evaluated by applying mathematical equations (7 and 8) adopted from Goulas *et al.* (2003). The membrane limited flux model was adopted from Koyuncu *et al.* (2004) to evaluate volumetric flux data for mixed carbohydrates.

3.2.2.5 Membrane characterization by TEM and FTIR

For TEM analysis, sheets of virgin and used nanomembranes were collected before and after crossflow filtration of 5% (w/v) carbohydrate mixture, ternary blend of lactose, glucose and galactose and with proportional ratio

adjusted to 40:30:30, and cut into 1-2 cm squares and placed into gelatin capsules with the membrane side down. The capsules were filled with LR White (Fisher Scientific, Edmonton) embedding resin and then placed in a 60 ±1^{o}C oven and allowed to harden overnight. Then they were removed by soaking in double deionized water at room temperature, and cut with a diamond knife on a Reichert-Jung Ultramicrotome (Model 701701, Leica, Milton Keynes, UK) in thicknesses of 60-70 nanometers, and collected on 300 mesh grids. After drying, the grids were examined with a Philips Morgangi 268 TEM (FEI Compagny France, Limeil-Brevannes, France) equipped with a CCD camera (FEI Compagny France, Limeil-Brevannes) to determine the size and distribution of the pores. The modified FTIR method of Cricenti *et al.* (2006) was used to evaluate the nanomembranes chemical composition. For FTIR analysis, sheets of virgin and used nanomembranes were analyzed with the Bruker Vertex 70 FTIR (Bruker Optics Inc., Billerica, Massachusetts, USA) with an attached Hyperion FTIR microscope (Bruker Optics Inc., Billerica) and equipped with a germanium micro-ATR objective with a contact area set at 100 microns in diameter. The spectra were acquired using 128 scans at a 4 wave number resolution with the Bruker OPUS software version 5.5 (Bruker Optics Inc., Billerica)**.**

3.2.2.6 Carbohydrate analysis by HPLC

Five milliliters of retentate and permeate were collected from the CFF nanomembrane system at the start of the filtration process and at 30 min intervals. The concentration of carbohydrates in the crossflow filtered samples and whey

was measured by the HPLC method (Anonymous, 2007), but the length of the total run time for each sample was modified to 19 min. The experiments were performed on an Agilent 1200 Series chromatograph equipped with a Model G1329A autosampler, a Model G1311A quaternary pump and a Model G1362A refractive index detector operated at 35^{o}C internal temperature (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). The analytical column was a Nucleogel Sugar Ca^{2+} column (300 mm x 6.5 mm) proceeded by a guard column (21 mm x. 4.0 mm) (Marcherey-Nagel Gmbh & Co. KG, Düren, Germany), and the column was heated at 87°C with an external column heater (Bio-Rad, Hercules, California, USA). The mobile phase was H_20, filtered by passaging through 0.5 μm filters, (Millipore, Bellerica, Illinois, USA), and degassed, while the 20 μL permeate sample was injected on the column with the flow rate set at 0.5 mL min^{-1}. The HPLC results for all samples were quantified using Agilent "ChemStation", version 2007, software, (Agilent Technologies).

3.2.2.7 Statistical analysis

All experiments were conducted in triplicate, the data averaged, and where applicable, evaluated by linear regression or by Student's T-test, using Microsoft Excel 2003 SP 2.0, Microsoft Co., Redmond, Washington, USA and the Statistica 5.0A software, Stat Soft Inc., Tulsa, Oklahoma, USA.

3.3 RESULTS AND DISCUSSION

The principal objective of this study was to demonstrate that it is possible to separate glucose and galactose from lactose in their proportional blends using the selected CFF nanomembranes. HPLC methods to do the same are used to separate carbohydrates but they are characterized by either costly equipment, materials and maintenance or low volume and efficiency (Pirisino 1983). Overall, the calculated average rejection values of lactose for retentate samples varied from 91 to 97%. The Koch TFC-SR3 nanomembranes showed the highest rejection rate of lactose for B, C, and D blends as their proportional lactose content declined. Its calculated average rejection value was only slightly affected by its concentration in the blended feed mixtures during the CFF process (**Table 3.1**).

Table 3.1 Rejections values (%) for lactose, glucose and galactose on nanomembranes. Their weighted amounts are expressed as a percent value (w/v) of the total for ternary mixture of lactose, glucose and galactose: A (80:10:10), B (60:20:20), C (40:30:30), and D (20:40:40)).

	Filmtec NF 270-400				**Koch TFC-SR3**			
Carbohydrate	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
lactose	92 ±2	92 ±3	96 ±3	94 ±2	91 ±3	97 ±3	98 ±2	97 ±2
glucose	88 ±3	76 ±3	78 ±4	70 ±3	70 ±3	81 ±3	77 ±3	70 ±2
galactose	89 ±3	77 ±4	78 ±4	71 ±3	71 ±2	82 ±3	79 ±3	72 ±4

On the other hand, the calculated average rejection values for glucose and galactose were lower than those estimated for lactose. It was also observed that the average rejection values for glucose were lower than the values for galactose. Similarly, Goulas *et al.* (2003) found that an increase in carbohydrate concentrations for mono-, di- and oligosaccharides in blended feeds during the CFF process affected their rejection values for nanomembranes. They concluded that the average rejection values calculated for the carbohydrate blends were different than the average rejection values for a single carbohydrate solution.

In our tests, the carbohydrate blends were concentrated and their calculated volume concentration ratios (VCR) evaluated at the end of the 5 h filtration for the retentate varied from 2.34 to 2.53 and from 2.29 to 2.52 for the Koch TFC-SR3 and the Filmtec NF270-400 nanomembrane respectively. Retentate and permeate samples collected during the CFF process for the proportional blends showed that with a gradual increase in the concentrations of glucose and galactose in a retentate, there was a gradual increase of glucose and galactose amounts in the permeate samples during the 5 h test runs for the Filmtec NF270-400 and the Koch TFC-SR3 nanomembranes, (**Figure 3.2A, B, C, D** and **3.3A, B, C, D**). There was a small increase in the concentration of lactose in the permeate samples for the NF270-400 membrane at the end of the filtration. It was also noted that the amount of glucose exceeded galactose in all permeate samples measured for B, C and D blends filtered on the Filmtec NF270-400 and the Koch TFC-SR3 nanomembranes. This concentration difference between glucose and galactose may be explained by the difference in their stereo-

chemical configuration or solubility which could affect their diffusion ability on the nanomembrane.

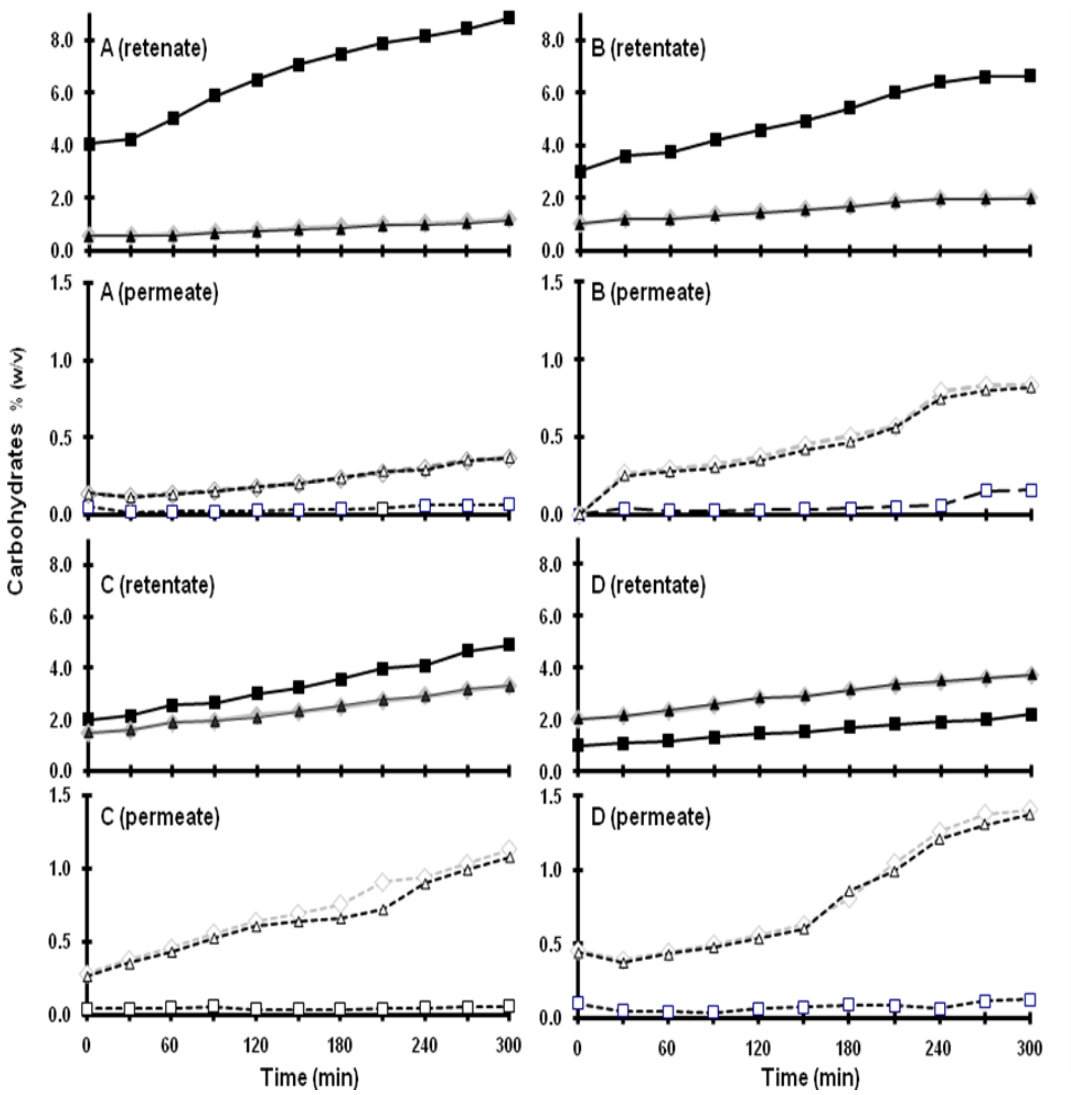

Figure 3.2 Concentration of lactose (—■—), glucose (—◆—), and galactose (—▲—) in retentate and lactose (- -□- -), glucose (– ◇ –), and galactose (– -△- -) in permeate for their blended ratios: A) 80:10:10, B) 60:20:20, C) 40:30:30, and D) 20:40:40, during CFF on Filmtec NF270-400 nanomembrane. Standard deviation values based on triplicate tests are smaller than icons.

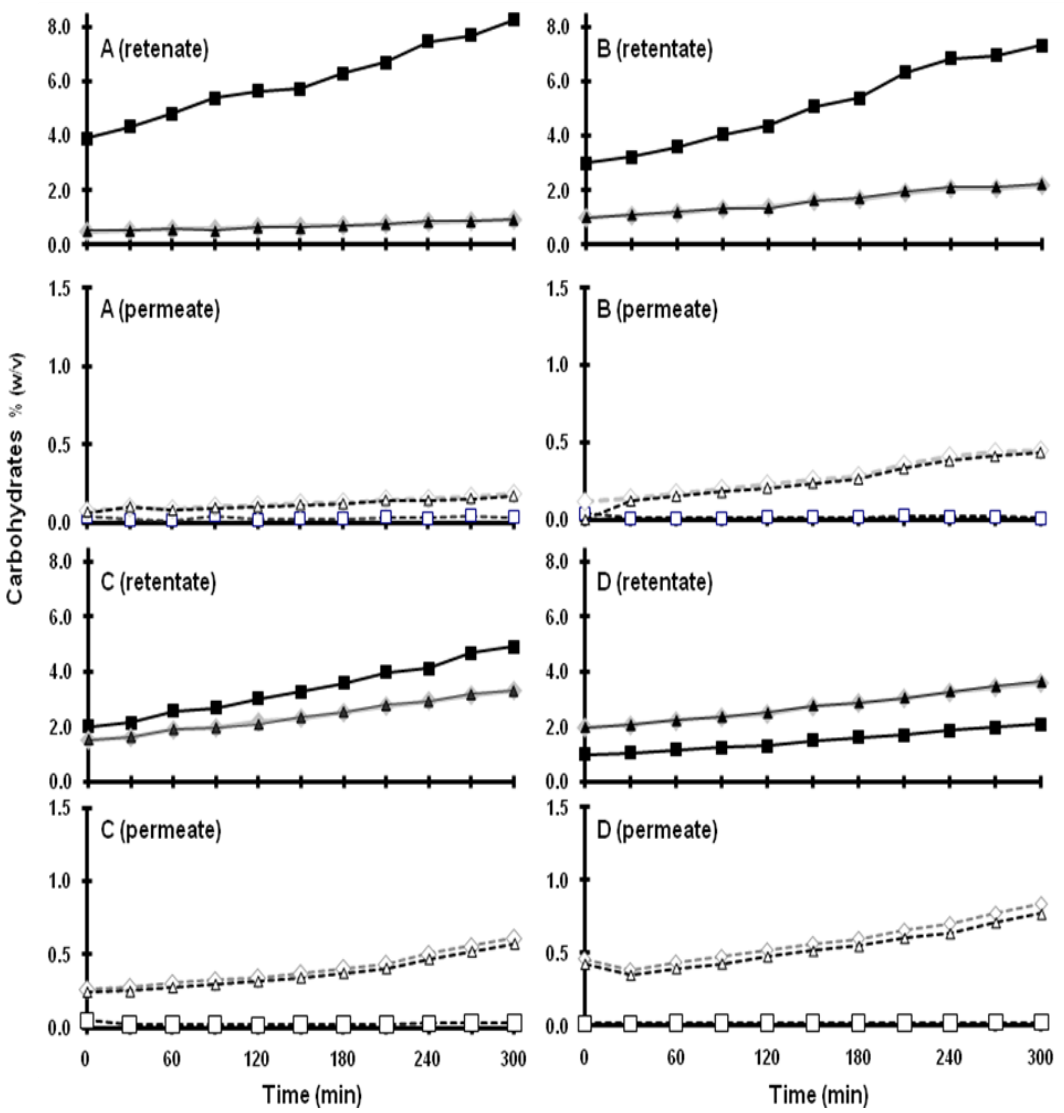

Figure 3.3 Concentration of lactose (—■—), glucose (—◇—), and galactose (—▲—) in retentate and lactose (- -□- -), glucose (– ◇ –), and galactose (– -△- -) in permeate for their blended ratios: A) 80:10:10, B) 60:20:20, C) 40:30:30, and D) 20:40:40, during CFF on Koch TFC-SR3 nanomembrane. Standard deviation values based on triplicate tests are smaller than icons.

The average volumetric flux for all proportional blends declined gradually as the concentration of carbohydrates increased during the 5 h test run. This observed volumetric flux decline was higher for the Filmtec NF270-400 nanomembrane than for the Koch TFC-SR3 nanomembrane. Next, the average

volumetric flux data for the tested membranes were evaluated with the membrane limited flux model, which was proposed earlier by Koyuncu *et al.* (2004), and revealed a rapid decline of the average volumetric flux rate for the Filmtec NF270-400 which was significantly (P >0.05) higher than for the Koch TFC-SR3 nanomembrane (**Figure 3.4**).

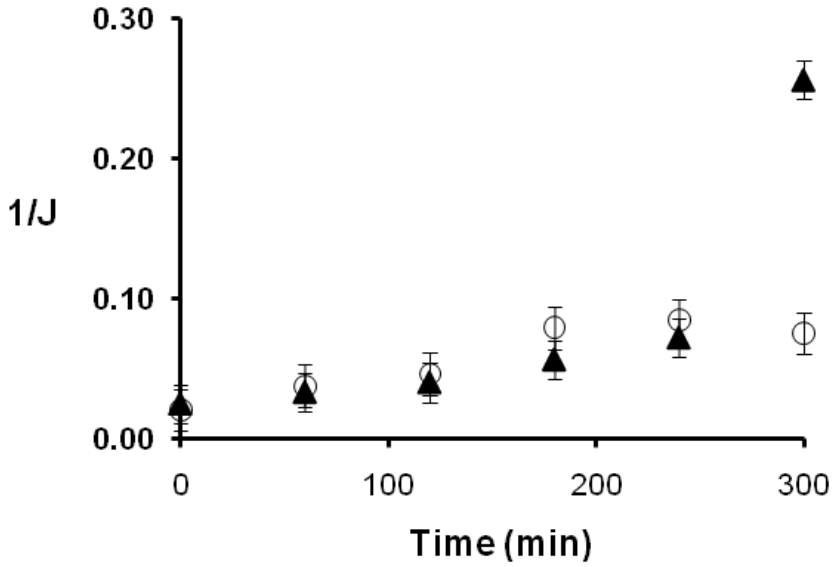

Figure 3.4 The membrane limited flux model shows the inverse of the average flux rate *versus* time during crossflow filtration of proportional blends of carbohydrates, dissolved in phosphate buffer at pH 6.7, at 37^{o}C, and at 1.38 MPa, on the Filmtec NF270-400 (▲) and the Koch TFC-SR3 (○) nanomembrane respectively. Error bars show standard deviation values based on triplicate tests.

Overall, the average volumetric flux values were higher for proportional blends with a higher concentration of glucose and galactose when applied to CFF on nanomembranes. The declining volumetric flux during the CFF process is an ongoing issue. Louie *et al.* (2006), in their evaluation of nanomembrane filtration potential, concluded that irreversible membrane fouling is a major factor affecting

the volumetric flux, and according to the authors, could only be remediated by either nanomembrane replacement or with better customized selection and modification in their polymer coating layer.

During CFF experiment, the back pressure in the nanomembrane system was monitored and controlled with the use of a PID mass flow rate and pressure controller which was interfaced with a computer. It should be noted that the back pressure, which was set at the start of each test at 1.38 MPa (200 psi) was not affected and only increased up to 1.52 MPa (220 psi) at the end of the filtration for both membranes. This small increase was caused by an increase in the volume concentration ratio of retentate in the feed. The analysis of data for the back pressure during the CFF process showed a lack of variation and demonstrated that a successful use of the PID mass flow/ pressure controller is possible for a similar or a larger pilot-scale CFF nanomembrane process. The control of the back pressure during the CFF process is important to optimize solute flux levels and prevent surface damage to the nanomembrane (Harrison et al., 2003). The authors elaborated that an increase in concentration of the solute caused an increase in the concentration polarization at the surface of the nanomembrane on the CFF, membrane which affected the flux and back pressure.

The structural evaluation of the virgin coupons of the nanomembranes with the TEM revealed that the Filmtec NF270-400 nanomembrane was characterized by high pore density, uniform pore distribution pattern and small size (**Figure 3.5A**). However, the Koch TFC-SR3 nanomembrane showed lower pore density, less uniform pore distribution but high variability in their size (**Figure 3.5C**).

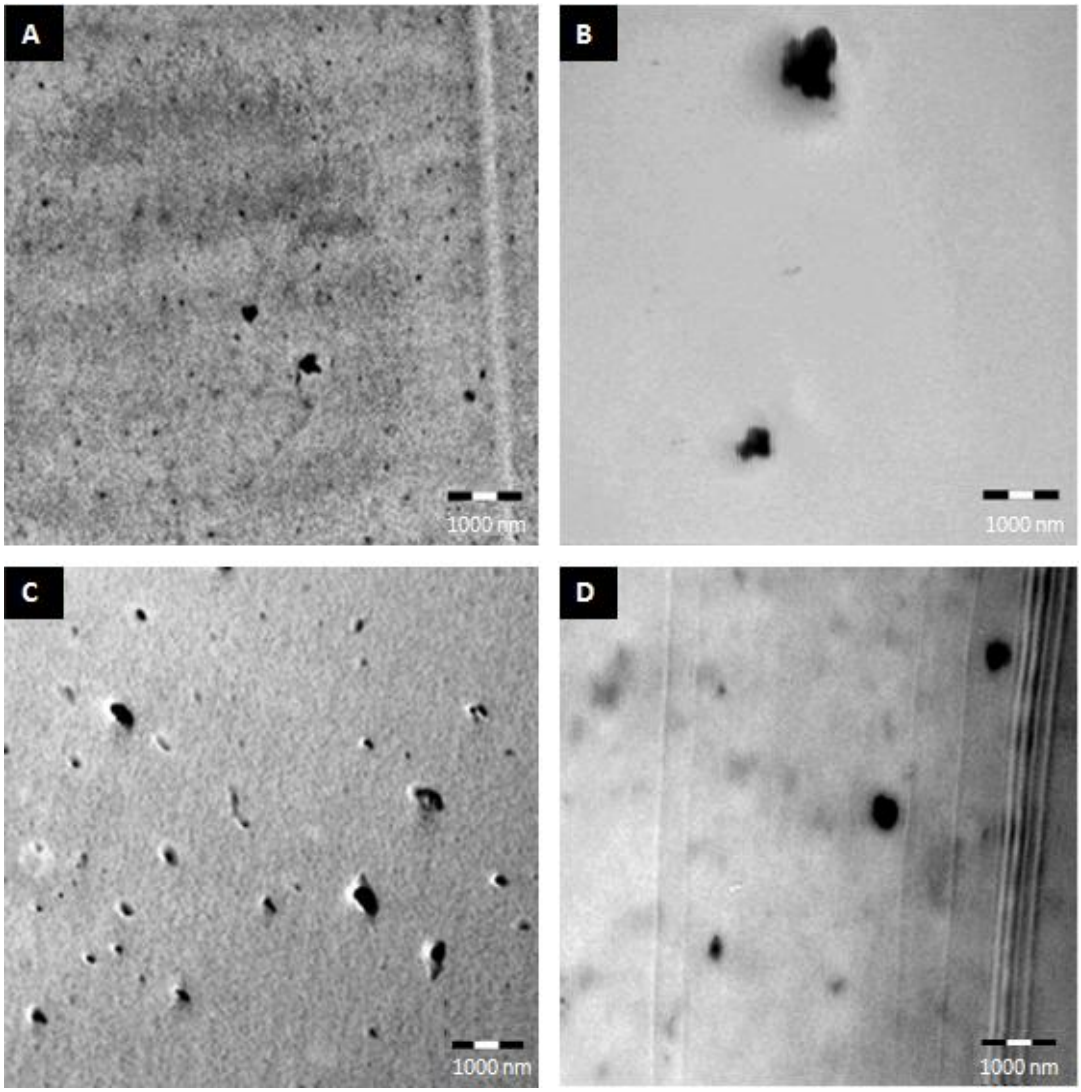

Figure 3.5 A) TEM of the virgin *versus* B) TEM used nanomembrane, for the Filmtec NF270-400, and C) TEM of the virgin *versus* D) TEM used nanomembrane for the Koch TFC-SR3.

It is important to note that the TEM analysis of nanomembranes collected after the CFF process of proportionally blended lactose, glucose and galactose, for tested nanomembranes, revealed a decline in the number of pores and an increase in the pore size for both nanomembranes (**Figure 3.5B** and **3.5D**).

It was reported earlier that lactose solubility is affected by its concentration and may in fact contribute to crystal formation in the solution (Holsinger 1997;

Mahoney 1997). Therefore, it was speculated that an increase in the concentration of buffer salts and carbohydrates could induce carbohydrate crystallization at the membrane polarization layer and cause membrane blinding. It was also suggested that high constant pressure used in the CFF process could be responsible for stretching in both nanomembranes, which could affect their pores.

The FTIR analysis for the new Filmtec NF270-400 and the Koch TFC-SR3 nanomebranes revealed that their membranes active layer was composed of aromatic polyamide (PA) and polyvinyl alcohol (PVA) layered over the polysulphone (PS) and polyester (PE) compounds. Tang *et al.* (2006) elaborated that it is necessary to evaluate the physicochemical properties of the membranes to determine and optimize their selection before their application in the CFF nanomembrane process. In addition, the comparative FTIR analysis of the spectra for virgin Filmtec NF270-400 and Koch TFC-SR3 *versus* used nanomembranes, sampled after the CFF process, revealed a lack of significant difference in their composition (data not shown).

Figure 3.6A and B demonstrates the comparative analysis of the FTIR spectra for: A) Filmtec NF270-400 and B) Koch TFC-SR3. The results revealed that the FTIR spectra of tested nanomembranes are similar to these reported by Freger *et al.* (2002). The FTIR nanomembranes spectra were also analyzed and compared to the spectra of pure lactose, glucose and galactose (**Figure 3.6C)**. This analysis revealed that individual carbohydrates for the wavelength from 1700 to 3650 cm^{-1} may contaminate the FTIR spectra of used nanomembranes.

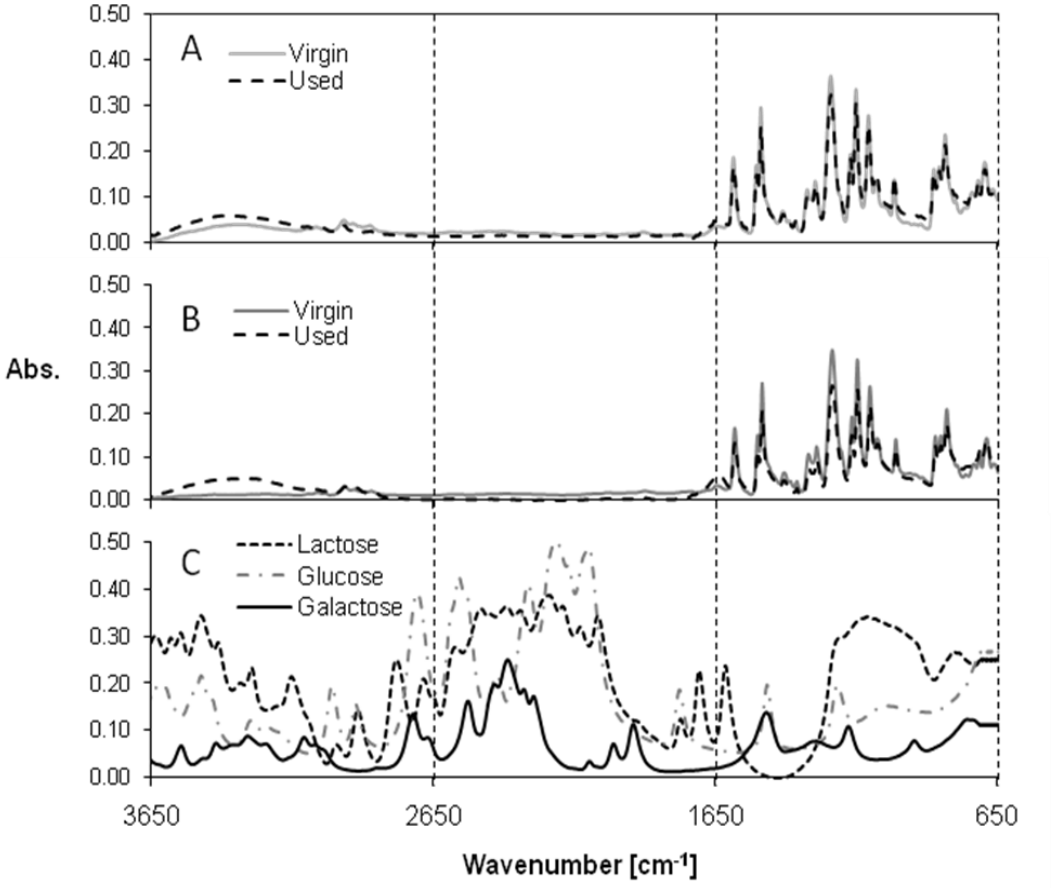

Figure 3.6 Comparison of the FTIR spectra of: A) Filmtec® NF270-400 and B) Koch® TFC-SR3 nanomembranes *versus* C) the FTIR spectra of carbohydrates (lactose, glucose and galactose).

Figures 3.7A and **B** demonstrate results for bioconversion of whey lactose (5.25% (w/v)) using β-galactosidase and the CFF process on the Filmtec NF270-400 and the Koch TFC-SR3 nanomembranes. Lactose, glucose and galactose concentrations in retentate and permeate are shown over 5 h of the filtration process. It was observed that the lactose degradation was rapid and almost complete at the end of filtration. However, the Koch TFC-SR3 nanomembrane showed a higher rejection value for lactose and lower rejection values for glucose

and galactose when compared to those calculated for the Filmtec NF270-400 nanomembrane.

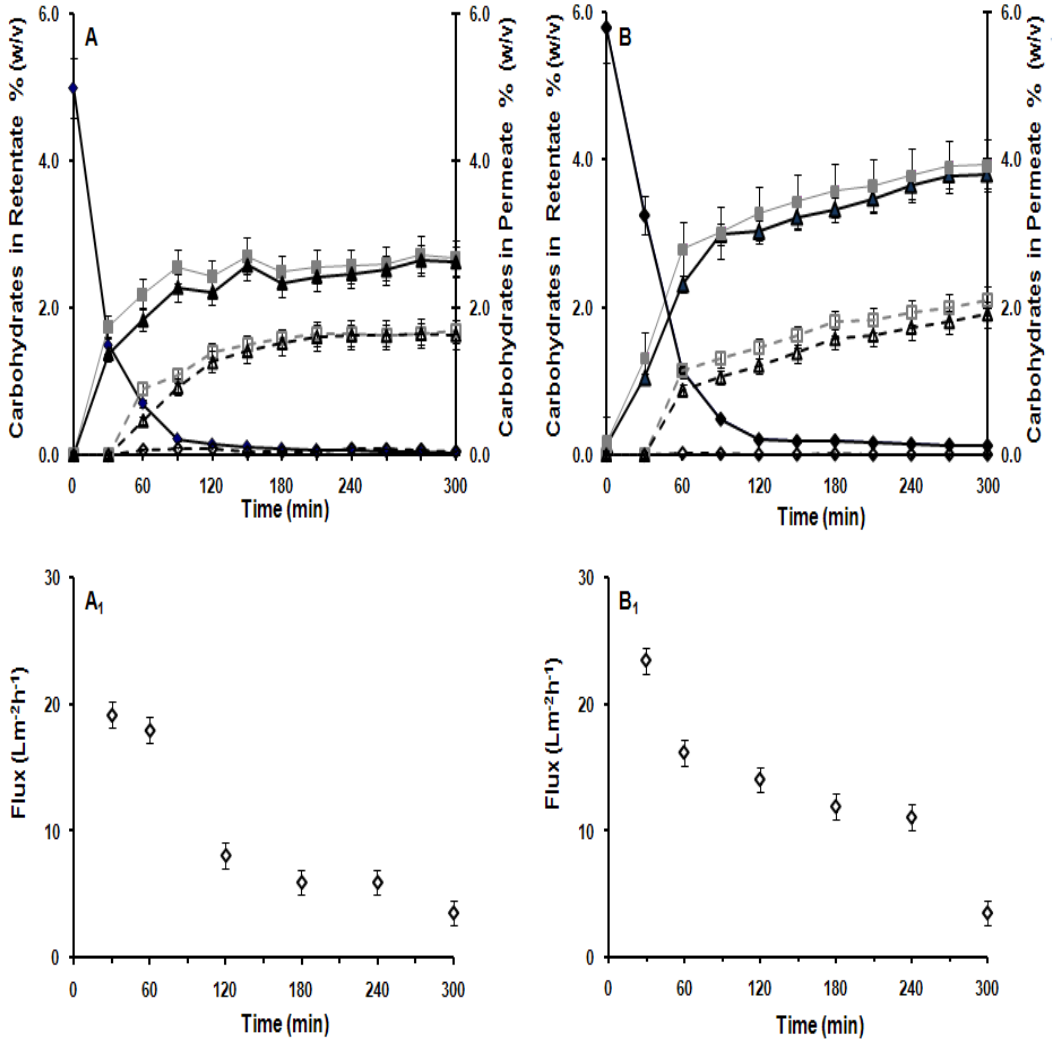

Figure 3.7 A) Sugar concentration values during CFF of bioconverted whey on the Filmtec NF270-400 nanomembrane and B) Sugar concentration values during CFF of bioconverted whey on the Koch TFC-SR3 for: lactose (—◆—), glucose (—■—) and galactose (—▲—) in retentate, and lactose (– ◇ –), glucose (– □ –) and galactose (– △ –) in permeate; A_1) Flux values during CFF of (◇) bioconverted whey on the Filmtec NF270 nanomembrane, and B_1) Flux values during CFF of (◇) bioconverted whey on the Koch TFC-SR3. Error bars show standard deviation values based on triplicate tests.

It was also observed that the addition of enzyme to whey at the start of bioconversion caused a small increase in the back pressure of filtration system. This could be attributed to the entrapment of the enzyme on the nanomembrane surface and to the volumetric flux decline which we observed in described tests. The comparative evaluation of data for permeates of the model solution of lactose, glucose and galactose *versus* the bioconverted whey solution showed that the concentration of glucose and galactose in whey permeates was higher at the end of CFF process. In addition, it was found that measured lactose concentration in permeate was very low, but surprisingly lower for a whey permeate stream.

Figures 3.7A_1 and **B_1** show that the volumetric flux declined faster for the Filmtec NF270-400 *versus* the Koch TFC-SR3 nanomembrane at the start of the filtration but this decline level was similar at the end. After the experiment, the CIP procedure was applied to clean the nanomembranes and conducted rechecks of volumetric fluxes for water. However, flux results showed lower values when compared to the water volumetric flux values measured for fresh nanomembranes (data not shown). Koyuncu *et al.* (2004) found in their tests with nanomembranes that flux values declined with an increase in the concentration of salts in the feedstock solution. The authors suggested that adsorption of solids on the membrane surface occurs readily for crossflow filtered solutions with a high ionic strength. Jeantet *et al.* (2000) reported that during the crossflow filtration of whey on membranes, a surface layer is formed, which regulates the permeability and the selectivity of the nanomembranes. They indicated that whey proteins and

minerals contributed significantly to the flux decline during the whey crossflow filtration process.

In this study, it was demonstrated that it is possible to separate monosaccharides from disaccharides in their proportional blends and whey feeds with selected nanomembranes. The experimental results show that the CFF process on nanomembranes offers an effective and alternative solution for the separation of carbohydrates from their feeds. It also demonstrates feasibility of recovering, standardizing, concentrating or fractionating carbohydrate-rich feeds or whey. The lactose was effectively rejected for applied proportional blends and whey feeds by the tested nanomembranes. However, the calculated average rejection values and flux revealed some differences, which varied subject to change in carbohydrate concentrations in the feeds. The use of the proportional-integral-derivative (PID) mass flow controller prevented an increase in the back pressure on nanomembranes, which may have prevented more stretching or damage to the nanomembrane structure during the cross flow filtration process.

3.4 CONCLUSIONS

Crossflow filtration of carbohydrates using nanomembranes offers an opportunity for separation or purification of carbohydrate-rich feedstocks. Tested nanomembranes could be effectively used to separate lactose from glucose and galactose, but the Koch TFC-SR3 nanomembrane demonstrated a higher average rejection value for lactose. The results from simultaneous bioconversion and

crossflow nanofiltration of whey carbohydrates in a stirred tank reactor showed that the separation of mono- and di-saccharides is also possible. Yet, to achieve the best separation results there is a need for careful consideration as to which nanomembrane should be selected before using it in the cross-flow filtration process.

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4. BIOCONVERSION OF LACTOSE USING SOLUBLE AND PVA IMMOBILIZED BIOCATALYSTS WITH FILTRATION ON NANOMEBRANES

4.1 INTRODUCTION

There is considerable interest in developing efficient and cost-effective processes for the bioconversion of lactose to produce milk for lactose-intolerant people and for generating value-added products from whey a by-product of cheese manufacturing containing about 5% lactose (Giorno and Drioli 2000). Mahoney (1997) indicated that effective hydrolysis of lactose is important for dairy manufacturers and consumers because reduced milks are sweeter and easier to metabolize by consumers. Effective partial or complete lactose bioconversion into monosaccharides (glucose and galactose) using stable immobilized biocatalyst would lower production costs and render the final products useful in numerous applications. Some of these applications include the production of lactose-reduced milk products, and glucose and galactose that can be used in food products (adjuncts), or for food and industrial fermentations (ethanol, butanol, platform chemicals etc.) (Elliot *et al.* 2001; Goulas *et al.* 2003).

Bioconversion of lactose is a major application of biocatalytic membrane reactors in the agro-food sector (Giorno and Drioli 2000). Overall, hydrolysis of lactose requires a reliable biocatalyst and specialized membranes to achieve efficient and effective degradation and fractionation of reaction products (IDF

1987; Cricenti *et al.* 2006). Stirred membrane bioreactors have the advantage of allowing for the separation and concentration of glucose and galactose from reduced lactose feedstocks. This separation is beneficial not only for the purification of the products, but also to prevent poisoning of the biocatalyst by competitive inhibition.

Efficient and cost-effective hydrolysis of lactose using a biocatalyst requires a robust, low production cost, and highly stable enzyme system. Immobilized biocatalysts are generally more stable and robust than conventional soluble biocatalysts. Immobilization of β-galactosidase and its activity on different supports have been investigated by several authors (e.g. Portaccio *et al.* 1998; Sun *et al.* 1999; Tanriseven and Dogan 2002; Sufang and Lingyun 2009). Grosová *et al.* (2008) summarized the published literature on immobilization of β-galactosidase using different immobilization supports. Their summary indicates that most of the supports had significant negative impacts on biocatalyst activity. Activity remaining after immobilization varied from 0.01% to 90% of original activity, depending on the immobilization method. In addition, Ladero *et al.* (2000) found that the specific activity for immobilized biocatalyst decreased significantly when compared to soluble biocatalyst.

Food grade β-galactosidase of *Kluyveromyces lactis* immobilized on polyvinyl alcohol (PVA) and modified with polyethylene glycol (PEG) is commercially available and manufactured by the LentiKat's® a. s. company in the Czech Republic. This novel biocatalyst is extruded in the form of hemispheric oval capsules with the LentiKat's® printer which was also developed by

LentiKat's® a. s. This novel immobilized biocatalyst is produced in the form of hemispheric lens-shaped capsules using a proprietary LentiKat's® printer and it is recognized by the U.S.A Food and Drug Administration (FDA) with GRAS (generally recognized as safe) status. The biocatalyst is characterized by high activity and offers good mechanical stability when used in a membrane filtration process (Grosová *et al.* 2009; Rebroš *et al.* 2007). However, these properties have not yet been verified during lactose reduction using nanomembrane filtration. In addition, the presence of glucose and galactose in the feedstock stream negatively affects the biocatalyst activity (Ladero *et al.* 2000). But, the authors found that the use of nanomembrane filtration was effective in reducing this negative effect.

To date, there is a lack of detailed information in published research literature that demonstrates the effective use of immobilized β-galactosidase using a stirred bioreactor equipped with nanomembranes. In comparison to other available methods, this method offers many advantages, such as: 1) lower processing costs by enhancing biocatalyst stability (Iorio *et al.* 2006); 2) increased biocatalyst reuse by adjusting the processing parameters during nanofiltration of the carbohydrates (Goulas *et al.* 2003); 3) formulation of novel lactose-reduced milk products or carbohydrate adjuncts for use in the production of human foods (Gänzle *et al.* 2008).

In this study, the major objectives were: 1) to measure β-galactosidase activity with LentiKat's® PVA-immobilized biocatalysts and compare it against soluble β-galactosidase, and 2) to evaluate the stability of the immobilized

biocatalysts after prolonged lactose hydrolysis in a stirred membrane bioreactor equipped with a nanomembrane.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The α-lactose monohydrate, D-glucose and D-galactose standards, each with 99.8% purity, were purchased from Sigma-Aldrich Canada Ltd. (Toronto, Ontario, Canada). Trichloroacetic acid (TCA), and citric acid, both 99.9% pure, were acquired from VWR Inc. (Toronto, Ontario, Canada). The potassium phosphate buffer, pH 6.5 ±0.1, was made by mixing 0.1 M K_2HPO_4, 0.1 M KH_2PO_4 and 2mM $MgCl_2$ into double deionized water which was produced with a resistivity of 18.2 MΩ · cm by filtering of tap water through the RO membrane/cation exchange system purchased from Millipore (Canada) Ltd.(Etobicoke, Ontario, Canada). All phosphate buffer components, 99.9% pure, were purchased from Fisher Scientific (Edmonton, Alberta, Canada). Solutions of 1 N NaOH and 1 N HCl used for the pH adjustment were acquired from VWR Inc. The soluble commercial preparation,

4.2.1.1 Enzyme

Yeast lactase, β-galactosidase (EC 3.2.1.23), was also used containing Lactozym 3000 L HP G, derived from *Kluyveromyces lactis*, with a declared activity of higher than 3000 LAU mL^{-1} (Lactase Activity Units), manufactured in

Novozymes A/S, Bagsvaerd, Denmark. According to the enzyme manufacturer, one LAU is the amount of commercial enzyme that can produce 1μmol of glucose per minute under standard conditions: 4.7% lactose concentration (pH=6.5), 30°C, 30 min, standard milky buffer. Polyvinyl alcohol (PVA) 17-99 and polyethylene glycol (PEG), both chemicals 99.8% pure, were donated by LentiKat's® a.s., Prague, Czech Republic. The soluble protein content in the commercial enzyme extract was 30.0 mg mL^{-1}. The Amido-Black "10B" staining reagent, with 99.8% purity, was purchased from ESBE Laboratory Supplies, (Toronto, Ontario, Canada).

4.2.1.2 Nanomembrane

Koch TFC-SR3 was used with a nominal pore size of 200-400 molecular weight cut off (MWCO) in a flat sheet configuration. The nanomembrane was donated by the Koch Membrane Systems, Inc.(Wilmington, Massachusetts, USA).

4.2.2 Methods and equipment

4.2.2.1 Enzyme immobilization

The β-galactosidase was immobilized according to the steps outlined in the proprietary procedure, which was provided by LentiKat's® a.s., Prague, Czech Republic. The 95 mL mixture, composed of PVA, 10% (w/v), PEG, 6% (w/v) in doubly deionized water, was heated for 20 min to the boiling point (98-

98.5°C) and cooled down to 35°C. The 5 mL of the soluble enzyme was added into the mixture and extruded on poly-carbon plates using the LentiKat's® printer in the form of oval beads, and dried down in an oven to achieve the mass specified by the LentiKat's® manufacturer (data not published). Next, the beads were swollen for 20-40 min in the stabilizing solution (0.1 M Na_2SO_4), and transferred into the 0.1M potassium phosphate buffer solution, pH 6.5, containing 2 mM of $MgCl_2$ and ethanol, 6% (v/v), and stored at 4°C.

4.2.2.2 Protein analysis

Two grams of beads with the immobilized biocatalyst were weighed and dried overnight at 70°C in a vacuum oven. After cooling to 20°C, the crude protein (CP) content was evaluated using a LECO nitrogen analyzer (Leco Corp., St. Joseph, Michigan, USA). The CP was determined by the Dumas method (AOAC 1995) and a conversion factor of 6.25 was used to convert the nitrogen content to crude protein content. The protein content in the soluble biocatalyst, and the retentate and permeate samples, collected at the start and at the end of the hydrolysis of the lactose experimental run, was measured according to Smith et al. (1985) using the Coomasie Plus® Bradford Assay and BSA protein standard (Pierce Biotechnology, Inc., Rockford, Illinois, USA).

4.2.2.3 Evaluation of biocatalyst activity and kinetic parameters

The β-galactosidase activity was assayed at 35°C by adding a 100 µL of soluble enzyme, which was diluted in a potassium phosphate buffer (1:10) or

20 g of immobilized biocatalyst into 10 mL of lactose solution with varying concentrations from 0 to 12% (w/v), and vigorously mixed in an orbital shaker for 10 min at 200 rpm. Samples of 100 µL were withdrawn after 4 min and the reaction was stopped by adding 100 µL of TCA 25% (w/v). The lactose and its hydrolysis products, glucose and galactose, were measured by HPLC. It should be noted that in experiments one enzyme unit is defined as the amount of enzyme activity which releases 1 mmol of glucose per minute from lactose 5% (w/v) in a potassium phosphate buffer pH 6.5, and at 35°C. The specific activity of the enzyme (SAE) was also calculated by dividing enzyme activity units by one gram of enzyme protein.

Kinetic parameters for the soluble and the immobilized biocatalyst were evaluated by initial rate studies using the conventional Michaelis-Menten model, equation 16, and the non-linear regression method included in the GraphPad Prism, version 5.02, software (GraphPad Software Inc., San Diego, California, USA):

$$Y = V_M * S_l / (K_M + S_l) \qquad (16)$$

where: Y is the initial reaction rate (mmol min^{-1} g^{-1} protein), V_M is the maximum enzyme velocity (mmol min^{-1} g^{-1} protein), S_l is the initial lactose concentration % (w/v) and K_M is the Michaelis-Menten constant in the units expressed as lactose concentration % (w/v). The immobilized biocatalyst activity was compared to the soluble form and its yield was calculated according to the formula adopted from Sun *et al.* (1999).

4.2.2.4 Evaluation of optimum pH and temperature profiles

The effect of pH for the soluble and the immobilized biocatalyst were evaluated (from 5.0 ±0.1 to 8.0 ±0.10) by the assay procedures as previously described using lactose 5 % (w/v) in a phosphate buffer at 30^{o}C. The pH value of the lactose solution was adjusted by the addition of NaOH or HCl solution. Similarly, the thermal profiles for both biocatalysts were evaluated at temperatures ranging from 20^{o}C to 60^{o}C during the lactose hydrolysis process. The samples of the lactose solution were preincubated to the desired temperatures and then the biocatalysts were added. Activities of both biocatalysts were evaluated during the four month storage at 4 ±1^{o}C.

4.2.2.5 Immobilized enzyme matrix characterization

The immobilized biocatalyst polymer beads were evaluated visually, before and after lactose hydrolysis in the membrane bioreactor and the crossflow nanofiltration with a light microscope (model Carl Zeiss Primo Star), equipped with a Plan Achromat phase contrast objective: 4 x /0.1 WD: 6.50 mm, Carl Zeiss Micro Imaging GmbH, Jena, Germany. The light microscope was connected to a Canon Power Shot camera (model A640, Canon Canada, Mississauga, Ontario, Canada), equipped with a Zoom Browser Ex., version 5.7 software for picture digital manipulation.

The distribution of the immobilized biocatalyst in the PVA LentiKat's® beads was also evaluated with transmission electron microscopy (TEM). Polymer spherical beads, containing the enzyme, were stained using the Amido-

Black "10B" reagent according to the method adopted from Jensen and Fisher (1981), and cut in half and placed into gelatin capsules. The capsules were filled with LR White embedding resin (Fisher Scientific, Edmonton) and then placed in a 60 $\pm 1^{o}$C oven and allowed to harden overnight. They were then removed by soaking in double deionized water at room temperature with a diamond knife on a Reichert-Jung Ultramicrotome (model 701701, Leica, Milton Keynes, UK), in thicknesses of 60-70 nanometers, and collected on 300 mesh grids. After drying, the grids were examined with a Philips Morgangi 268 TEM (FEI Compagny, Limeil-Brevannes, France), equipped with a CCD camera (FEI Compagny France), to determine the size and distribution of the pores.

4.2.2.6 Batch-stirred membrane bioreactor system and membrane

The details of the Batch- stirred membrane bioreactor are shown earlier in **Figure 3.1** and described in paragraph 3.2.1.6 of Chapter 3. However, it is important to note that to avoid plugging the PID mass flow controller loop, the immobilized biocatalyst was retained in the feed tank on the stainless steel screen (with 0.5 mm in diameter holes).

The new nanomembrane was preconditioned prior to installation in the SEPA CFII membrane unit by immersing it in the fresh doubly-deionized water and by holding it immersed at room temperature for 24 h. After the membrane installation was completed, the water was recycled in the membrane bioreactor system at a flow rate set at 72 L h^{-1} and flushed through the membrane at a back pressure set at 1.38 MPa (200 psi), which was controlled by a PID mass/flow

controller interfaced via RS 232 with a computer. The temperature of the feed solution and stirrer speed in the membrane bioreactor were adjusted manually with available controls and set up at 35 ±1^{o}C and 200 rpm respectively. The water volumetric flux for the nanomembrane was measured after 15 min and monitored from the start up time of the crossflow filtration process on the membrane to assure that the installed nanomembrane was without defects. The collected data for the volume concentration ratios (VCR) were evaluated at the end of the filtration process by applying mathematical equations 7 and 8 which were adopted from Goulas *et al.* (2003). It should be noted that a new nanomembrane was installed in the membrane bioreactor system prior to each lactose bioconversion test.

4.2.2.7 Lactose bioconversion in the stirred membrane bioreactor

The lactose solution, 5.0% (w/v), was prepared by mixing it with the phosphate buffer solution (pH 6.5 ±0.1). It is important to note that the weighed amount of α-lactose monohydrate was recalculated and reported as lactose and the pH value and lactose solution concentration was reconfirmed immediately prior to the nanomembrane filtration process.

At the start of the lactose bioconversion, water was drained from the bioreactor feed tank which was then filled with 4 L of 5 % (w/v) lactose feed solution. Next, the feed solution was recycled through the whole system and back to the feed tank at a constant feed flow rate of 72 L h^{-1} set at the Hydra-Cell pump with a nanomembrane back pressure set at 0 MPa (0 psi). The lactose feed solution temperature was adjusted to 35 ±1^{o}C, and either 400 g of immobilized or

20 mL of soluble biocatalyst were added directly into the bioreactor tank and mixed with a lactose feed solution at 200 rpm. The lactose feed solution free of the immobilized biocatalyst was recycled in the membrane bioreactor. The soluble biocatalyst in the bioreactor was rejected by the membrane and recycled continuously back to the bioreactor vessel. After 15 min of the recycling in this process, the lactose solution was filtered on the nanomembrane for 5 h with a back pressure set to 1.38 MPa (200 psi) and the retentate solution recycled back into the feed tank with a constant flow rate of 72 L h^{-1}, at 35 $\pm 1^{o}$C. The permeate solution volume was collected continuously in a graduated cylinder and its volume was recorded. Its mass was measured with a digital balance, at the start and every 15 min for the first three hours and every 30 min for the last two hours of lactose degradation and crossflow filtration on the nanomembrane. The cleaning in place process of the stirred membrane bioreactor system was completed after each experiment according to the procedure described earlier in paragraph 3.2.2.2 in Chapter 3.

4.2.2.8 Carbohydrate analysis

The HPLC method was adopted with a run time for each sample modified to 16 min (Anonymous 2007). The experiments were performed on an Agilent 1200 Series chromatograph equipped with a Model G1329A autosampler, a Model G1311A quaternary pump and a Model G1362A refractive index detector operated at 35^{o}C internal temperature (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). The analytical column was a Nucleogel

Sugar Ca^{2+} column (300 mm x 6.5 mm) proceeded by a guard column (21 mm x. 4.0 mm) (Marcherey-Nagel Gmbh & Co. KG, Düren, Germany), and the column was heated at 87°C with an external column heater (Bio-Rad, Hercules, California, USA). The mobile phase (isocratic solvent) was H_20, filtered on a 0.5 µm membrane filter purchased from Fisher Scientific, Edmonton, and degassed. The 20 µL of retentate and permeate sample, collected during the lactose hydrolysis process, was injected on the column with a flow rate set at 0.5 mL min^{-1}. The HPLC results for all samples were quantified using Agilent "*ChemStation*", version 2007, software, (Agilent Technologies). The phosphate buffer solution was used as a blank with a zero value reference.

4.2.2.9 Statistical Analysis

All experiments were conducted in triplicate, the data averaged, and where applicable were evaluated with Student's t-test using Microsoft Excel 2003 SP 2.0, Microsoft Co. (Redmond, Washington, USA) and Statistica, version 5.0A software (Stat Soft Inc., Tulsa, Oklahoma, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Evaluation of enzyme activity, kinetic parameters and activity yield

Our test results demonstrate that the biocatalyst immobilized in the PVA LentiKats® beads has a lower activity relative to the soluble enzyme when hydrolyzing lactose. The maximum value for the average specific activity of

enzyme (SAE) for the immobilized biocatalyst was measured at 247.8 mmol min^{-1} g^{-1} *versus* 263.1 mmol min^{-1} g^{-1} soluble biocatalyst. The activity tests conducted during four months of storage at 4°C showed a decline for both the immobilized and the soluble biocatalysts in the third month of storage (**Figure 4.1A** and **4.1B)**. This decline trend was similar for both biocatalysts and indicated a calculated average activity value in the fourth month of storage to be at 68.9% of that for the fresh immobilized biocatalyst *versus* 68.5% for the soluble one.

The SAE values for the immobilized biocatalyst at different temperatures, in the range from 20 to 60°C, revealed a trend, which indicated that its activity increased as the temperature increased up to 50°C, and then declined. In contrast, the SAE values for the soluble biocatalyst peaked in the temperature range from 30 to 35 °C, but they were lower at higher and lower temperatures.

(**Figure 4.1C)**.

This observed higher activity of the immobilized biocatalyst at higher temperatures could be beneficial when using it in the crossflow filtration process because of the possible increase in the permeate flux at higher temperature (Sjöman *et al.* 2008). The immobilized biocatalyst activity was calculated at 94.2% of that for the soluble enzyme was higher when compared with the research results using other immobilization systems (Sun *et al.* 1999). Sun *et al.* (1999) in their report described β-galactosidase extracted from gram chicken beans, which was immobilized in polyacrylamide and reported an activity yield at 72%. However, it is difficult to compare our results with theirs because in their

experiments they used a biocatalyst originating from a different source as well as another immobilization method.

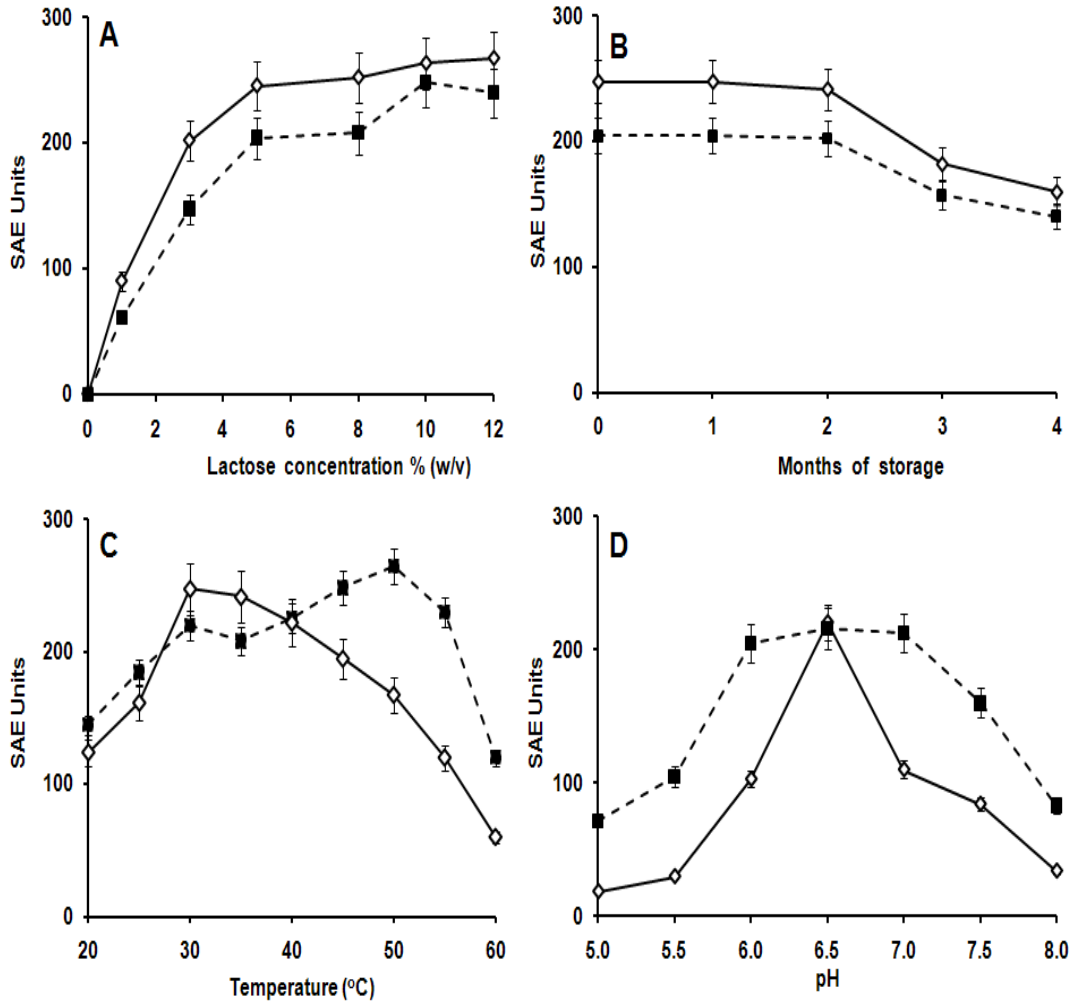

Figure 4.1 Comparative analysis of SAE for the immobilized (– ■ –) *versus* the soluble biocatlyst (—◇—) for: A) different lactose concentrations at pH 6.5 and temperature at 35°C; B) during four month of storage at 4°C, and lactose solution concentration at 5% (w/v); C) different temperatures, lactose concentration at 5% (w/v), at pH 6.5, and D) different pH values for lactose concentration at 5% (w/v), and temperature at 35°C. Error bars show standard deviation values based on triplicate tests.

The kinetic parameters and the activity of biocatalysts were evaluated with the conventional Michaelis-Menten-type reaction kinetics equation. The data were fitted into the non-linear regression model and the results revealed the Michaelis-Menten reaction kinetics constants for the soluble biocatalyst at: V_{max} = 327.4 mmol min^{-1} g^{-1} protein and K_M = 2.3 (lactose % (w/v), with the goodness of fit of $R^2 \geq 0.97$, and for the immobilized biocatalyst at: V_{max} = 326.0 (mmol min^{-1} g^{-1} protein) and K_M = 3.9 (lactose % (w/v)), with the goodness of fit of $R^2 \geq 0.97$. Noticeably, the V_{max} value of the immobilized biocatalyst (326.0 (mmol min^{-1} g^{-1} protein) was lower than the value calculated for the soluble biocatalyst (327.4 mmol min^{-1} g^{-1}) but the K_M value was higher (3.9 *vs.* 2.3 as lactose % (w/v)).

It was attempted to compare obtained experimental results against other published in research reports (Juardo *et al.* 2006; Grosová *et al.* 2009b). However, the experimental design or method was lacking that would match exactly either our biocatalyst activity or its immobilization method. In addition, the majority of researchers used *o*-nitrophenyl β-D-galactopyranoside (ONPG) reagent to evaluate the activity of the β-galactosidase, and they also used the Michaelis-Menten-type reaction kinetics that account for the competitive inhibition of the biocatalyst with products of its reaction. For example, Zhou *et al*., 2003, in their report proposed and used the mathematical model for Michaelis-Menten type reaction kinetics with product competitive inhibition and side-reactions to evaluate their new immobilization method for the β-galactosidase of *Kluyveromyces lactis.*

4.3.2 Evaluation of optimum pH and temperature profiles

Analysis of the experimental data describing the activity of both biocatalysts in the pH range from 5.0 to 7.5 and at 35°C showed that their pH optimum value was at pH = 6.5 (**Figure 4.1D**). But, it was also observed that the activity of the immobilized biocatalyst at the lower pH values was trending higher. This trend was also characterized by an extended pH optimum in the higher pH values when compared to the soluble biocatalyst. This broadening effect of activity could be of some advantage for food processors who would intend to use the immobilized biocatalyst to degrade lactose in feed streams whose pH optimum would fall outside the pH range set up for the soluble biocatalyst. Rebroš *et al.* (2006) also noted that a broader pH optimum could benefit the fermentation industry that could use immobilized biocatalysts for subsequent degradation of carbohydrates and fermentation process. Recently, Rebroš *et al.* (2007) reported on using the PVA LentiKat's® immobilization matrix for invertase and demonstrated that its activity, at a tested pH range, was higher when compared to that of soluble enzyme. They also suggested that such an effect was a consequence of secondary interactions that occurred between the biocatalyst and polymer gel matrix.

4.3.3 Immobilized enzyme matrix characterization

Figures 4.2A and **4.2B** demonstrate the results for the **s**tructural evaluation with a light microscope of immobilized PVA LentiKat's® biocatalyst beads with four fold magnification. The beads' shape is similar to the hollow

sliced in a half sphere. Comparative evaluation of the beads before and after use in the lactose hydrolysis process in the bioreactor clearly demonstrates that their structure have not changed after this process. In addition, samples of the hydrolyzed lactose solution were evaluated for a possible protein leak out of the polymer matrix during processing, but there was no soluble enzyme detected before or after the process.

The evaluation of immobilized biocatalyst kinetics also requires additional information about the biocatalyst distribution within the immobilizing polymer. Results of TEM investigation of the immobilized biocatalyst interior matrix at 18,000 magnification revealed that the uniform distribution of pores within the bead polymer matrix contained the trapped biocatalyst (**Figure 4.2C**). The high porosity of this matrix is advantageous because it improves the process of carbohydrate mass transfer that otherwise would be severely limited and could affect the productivity of the bioreactor using the immobilized biocatalyst (Zhang and Franco 1999). **Figure 4.2D** illustrates the biocatalyst interior matrix at 3,500 magnification after staining polymer beads with the Amido-Black "10B". The uniform distribution of the biocatalyst protein was observed within the sliced polymer matrix and outside pore structure, in the form of black oval circles.

Pinto and Macias (1995) elaborated that the lack of uniform distribution of the biocatalyst within the support could cause its increased activity and selectivity but could decrease its stability. They emphasized that it is important to learn exactly about the distribution of the biocatalyst within the support to be able to distinguish properly between kinetic and mass transfer effects.

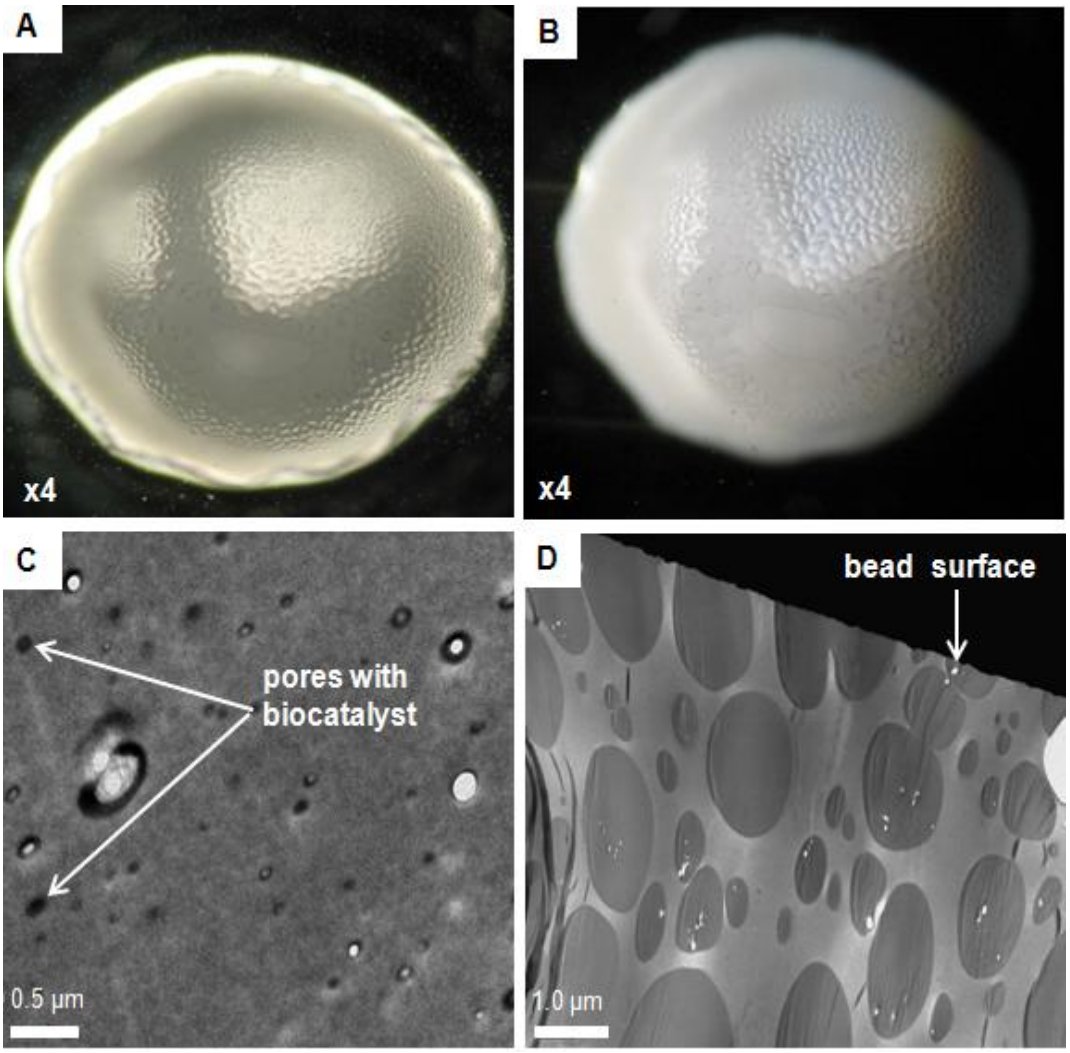

Figure 4.2 Structural evaluation of immobilized PVA LentiKat's® biocatalyst: A) fresh biocatalyst bead picture taken with the digital camera attached to the light microscope, B) biocatalyst bead picture taken after hydrolysis of lactose in the membrane bioreactor, C) TEM of biocatalyst bead cross section showing pores in polymer matrix with trapped enzyme, x18,000 magnification, and D) TEM of biocatalyst bead interior after application of dye, the stained in dark oval shapes are enzyme proteins; the arrow shows immobilized biocatalyst bead surface, x3,500 magnification.

Therefore, they suggested that the full evaluation of immobilized biocatalyst requires additional information about the biocatalyst distribution within the immobilizing polymer.

4.3.4 Lactose bioconversion in the stirred membrane bioreactor

Figures 4.3A and **4.3B** show the comparative results for 5% lactose degradation by the immobilized and soluble biocatalyst respectively in the stirred batch reactor equipped with the crossflow filtration nanomembrane. It should be noted that the soluble enzyme hydrolyzed lactose at a much faster rate and almost completed its degradation, 99.9% (w/v), after 60 min of reaction. On the other hand, the degradation of lactose with the immobilized enzyme was not completed during five hours of processing. It was reported earlier in this manuscript that the lower activity of the immobilized biocatalyst was one of the major contributing factors to slower degradation of lactose. Another contributing factor to slower hydrolysis of lactose was a lack of good control over the stirring process in the bioreactor. It was observed that during the lactose degradation process some beads attached to the cylindrical wall of the stainless-steel strainer, inserted inside the bioreactor tank, and formed an approximately 1 cm thick layer. To remediate this we attempted to vary the speed rate of the stirrer between 100 rpm and 300 rpm, but we were not successful in avoiding the accumulation of beads on the side wall. Therefore, more research to prevent this from occurring is needed.

After 60 min of conversion time, on average 83.1% (w/v) of lactose was degraded to glucose and galactose, but 7.0% (w/v) of lactose was still available at the end of the process. The analysis of retentate and permeate samples during 5 h crossflow trial test runs with the immobilized and the soluble biocatalyst revealed a gradual increase in glucose and galactose concentrations.

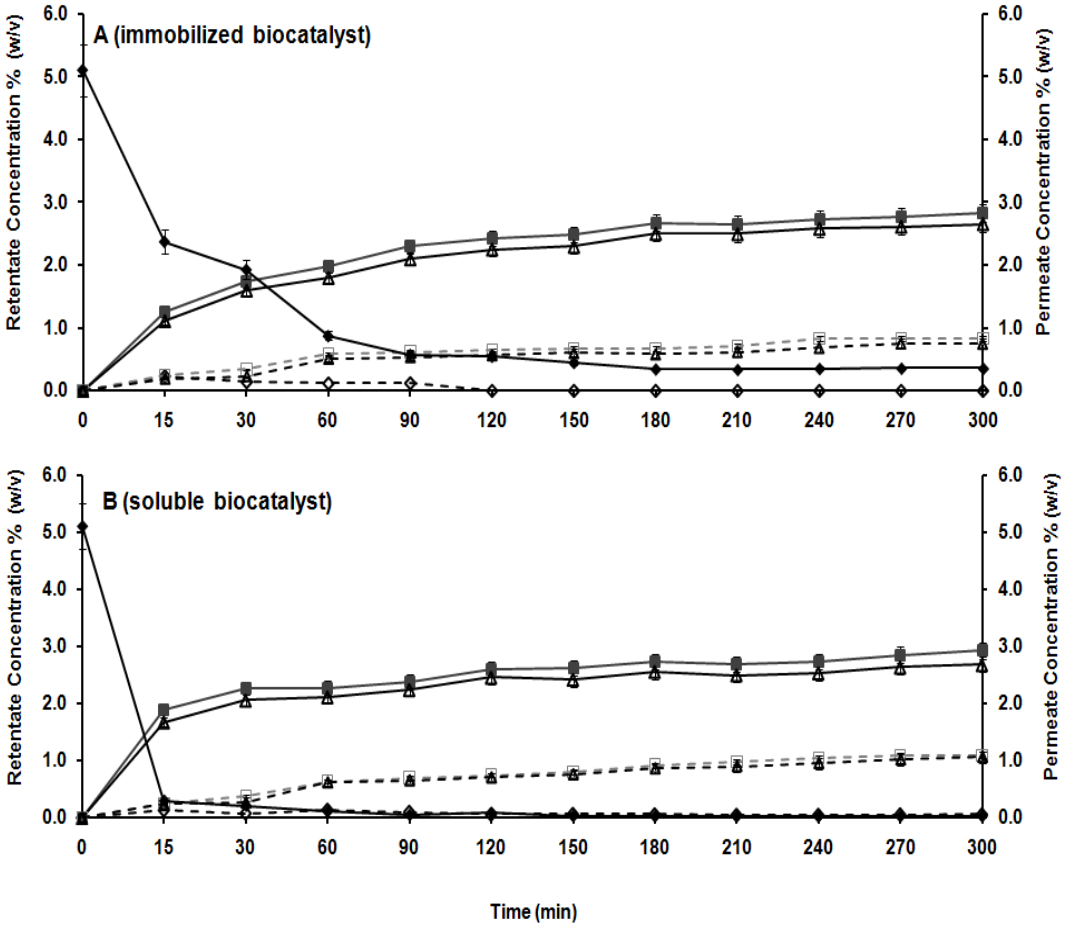

Figure 4.3 Hydrolysis and nanofiltration of lactose 5% (w/v) in the stirred membrane batch reactor at 35°C, pH 6.5 with: **A**) with the immobilized biocatalyst (the PVA LentiKat's®), and **B**) the soluble biocatalyst; reaction products in retentate (lactose (—◆—), glucose (—■—), and galactose (—△—)), and in permeate (lactose (- ◈ -), glucose (- ⊟ -) and galactose (- ▲ -)); Triplicate standard deviation for permeate values are smaller than icons.

The measured volume concentration ratios (VCR), the ratio of the initial feed volume to the retained feed volume, during cross-flow filtration, averaged 1.27 for the immobilized versus 1.36 for the soluble biocatalyst. The assayed average concentration of lactose in permeate was at 0.28% (w/v) after the first hour of the feedstock processing with the immobilized biocatalyst, and at 0.10% (w/v) in permeate for the soluble biocatalyst. Presence of small amounts of

lactose in permeate during the first hour of filtration when using immobilized or soluble biocatalyst agrees with earlier reported results shown in **Table 3.1** in Chapter 3 in which the evaluated lactose was rejected on the Koch TFC-SR3 nanomembrane.

At the end of experimental test runs, it was observed that the lactose concentration in permeate declined completely both for feeds treated with the immobilized and soluble biocatalyst. In addition, it was noted that the measured average volumetric flux for the tested feedstocks also declined by 50% as the concentration of carbohydrates increased during the 5 h test run. Goulas *et al.* (2003) found that an increase in carbohydrate concentrations for mono-, di- and oligosaccharides in blended feeds during the filtration process on nanomembranes affected their rejection values and fluxes. Moreover, they elaborated that the lactose, glucose and galactose rejection varied for different nanofiltration membranes due to increased concentration polarization layers and the retentate volume concentration ratio. It is also well documented that a change in lactose, glucose, and galactose concentrations in retentate affects the activity of free or immobilized forms of biocatalysts during the lactose hydrolysis process. Mahoney (1997) and Ladero (2000) reported that maintenance of the biocatalyst activity at the optimum is very important during the lactose degradation process in concentrated feeds.

To our knowledge, this is the first demonstration of results showing the biodegradation of lactose with a novel immobilized biocatalyst using a batch stirred bioreactor equipped with nanomembranes. Although the assayed activity of this immobilized biocatalyst is lower than the soluble one, it offers some unique advantages during the lactose degradation process such as a broader pH

and temperature range or good storage and mechanical stability. There are other possible potential benefits for use of this immobilized biocatalyst. A noteworthy example would be its possible application during the continuous bioconversion of lactose in the bioreactor equipped with nanomembranes.

4.4 CONCLUSIONS

In this study, it was found that the evaluated biocatalyst, immobilized in the PVA LentiKat's® matrix was characterized by high stability and high activity, which was also lower from the activity measured for the soluble form but still higher when compared with other known immobilization methods. The results revealed that this novel immobilized biocatalyst offered an effective and alternative solution when was used to degrade lactose and possibly other sugars in industrial fermentation or food processing. It was demonstrated that the application of this novel biocatalyst during the hydrolysis of lactose process in the stirred bioreactor and crossflow filtration on nanomembranes was possible.

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5. RAPID BIOCONVERSION OF WHEY LACTOSE IN NANOMEMBRANE BIOREACTOR WITH THE β-GALACTOSIDASE IMMOBILIZED ON THE PVA LENTIKAT'S®

5.1 INTRODUCTION

The cost effective bioconversion of whey permeate containing lactose to simple sugars is an area of extensive and ongoing research based on new available technological solutions (Mahoney 1997; Gänzle *et al.* 2008.). Currently, new methods for lactose bioconversion include the use of membrane bioreactors in different configurations (Shahbazi *et al. 2*005; Juardo *et al.* 2006; Saddoud *et al.* 2007). To obtain optimum functionality, an understanding of the membrane bioreactor design is required.

Williams (2002) reviewed key issues that are important in bioreactor design and development. The author noted that the major functions of an effective bioreactor are: control, containment of biocatalyst and the increased efficiency of the catalytic processes. He described two key issues that need to be considered when designing the bioreactor. The first, concerns the optimization of the bioconversion process through the selection of a suitable biocatalyst. The second refers to the need for control of bioreactor reaction parameters like: 1) temperature, 2) pH, 3) sufficient feedstock amount (volume and concentration), and 4) product or byproduct removal.

Shahbazi *et al.* (2005) and Curcio *et al*. (2006) indicated that the application of membrane bioreactors was unique and could improve whey permeate lactose utilization. In addition, they both suggested that there is an opportunity for dairy processors to generate novel products from whey permeates and eliminate waste. Gänzle *et al.* (2008) in thier review offered some advice about the selection of the biocatalyst for optimal lactose bioconversion (β-galactosidase). The authors described that the β-galactosidase activity was negatively impacted during lactose hydrolysis because of released galactose. Earlier, Goulas *et al.* (2003) demonstrated that separation of more complex carbohydrates (including lactose) from simple carbohydrates (e.g. glucose or galactose) through the application of nanomembranes was possible.

These reports offer their own unique insights into the design and development of the processes for lactose hydrolysis. The common major issues relate to: lactose degradation in the bioreactor, measurement of substrates, evaluation of the selected membrane, and the reaction kinetics of the used biocatalyst. All these issues are important for optimal use of the bioreactor, but more knowledge is needed about the design of the nanomembrane bioreactor for the whey lactose bioconversion.

Therefore, based on the evidence from earlier studies, the major objective of this study was to design and develop a functional model of a small batch-stirred nanomembrane bioreactor for the bioconversion of whey permeate lactose with the β-galactosidase. The steps required for the completion of this process were described in terms of: 1) process description (mass balance, process flow

diagram), 2) equipment (the membrane bioreactor design), and 3) process cost analysis. In addition, a detailed description of the equipment and cost analysis were provided. A detailed description of the bioreactor equipment and results demonstrating the bioconversion of whey permeate lactose follow.

5.2 MATERIALS AND METHODS

5.2.1 Materials

5.2.1.1 Whey permeate

A batch of pasteurized skim milk 5 L, a product of Saputo Inc. (Montreal, Quebec, Canada), was purchased at Safeway Canada Inc. (Edmonton, Alberta, Canada), and poured into the five 1 L glass bottles. Next, the bottles were heated up to 32^{o}C in a water bath (model Haake F3, Fischer Scientific, Edmonton). One mL of a 1:20 dilution of rennet enzyme (strength 580 International Milk Clotting Units mL^{-1}), 92.0% pure, acquired from Renco Inc. (Eltham, New Zealand), was added to each milk bottle, mixed thoroughly and kept at 32^{o}C for 45 min. The formed cheese curd was stirred thoroughly for 2 min and fresh whey, free of cheese curd particles, was separated with cheese cloth (Safeway Inc. brand, Edmonton, Alberta, Canada), and was filtered on the Koch HFK 131 ultrafiltration membrane. The whey permeate was poured into a set of four sterile 1 L glass bottles, and stored at 4^{o}C for 12 h. The protein in the resulting milk retentate and permeate was measured with a Coomasie Plus® Bradford Assay using BSA protein standards (Pierce Biotechnology, Inc., Rockford, Illinois, USA). The protein rejection value of the Koch membrane was

calculated according to the equation (8) which was described by Goulas *et al.* (2003) and explained in Chapter 1.

5.2.1.2 Membranes

The ultrafiltration and nanomembranes used in the process are described below. The Koch HFK 131 ultrafiltration membrane in a flat sheet configuration with a nominal molecular weight cut-off (MWCO) of 10,000 (made by Koch Membrane Systems Inc. (Wilmington, Massachusetts, USA), was purchased from Sterlitech Co. (Kent, Washington, USA).

The Koch TFC-SR3 nanomembrane (nominal pore size 200-400 MWCO), in a flat sheet configuration, was donated by the Koch Membrane Systems Inc. (Wilmington, Massachusetts, USA).

Before use, the new membranes were preconditioned by immersing in fresh double deionized water and held at room temperature for 24 h. Double deionized water was recycled through the membrane system after installing the preconditioned membrane at a flow rate set at 72 L h^{-1} with a membrane back pressure set at 0.97 MPa (140 psi). A new nanomembrane was applied each time for each trial of the repeated cross-flow filtration experiments.

5.2.1.3 Immobilized PVA biocatalyst

The soluble β-galactosidase (EC 3.2.1.23) used in experiments contains a commercial enzyme (Lactozym 3000 L HP G) derived from *Kluyveromyces lactis*, which had an activity higher than 3000 LAU mL^{-1} (Lactase Activity

Units). The biocatalyst was manufactured by Novozymes A/S, Bagsvaerd, Denmark. According to personal correspondence with the enzyme manufacturer, one LAU is the amount of commercial enzyme that can produce 1 μmol of glucose per minute under the following standard conditions: 4.7% lactose concentration, pH= 6.5, 30°C, 30 min, in milk. The soluble protein content in the commercial enzyme extract was 30.0 mg mL^{-1}.

Polyvinyl alcohol 17-99 and polyethylene glycol, both 99.8% pure, were donated by LentiKat's® a.s., Prague, Czech Republic. Soluble β-galactosidase was immobilized according to the steps outlined in the proprietary procedure which was provided by LentiKat's® a.s.. Ninety five milliliters of mixture, containing polyvinyl alcohol ((10% (w/v)) and polyethylene glycol (6% (w/v)) in double deionized water, was heated for 20 min to boiling point (98-98.5°C) and cooled down to 35°C. Next, 5 mL of the soluble enzyme was added into the mixture. The whole mixture was extruded on poly-carbon plates in the form of oval beads using the LentiKat's® printer, and dried down in an oven to a standardized mass as described in the company confidential method. Next, the beads were hydrated for 20-40 min in the stabilizing solution, 0.1M Na_2SO_4, and transferred into a 0.1M potassium phosphate buffer solution, pH 6.5, containing 2 mM of $MgCl_2$ and ethanol, 6% (v/v), and stored at 4°C.

5.2.2 Methods

5.2.2.1 Evaluation of biocatalyst activity during processing

The activity of the immobilized biocatalyst was assayed before its addition to the bioreactor, at the start of the whey lactose bioconversion and after the bioconversion of whey lactose (at 5 h). Twenty grams of biocatalyst was weighed and added into two vials with 10 mL of lactose solution at a concentration of 5% (w/v), and vigorously mixed in an orbital shaker for 10 min at 200 rpm. A 100 µL sample was withdrawn after 4 min and the reaction was terminated through the addition of 100 µL of TCA 25% (w/v). The lactose and its hydrolysis products, glucose and galactose, were measured by HPLC. It should be noted that in our experiments, one enzyme unit is defined as the amount of enzyme activity which releases 1 mmol of glucose per minute from a 5% solution of lactose (w/v) in a potassium phosphate buffer (pH 6.5) at 35°C. The specific activity of the biocatalyst (SAE) was calculated by dividing enzyme activity units by one gram of enzyme protein.

5.2.2.2 Evaluation of biocatalyst colour after oven drying

Biocatalyst bead samples (10 ± 0.1 g), were dried before and after use, for 12 h at a temperature set at 80 ± 1^{o}C. Then, the beads were cooled to room temperature and their CIE L* a* b* colour space values were evaluated using the Chroma meter (the CR-400/410 model, purchased from the Konica Minolta, Mississauga, Ontario, Canada). The calibration of the instrument was verified with the “white calibration standard” which was provided by the equipment

manufacturer and described by the following CIE L* a* b* space colour values: L* = 97.69, a* = -0.05, and b* = 1.92.

CIE L* a* b* colour space envisions a three dimensional space colour model with three axes which represent the lightness of the colour. When evaluated together CIE L* a* b* colour space values represent one particular standardized colour (Fairman *et al.* 1997). The L* value represents the light and dark axis, and is recognized as the average value of a spectral curve. The "0" value yields black colour and the "100" value yields white colour. The a* value represents the red and green axis while the b* value represents the yellow and blue axis. The positive a* value indicates that a measured colour is red while a negative a* value indicates that a measured color is green. On the other hand, the positive b* value indicates that the measured colour is yellow and a negative b* value indicates that a colour is blue.

5.2.2.3 Carbohydrate analysis

Carbohydrates concentration values were evaluated with HPLC method described in paragraph 2.3.2 of Chapter 2.

5.2.2.4 Whey treatment process description

Five liters of a 5% sweet whey lactose feedstock was pre-filtered through a membrane with a nominal molecular weight cut off (MWCO) of ≥ 10,000, to remove casein fines and whey proteins. Four liters of filtered whey permeate (containing lactose) was adjusted to pH 6.5 ±0.1 and was transfered into

the batch-stirred membrane bioreactor. Next, the whey lactose was hydrolyzed with the immobilized β-galactosidase into a glucose and galactose mixture at 35 ±1^{o}C. The mixture of lactose, glucose and galactose was crossflow filtered through the nanomembrane. The retentate stream was concentrated and separated from the permeate stream, which contained primarily glucose and galactose. Both the whey retentate and permeate streams were collected during 5 h bioconversion process, cooled and stored at 4 ±1^{o}C for analysis.

5.2.2.5 Whey permeate bioconversion and nanofiltration

The membrane bioreactor feed tank was drained of water and filled with 4 L of whey lactose solution (5.1 % (w/v)). Next, the feed solution was recycled through the whole system and back to the feed tank at a constant feed flow rate of 72 L h^{-1} set at the Hydra-Cell pump with the nanomembrane back pressure set at ambient pressure. The whey lactose feed solution, temperature adjusted to 35 ±1^{o}C, and 400 g of immobilized biocatalyst were added directly into the bioreactor tank and mixed at 100 rpm. It should be noted that to avoid plugging the proportional integral derivative (PID) mass flow controller loop, the immobilized biocatalyst was retained in the feed tank containing the stainless steel porous screen with 0.5 mm holes, while the whey lactose (feed) solution was recycled in the membrane bioreactor. After 30 min of feed recycling, the whey lactose solution was filtered through the nanomembrane for 5 h with a back pressure set to 2.06 ± 0.01 MPa (300 psi).

The retentate solution was recycled into the feed tank after its separation from the permeate solution with a constant flow rate of 72 L h^{-1}, at 35 ±1^{o}C. The permeate solution volume was collected in a graduated cylinder and its volume was noted and mass measured on the digital balance, during and at the end of the CFF process. The permeate volume, weighed mass and the back pressure, were all recorded at the start, and every 30 min during the CFF nanomembrane process. The protein in the whey retentate and permeate was measured with the Coomasie Plus® Bradford Assay as described earlier in the paragraph 5.2.1.1. The cleaning in place process of the stirred membrane bioreactor system was completed after each experiment according to the procedure described earlier in paragraph 3.2.2.2 in Chapter 3.

5.2.2.6 Mass balance and summary flow sheet

Knowledge about mass balance equations specific to the planned process was necessary to properly optimize the kinetic reaction rates and the flow rates of the substrates and products. The process mass balance and flow diagram for the whey lactose bioconversion process was evaluated according to the following reaction:

$$H_2O + \text{Lactose} \xrightarrow{\beta\text{-Galactosidase}} \text{Glucose} + \text{Galactose}$$

The flow diagram for the whey lactose bioconversion process is shown in **Figure 5.1**.

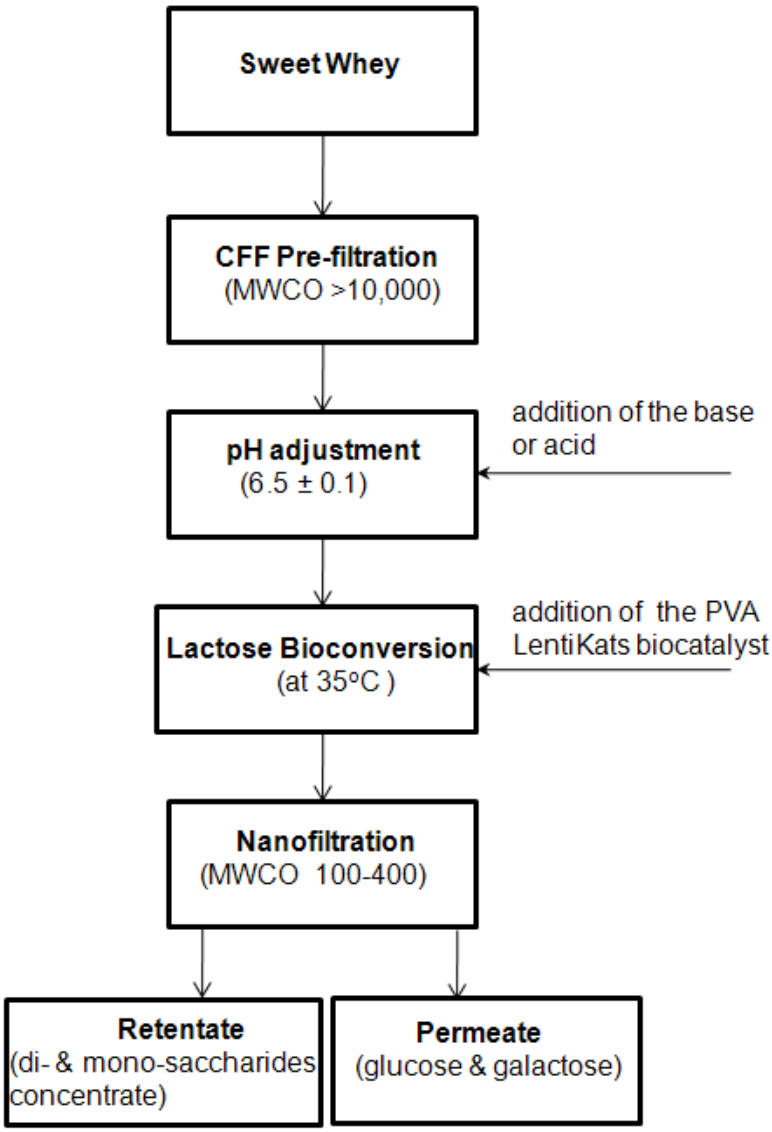

Figure 5.1 The process flow diagram for the bioconversion of the whey lactose with the polyvinyl alcohol (PVA) LentiKats in the batch-stirred nanomembrane bioreactor.

5.2.3 Equipment

The major equipment components for the small batch-stirred nanomembrane bioreactor system developed are shown in **Figure 5.2**.

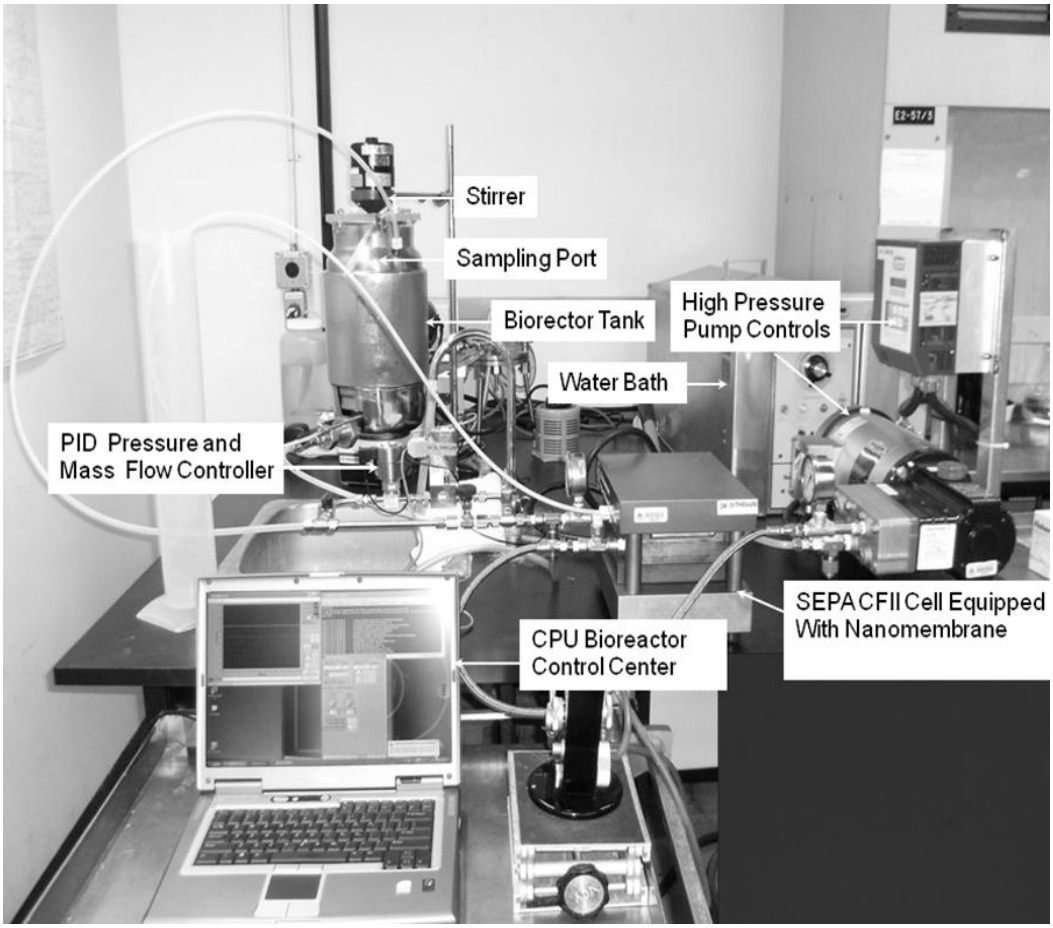

Figure 5.2 The batch-stirred nanomembrane bioreactor for the bioconversion of lactose.

5.2.3.1 Internal temperature probe

A platinum "two-wire" Pt-100 probe was used for measuring the feed temperature in the bioreactor tank. The resistance of this probe (R) demonstrated a linear correlation to the indicated temperature (T) in the range from 0 to 100^{o}C according to the following equation:

$$R = 100 + 0.385\ T \qquad (17)$$

The probe was calibrated through immersion in the temperature-controlled water bath. Its output was compared to the water temperature, which was displayed in the water bath (model Haake F3, Fischer Scientific, Edmonton). For data

acquisition and display, the platinum "two-wire" Pt-100 probe was connected to the Fluke 701 process calibrator (Fluke Electronics Canada Inc., Mississauga, Ontario, Canada). The temperature of the feed in the bioreactor tank was adjusted manually.

5.2.3.2 Pressure gauges, feed temperature and mixing speed

Two analog pressure gauges (NoShok Inc., Berea, Ohio, USA) were installed in the feed inlet and retentate outlet line and were used for pressure measurement in the crossflow process. In addition, the PID digital pressure and mass flow controller valve, purchased from the Bronkhorst High-Tech BV (Nijverheidsstraat, AK Ruurlo, Netherlands), were used to achieve automatic control of the back pressure in the nanomembrane unit. All gauges were calibrated on line with the pressure calibrator purchased from the VWR (Toronto, Ontario, Canada) before the start of the bioconversion process. The feed temperature and stirrer mixing speed in the bioreactor tank was adjusted manually with available controls and set up at 35 ±1 ^{o}C and 100 rpm, respectively.

5.2.3.3 Water volumetric flux and volume concentration ratio

The volumetric flux of water was used to check the integrity of the nanomembrane before processing. The new nanomembrane was preconditioned by immersion at room temperature for 24 h in double-deionized water. Then, the membrane was installed with an effective flat membrane filtration area of 140 cm^2 in the nanomembrane unit. Then, the double-deionized water (at room

temperature) was recycled with the pump volumetric flow rate set at: 36, 72, and 144 L h^{-1}. The back pressure value of the feed solution was set with a manual needle valve at 0.68; 1.38; 2.07; 2.76 and 3.44 MPa and was controlled by a PID mass/flow controller interfaced via RS232 with a computer. The volumetric flux for each nanomembrane was measured after 15 min, counted from the start-up time of the crossflow filtration process on the membrane, in order to ensure that the installed nanomembranes were without defect. This procedure was adopted as a standard for each experiment.

The volumetric flux for the whey permeate lactose was evaluated during the bioconversion and crossflow filtration processes. Process parameters such as temperature, pressure and flow rates were set up according to the methodology reported earlier in paragraph 3.2.2.3 in Chapter 3. It should be noted that the volumetric permeate flux for whey was measured after 30 min and counted from the start-up time of the crossflow filtration process on the nanomembrane. This allowed some time for the biocatalyst to begin to degrade lactose. The data on the volumetric flux of water and whey, and on the volume concentration ratio (VCR), the yield of monosaccharides were evaluated by applying mathematical equations 6, 7 and 9 adopted from Goulas *et al.* (2003) which were shown in the subchapter 1.4.4 in Chapter 1.

5.2.4 Statistical Analysis

All tests were performed in triplicate, the data were averaged, and where applicable they were evaluated with the linear regression and Student's t-tests

using Microsoft Excel 2003 SP 2.0, Microsoft Co. (Redmond, Washington, USA) and Statistica, version 5.0A software, Stat Soft Inc.(Tulsa, Oklahoma, USA).

5.3 RESULTS AND DISCUSSION

5.3.1 Whey permeate lactose bioconversion

The produced whey permeate, lactose concentration of 5.10 ± 0.1% (w/v), pH 6.5 and free of milk fat and protein, was bioconverted with the β-galactosidase immobilized on the PVA biocatalyst (PVA LentiKat's®). **Figure 5.3** shows the results of the whey permeate lactose bioconversion with the β-galactosidase immobilized on the PVA in the nanomembrane bioreactor, which was equipped with the Koch TFC-SR3 nanomembrane**.**

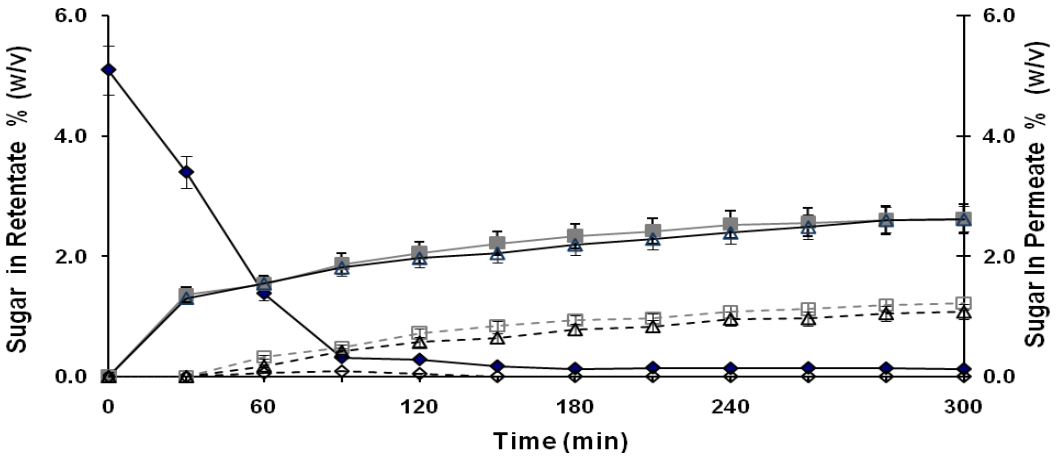

Figure 5.3 Whey lactose, 5.1% (w/v), bioconversion and nanofiltration in the batch-stirred membrane batch reactor with the immobilized biocatalyst (the PVA LentiKat's®) at 35°C, pH 6.5. Concentration of rection products in retentate: (lactose (—◆—), glucose (—■—), and galactose (—△—), and in permeate (lactose (- ◈ -), glucose (- ⊟ -) and galactose (- ▲ -). Triplicate standard deviation for permeate values are smaller than icons.

The evaluation of the HPLC results for retentate and permeate streams revealed that there was rapid degradation of lactose. Whereas, the concentration of glucose and galactose in permeate increased gradually during bioconversion and crossflow filtration process of lactose. Small amounts of lactose crossed initially from the retentate to permeate at the start of the crossflow filtration. But, the measured average value of lactose in the total volume of permeate was at 0.02% (w/v). Knowledge about possible contamination of permeate with lactose could be of significance when attempting to use this method in the production of lactose free whey beverages or applying it towards selective purification of carbohydrates (Sjöman *et al.* 2007; Abd EL-Khair 2009).

It was also determined that the amount of assayed glucose in permeate was higher than that of galactose. This measurement was in agreement with the results obtained in our earlier bioconversion trials involving experiments with carbohydrate mixture solutions including lactose and reported in Chapter 4. Sjöman *et al.* (2007) who reported on the process of separating glucose from xylose with nanomembranes, indicated that their method could be an effective and simple step to enhance the separation and purification of monosaccharides. However, they indicated that the selection of appropriate nanomembranes and crossflow control of the back pressure was necessary to optimize this process.

The evaluated specific activity of the immobilized biocatalyst during 5 h of processing declined on average from 192.3 ± 6.2 mmol min^{-1} g^{-1} to 185.3 ± 3.36 mmol min^{-1} g^{-1}, but this decline was not significant ($P > 0.05$). However, it was observed that the immobilized PVA LentiKat's® biocatalyst was inactivated

after its use in storage (24 hours in the fridge at 4^{o}C). Therefore, a new batch of the biocatalyst was used for the bioconversion of whey permeate lactose in each experiment. One possible cause of the observed inactivation of the immobilized PVA LentiKat's® biocatalyst after processing is its reaction with entrapped carbohydrates within its hydrogel matrix **(**Pinto *et al.* 1995). Mahoney (1997) noted that the presence of galactose affected the β-galactosidase activity during bioconversion of lactose.

Figure 5.4 shows two batches with the biocatalyst, before use (fresh beads) and after use (used beads) and after drying in the oven. It should be noted that the batch of

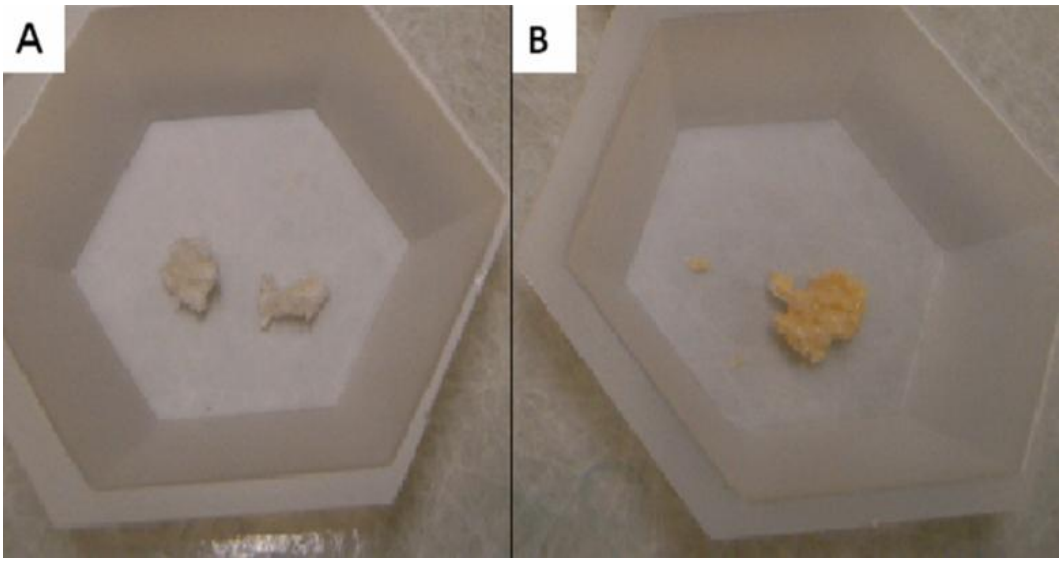

Figure 5.4 Biocatalyst fresh and used after drying for 12 h in the oven at 80^{o}C: A) average CIE L* a* b* values for fresh beads (L* = 97.13, a* = -0.41, and b* = 6.01) and B) average CIE L* a* b* values for used beads after bioconversion of whey permeate lactose (L* = 92.97, a* = 0.10, and b* = 18.41).

used beads was characterized by the CIE L* a* b* colour space average values of L* = 92.97, a* = 0.10, and b* = 18.41. These average values were different from

the colour space values for the fresh beads, which were L* = 97.13, a* = -0.41, and b* = 6.01. The calculated difference between the colour space average values for the fresh beads samples and the standard plate average values (ΔL* = - 0.56, Δa*= - 0.36, and Δb* = 4.09) showed that those samples were characterized by the light dark, light green and light yellow colour space attributes. In contrast, the calculated difference between the colour space average values for the used beads and the standard plate average values (ΔL* = - 4.72, Δa*= 0.15, and Δb* = 16.49) indicated that the used beads were characterized by the dark, light red and dark yellow colour space attributes. It is known that carbohydrates when dried at high temperatures commonly take on a dark yellow colour due to caramelization or oxidative browning reactions (Mahoney, 1997). Therefore, it could be argued that this test demonstrated that glucose and galactose are partially retained by the biocatalyst beads during the lactose bioconversion.

5.3.2 Water and whey lactose volumetric flux

The calculated volumetric flux of water revealed a linear correlation ($R^2 > 0.99$) with the increasing test pressure values (**Figure 5.5**). The average water volumetric flux values were similar to those reported by Koch TFC-SR3 nanomembrane manufacturer. The optimal flow rate and pressure parameters were used as a guide for this nanomembrane selection and used in the whey lactose bioconversion process. Similarly, Pontié *et al.* (2008) used a water volumetric flux test to verify the permeability of their selected nanomembranes.

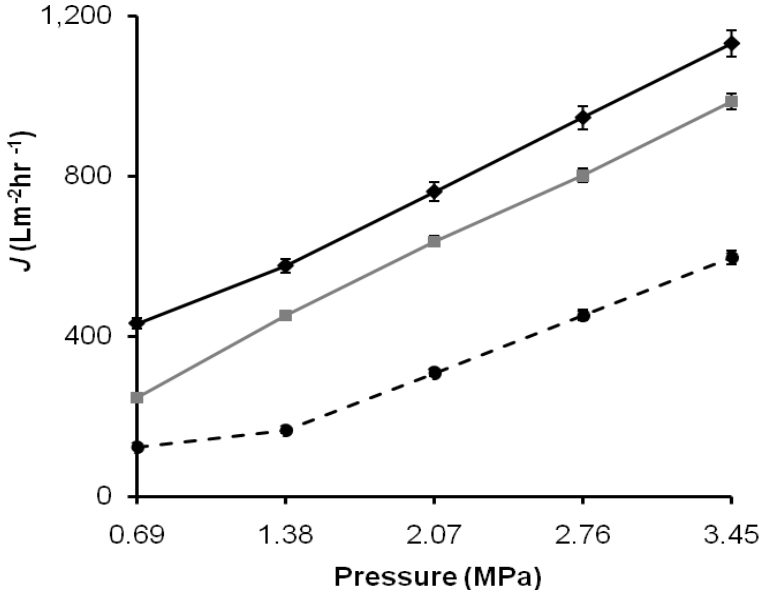

Figure 5.5 Correlation between the volumetric water flux test and pressure on the Koch TFC-SR3 nanomembrane. Tests carried out at different water flow rates: – ● – 36 L hr^{-1}, —■— 72 L hr^{-1}, and —◆— 144 L hr^{-1}. Error bars show standard deviation values based on triplicate tests.

The authors indicated that the increase in the water volumetric flux value was accompanied by an increase in the retention of salt solutes on the nanomembranes. They also noted that the diffusive flux of the solute was negligible at the high water volumetric flux values.

Figure 5.6 shows the average volumetric flux values measured during whey lactose bioconversion and crossflow filtration in the membrane bioreactor. The average volumetric flux declined gradually as the concentration of carbohydrates across the Koch TFC-SR3 nanomembrane increased during 5 h test.

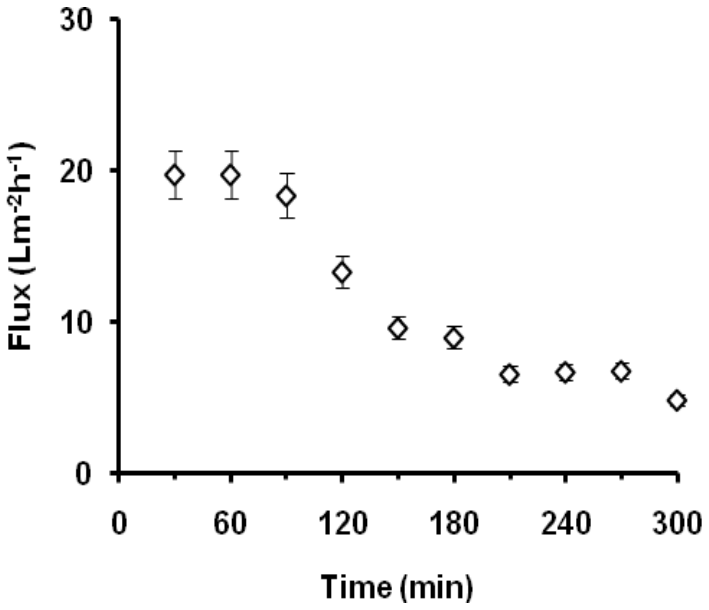

Figure 5.6 Bioconverted whey lactose volumetric flux rate values during CFF (◇) on the Koch TFC-SR3 nanomembrane. Error bars show standard deviation values based on triplicate tests.

Goulas *et al.* (2003) demonstrated the potential for nanofiltration membranes used in the separation of di- and oligosaccharides from monosaccharides. In addition, the authors indicated a lack of irreversible fouling during the crossflow process. They noted that the permeate flux was dependent on the concentration of carbohydrates in the solution during the nanomembrane filtration process and affected by the polarization layer generated at the membrane's surface.

In this experiment, it was observed that the permeate flow across the membrane declined during the bioconversion of lactose. An adjustment of the back pressure of the feed to 2.06 MPa (300 psi) was required at the start of the crossflow filtration process to obtain crossflow filtration of carbohydrates. The

calculated volume concentration ratio (VCR), the volume of initial feed divided by the volume of retentate after 5 h of filtration was at 1.2 at the end of filtration run.

5.3.3 Bioreactor Controls

All nanomembrane bioreactor controls (temperature, the PID digital pressure and mass flow controller valve) worked without malfunction. The temperature measured with the platinum "two wire" Pt-100 probe showed no variation during the processing of whey permeate lactose (data not shown). The use of the PID and mass flow controller valve showed a lack of rapid fluctuations in back pressure and only a small but steady increase in the back pressure during 5 h of lactose bioconversion and crossflow processing. However, it was noted that the PVA biocatalyst agglomerated during the bioconversion process on the walls of the stainless steel strainer at a stirrer speed of 100 rpm, which could affect the mass transfer of carbohydrates to the biocatalyst and slow down the bioconversion process of lactose.

5.4 Conclusions

This study demonstrates that the bioconversion of the lactose to monosaccharides with a novel immobilized biocatalyst in the batch-stirred nanomembrane bioreactor equipped with the cross-flow filtration nanomembrane is possible. It offers some advantages such as: the fast lactose degradation and separation of lactose during processing. Therefore, offers opportunity for

standardization and formulation of new feedstock streams containing lactose, and their fractionation. In this research, the applied analytical tests and the bioreactor control methods were used to monitor the whey lactose bioconversion process.

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6. OVERALL DISCUSSION AND SUGGESTIONS FOR FURTHER RESEARCH

6.1 OVERALL DISCUSSION

The goal of this thesis was to develop methodologies for: 1) the evaluation of milk carbohydrates during bioconversion and the separation of carbohydrates by crossflow filtration (CFF) through nanomembranes, 2) the characterization of nanomembranes, 3) the evaluation of a method for the production of a stable immobilized biocatalyst applicable to the lactose degradation process, and 4) the development of the stirred nanomembrane bioreactor used for the bioconversion of carbohydrate feedstocks (e.g. whey lactose bioconversion). Furthermore, this research has the potential to enhance the design and development of the fed-batch or continuous membrane bioreactor. Such a unique bioconversion technological platform would be flexible and robust, and would accommodate different types of carbohydrate feedstocks.

Several of the literature publications offer details on the lactose bioconversion process. The recurring theme in all of these studies is the development of effective bioconversion methods which focus on: 1) different measurement methods for carbohydrates **(**Lanza and Li 1984; Kearsley 1985; Zaitoun 2006), 2) different membrane bioreactor models (Curcio *et al.* 2006; Jurado *et al.* 2006; Li *et al.* 2008), 3) methods for the evaluation of the transport mechanism of carbohydrates on membranes (Jeantet *et al.* 2000; Goulas *et al.* 2003; Sjöman *et al.* 2007), 4) models for cost analysis of developed methods

(Mahoney 1997, Bury and Jelen 2000), and 5) new methods for the immobilization of enzymes (Grosová *et al.* 2008; Illanes *et al.* 1990). However, in the literature, there is a lack of detailed information that would demonstrate methods for the rapid measurement of lactose, glucose and galactose in their solutions, or the separation of lactose from glucose and galactose on nanomembranes. Therefore, in this dissertation project new methods were designed, developed and tested. These offer new solutions and the capacity to improve the bioconversion of lactose in small batch-stirred membrane bioreactors.

Overall, the information described in this thesis is new and in its scope offers several new methods applicable to the bioconversion of lactose into glucose and galactose. The major methods described in this research are: 1) a new polarimetric method for the assay of carbohydrates during the bioconversion of lactose, 2) a method for the separation of monosaccharides from disaccharides on nanomembranes, 3) a method for the bioconversion of lactose with β-galactosidase immobilized on the PVA hydrogel, and 4) a method which demonstrates feasibility of using the batch-stirred membrane bioreactor for lactose bioconversion to simple carbohydrates.

Food manufacturers, including those in the dairy and beverage industries, search for low-cost methods to assay carbohydrates in raw materials, process ingredients, and finished products (Mahoney 1997; Williams 2001). The desired method of choice should be accurate, rugged and simple to use. In addition, such an assay should perform a quick (on line) measurement of carbohydrates.

The modified polarimetric method which was developed and tested in our research lab addresses all of the requirements noted above. Chapter 2 describes this new method, which incorporates the lactose hydrolysis model generated with the polynomial equation available in the Design Expert®, DOE 7.1.6 software. The method could be used as an alternative to other time consuming and more expensive methods and would facilitate the accurate evaluation of carbohydrates in feeds during lactose reduction in milk. The possible use of this polynomial equation to evaluate analytical components in complex mixtures (eg. ternary mixtures) was indicated earlier by Scheffe (1958). However, a practical application, which demonstrates its use for the evaluation of carbohydrates, as is described in this research has not yet been published.

The polarimetric method offers an opportunity for the automation of the measurement process and the on-line optimization of lactose bioconversion process. It has a limitation however, when using this method to evaluate the bioconversion of lactose, it is necessary to adjust the input of the initial lactose, glucose and galactose and galacto-oligosaccharides (GOS) concentration values in the DOE model. This however can be counterbalanced by a rapid measurement process and a relatively low cost of the measuring equipment and the DOE software. For example, some improvements for calibration of analytical methods are suggested by Bro (2003). Bro (2003) reviewed the use of univariate and multivariate calibration techniques that led him to propose the design of a successful calibration model suitable for some analytical methods. The author indicated that to achieve the high accuracy of working models needed in multi-

component evaluation, the following two criteria are required: 1) a high selectivity of the specific analytical component (only the analyte of interest contributes to the measured signal) and 2) a high linear correlation between the analyte concentration and the instrument signal.

Furthermore, during the evaluation of the new modified polarimetric method the kinetic characterization of enzymatic lactose hydrolysis was conducted with 1) a simple Michaelis-Menten model, 2) a Michaelis-Menten model with competitive product inhibition by galactose, and 3) a more complex model that considers the formation of di- and tri-saccharides, adapted from Cupples *et al.* (1990). As an outcome of this evaluation, the predicted values for the kinetic parameters agreed with the values reported by Jurado *et al.* (2002). However, the experimental data reported in our research did not provide evidence of inhibition by galactose.

To summarize: in Chapter 2, a new rapid polarimetric method was developed and used to measure the bioconversion of lactose to glucose and galactose in the bioreactor. The data generated using this method were also successfully used to evaluate the kinetics of the GOS formation.

Chapter 3 describes the results of the separation of lactose, glucose and galactose on nanomembranes in the crossflow filtration (CFF) process. In this research, feedstocks, containing different concentrations of carbohydrates, were filtered through two new nanomembranes for an extended time (5 h). The test results demonstrated that the carbohydrate separation experiments, conducted on concentrated mixed feedstocks containing lactose, glucose and galactose, as well

as with bioconverted whey, showed similar rejection values to those reported earlier (Goulas *et al.* 2003; Sjöman *et al.* 2007).

In addition, this research found, that the Koch TFC-SR3 nanomembrane offered a higher average rejection value for lactose than the Filmtec NF270-400 nanomembrane, and a slightly lower rejection value for glucose and galactose. It was also observed during crossflow experiments that the amount of carbohydrates increased in the permeate and in the retentate feeds whereas the volumetric flux declined. Overall, the disaccharide lactose was rejected on nanomembranes at a higher rejection value (91-98%) than that of glucose and galactose (71-89%), whereas the volume concentration ratio of retentate (VCR) increased from 1.0 to approximately 2.5.

The selection of an appropriate nanomembrane is necessary to achieve the most efficient separation of selected carbohydrates by use of the CFF method; some commercial nanomembranes differ in the way that they separate carbohydrates. Therefore, before their application in the membrane bioreactor, to show that the separation of carbohydrate-rich feedstocks is feasible, some trial tests are needed (Aydogan *et al.* 1998; Goulas *et al.* 2003; Sjöman *et al.* 2007). Aydogan *et al.* (1998) found that separation of the binary mixtures of sucrose into singular component feeds with nanomembranes is possible. Goulas *et al.* (2003) used their test set up to show the potential of thin film composite nanomembranes for the separation of oligosaccharides from a mixture of mono- and disaccharides. However, in their tests they used the dead-end filtration system instead of the crossflow one. Sjöman *et al.* (2007) used a crossflow filter and concluded that the

application of nanomembranes to the separation of monosaccharides like xylose and glucose from disaccharides (lactose) is possible. One of the nanomembranes they used for testing was the Filmtec NF 270 membrane. Their results, for this particular nanomembrane, agreed with ours and showed that the lactose rejection on this nanomembrane was at 97%. Yet, they did not show results which would demonstrate the cross-flow of carbohydrates over an extended period of time.

Sjöman *et al.* (2007) also reported that the concentration of the feed solution had only a minor effect on the rejection values of monosaccharides. They explained that carbohydrates in water solutions are charge-neutral molecules and suggested that the transport of carbohydrates through membranes depends on their molecule structure and nanomembrane type. The authors also suggested that the increase in permeate flux was a major factor contributing to the rejection of monosaccharides on nanomembranes. In addition, they elaborated that the convection process was a major factor contributing to the separation mechanism. It was also noted that changes in the nanomembrane structure were due to several other factors such as: swelling, caking, compacting, filtration temperature, pressure and pH. They also used different feedstocks, specialized nanomembranes and their own system configurations to achieve their own research objectives. Furthermore, their test methods for the evaluation of nanomembranes showed that their membrane separation parameters, i.e. the volumetric flux and solutes rejection values, varied. Reasons for the observed variations were unclear.

Koyuncu *et al.* (2004) reported that the volumetric flux (permeate flux) was affected by several factors including: feed pressure, temperature, crossflow velocity, and feed composition. They presented a few equations that could be used to evaluate permeate fluxes subject to the type of fouling occurring during the crossflow filtration process and they used a volumetric water flux test method to evaluate and compare nanomembranes before their evaluation during the filtration of mixed solutions. In this test method, the known water volume is crossflow filtered across the nanomembrane and the volume of permeate (filtrate) is measured at a specific time interval and at assigned pressure and temperature conditions. Commonly, a high volumetric flux indicates that a selected membrane is characterized by high porosity, permeability and a lack of restricting components, such as scale or cake. This method was adopted and used in this research project to evaluate the permeability of nanomembranes. It was found that the high water flux were measured for the Koch TFC-SR3 *versus* Filmtec NF270-400 nanomembrane, which indicated that the Koch nanomembrane's porosity and permeability was higher than those of the Filmtec nanomembrane's. These results were confirmed through the material analysis of nanomembranes using the TEM microscopy method.

Tang *et al.* (2006) indicated that an understanding of the physicochemical structure of nanomembranes was necessary for their selection before their application to the crossflow filtration process. Therefore, in this research, in addition to the transmission electron microscopy method, which was used for the physical evaluation of nanomembranes, the modified Fourier Transform Infrared

method (FTIR) was applied to evaluate the nanomembranes' chemical composition (Freger *et al.* 2002; Cricenti *et al.* 2006).

Freger *et al.* (2002) found that the polyamide compound, known as "Amide I", was a major component in the active layer of the nanomembrane and was characterized by a small peak at 1650 cm^{-1} wavelengths. This peak was separable from the group of peaks corresponding to the polysulphone compound in the support layer at 1487, 1503 and 1584 cm^{-1} wavelengths, which was found both in the Filmtec® NF270-400 and Koch® TFC-SR3 nanomembranes surface layer.

The PID mass flow controller was also successfully used as a preventive guard against rapid jumps in the back pressure of the CFF membrane unit. It was found that the control of the back pressure is critical to the nanomembrane's physical stability. It was shown in this research that nanomembranes' pores are prone to stretching and tearing due to the pressure applied during crossflow filtration of carbohydrates.

In Chapter 3, a comparative evaluation of two commercial nanomembranes showed that the Koch TFC-SR3 nanomembrane offered a higher average rejection value for lactose and a higher permeability than the Filmtec NF270-400 nanomembrane and a slightly lower rejection value for glucose and galactose. It was also observed that as the amount of carbohydrates increased in permeate and in their retentate feeds, the volumetric flux decreased, and the flux decline was higher for the Filmtec NF270-400 nanomembrane.

In Chapter 4, the immobilized biocatalyst activity results were demonstrated for a new commercial biocatalyst (β-galactosidase immobilized in the PVA LentiKat's® matrix), which was acquired from the biotechnological company in the Czech Republic. It was found that the biocatalyst PVA LentiKat's® was characterized by high stability and high specific activity. Park (1993) reported a similar high specific activity of β-galactosidase when using his entrapment method. The author used a cross-linked poly (N-isopropylacrylamide-co-acrylamide) hydrogel that exhibited a lower critical solution temperature. It was demonstrated that the immobilized enzyme showed a specific activity comparable to that of soluble β-galactosidase. In addition, he hypothesized that mass transfer rates of "substrate in" and "product out" of the immobilized enzyme may have been changed with the adjustment of the temperature-dependent gel-swelling behaviors. Park (1993) also suggested that an increased restriction of the soluble enzyme, due to its entrapment in the hydrogel matrix, was a major cause of its enhanced stability at higher temperatures. However, in his work, the author did not offer a detailed description of the source of the soluble β-galactosidase. Klibanov (1983) reviewed different immobilization methods and indicated that some entrapment methods for immobilized enzymes were affected by the leakage of enzymes from their entrapment supports. But this could not be confirmed for the PVA LentiKat's® matrix; our tests showed no leak of the enzyme with this novel immobilization method.

In Chapter 4, the results of our experiments revealed that this novel immobilized biocatalyst is an effective solution when used for the bioconversion

of lactose. It also demonstrated that this novel biocatalyst worked well when applied to the hydrolysis of lactose in the stirred bioreactor and to crossflow filtration on nanomembranes. Nonetheless, the results of this project demonstrated that some loss of activity of the biocatalyst could be expected because of its agglomeration and retention on the walls of the bioreactor during stirring.

In Chapter 5, the experimental results are described showing the bioconversion of whey lactose with a novel immobilized biocatalyst in the batch-stirred membrane bioreactor equipped with the Koch TFC SR3 crossflow nanomembrane in the flat configuration. The bioreactor described in this chapter includes: the biocatalyst (enzyme entrapped on the PVA LentiKat's® matrix), a stainless steel screen for the retention of the biocatalyst in the bioreactor tank, a cross-flow unit with the nanomembrane (nanomembrane that rejects lactose), and a PID pressure and mass flow controller for the automatic adjustment of the back pressure during the crossflow filtration. The experimental results revealed that lactose was quickly bioconverted with the PVA LentiKat's biocatalyst into glucose and galactose during 5 h of the experiment. The lactose bioconversion was almost complete, and only 0.13% (w/v) lactose was measured in the retentate after 5 h of the bioconversion. It was also found that the biocatalyst activity was not affected immediately at the end of experimental run. However, that activity decreased almost completely after the biocatalyst was stored at 4^{o}C. Finally, a small amount of lactose was measured as it leaked across the nanomembrane at the start of the crossflow filtration process. This leak was stopped after two hours.

The possible explanation for this leak was that the nanomembrane pores stretched and narrowed later, during the crossflow filtration. This narrowing effect may be caused by the polarization layer and soluble solids present in the recycled retentate. With an increase in the concentration of glucose and galactose in retentate, there was also a proportional increase in their concentration in permeate. This contributed to a decline in the volumetric flux in permeate during the cross-flow filtration process. A possible key factor to this decline was the presence of soluble solids in the form of salts which naturally occurred in the whey lactose feedstock.

To summarize Chapter 5, the experimental results revealed that the bioconversion of lactose in the batch-stirred membrane bioreactor offered nearly complete degradation of lactose with a simultaneous separation of glucose and galactose. It is also suggested that the increase in the concentration of these carbohydrates in retentate is a primary cause for the decline in activity of the used biocatalyst.

In all of the components of these research projects detailed in Chapters 2-5 the modified HPLC method was used to evaluate carbohydrate concentrations in feedstocks (Anonymous 2007). This adopted method was fast and permitted a quick measurement of mixed carbohydrate solutions containing lactose, glucose and galactose. No interference was observed between glucose and galactose peaks. Richmond *et al.* (1982) reported that some compounds (e.g. salts, fats, proteins, acids etc.) may affect the resolution of individual carbohydrates. They suggested using additional guard columns to improve the resolution of separated

components as a solution for extending the life of the column. In addition, they described that some HPLC methods may suffer due to the poor separation of individual carbohydrates, and they indicated that the choice of an adequate HPLC method for separation and quantification of mixed solutions of glucose, galactose and lactose is important. This method was also used for the evaluation of new methods such as polarimetry, crossflow filtration of carbohydrates, and used to study the reaction kinetics of soluble and immobilized β-galactosidase.

Overall, the research clearly demonstrated that new methods developed and described offer a good alternative to presently used methods and provides an opportunity for the efficient bioconversion and evaluation of lactose. The methods described here are useful to evaluate the following:

- the bioconversion of lactose in the batch, fed-batch or continuous nanomembrane bioreactor;
- the standardization of concentration for lactose, glucose and galactose feeds in filtered permeate;
- the purification of lactose, glucose and galactose feeds;
- the development of a new categories of adjuncts or food products (beverages);
- the bioconversion of other complex carbohydrates.

Further investigation, improvement and optimization of methods described in this project is possible by developing application-oriented models. This approach could lead to the development of a more efficient stirred membrane

bioreactor for the bioconversion of carbohydrates in a fed-batch or a continuous configuration.

6.2 SUGGESTIONS FOR FURTHER RESEARCH

It was noted during this dissertation project that there are some key issues that needed more research such as:

1. Testing of the suitability of the polarimetric method to measure carbohydrates in concentrated whey lactose permeates and non-dairy feeds.

It is not clear yet if the polarimetric method, which was discussed in Chapter 2, would be of use when monitoring the bioconversion of lactose at high concentrations. Therefore, experiments with more concentrated whey lactose feeds are required to clarify this issue. In addition, upgrading the polarimetric instrument used in this research from the single-wavelength polarimeter to a new state-of-the-art multi-wavelength polarimeter could very likely improve measurement accuracy and enable the evaluation of non-dairy carbohydrate solutions and feedstocks (Rudolf Research Analytical 2006).

2. Evaluate nanomembranes during the crossflow filtration of the concentrated carbohydrates feedstocks.

In this research, the nanomembranes tests were carried out for feedstock solutions containing lactose concentrations similar to those of milk or whey. The processing of feedstocks with increased concentration of carbohydrates could

result in volumetric flux and rejection characteristic, which are different from these obtained during this research. Therefore, the ability to process feedstocks containing higher concentrations of lactose or feeds with different carbohydrates has yet to be verified.

3. Verify the applicability of nanomembranes in the spiral wound configuration for their possible use in the bioreactor scale up.

During the crossflow filtration of whey, the volumetric flux declined rapidly. One of the major reasons contributing to the flux decline was a small surface area of the tested nanomembrane. The applied flat nanomembrane crossflow configuration is perfectly suitable for testing characteristic of feedstocks during crossflow filtration in short time applications. However, for a nanomembrane bioreactor accommodating a larger volume of bioconverted carbohydrate feedstocks, a spiral wound nanomembrane configuration is recommended (**Figure 6.1).**

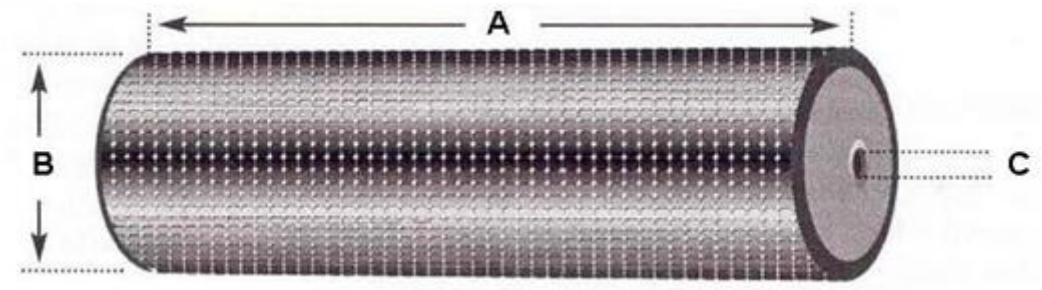

Figure 6.1 Koch KMS SR3 nanofilter (spiral wound element:Model # 8383808 3838 SR3-NYV ((A) 38.0 inches (965 mm), B) 3.8 inches (96.0 mm), and C 0.83 inches (21.1 mm)), 200 MWCO, and 7.1 m^2 active surface area, adapted from www.kochmembrane.com.

In the author's opinion the test of this nanomembrane configuration is a desirable option for a future membrane bioreactor scale up.

4. Evaluate the long-term stability of the PVA immobilized β-galactosidase.

Importantly, the long-term stability of β-galactosidase immobilized on the PVA needs verification when used in the fed-batch or continuous lactose bioconversion process. In this research it was found that the used PVA immobilized enzyme lost its activity in refrigerated storage. Therefore, it is suggested that in the future, research projects should apply the washing procedure with a phosphate buffer solution, at a biocatalyst optimum pH. This may remove carbohydrate residue retained in the PVA biocatalyst matrix and extend the biocatalyst's long-term activity.

5. Improve and test the nanomembrane bioreactor controls and apply them in the fed-batch or continuous configuration.

In order to scale up the nanomembrane bioreactor, or to modify it to the fed batch or to the continuous system, some additional design and optimization of the control instruments are necessary. For example, one proposal would be to introduce an on-line central computer control system to gather and adjust control signals for measurements of: a) feed concentration, temperature and stirring rate at the bioreactor tank, b) pressure and flow rate of feed pumped from the bioreactor to the nanomembrane unit, and c) level controls in the bioreactor tank.

Sonnleitner (2006) in his review offered a new concept (a hierarchical bioprocess automation system) for process documentation which, when implemented, could provide a unique flexible platform for the on-line interface between simple control loops (e.g. pressure control, temperature control, flux control or flow control) and a digital supervisory data management system. He

described two types of on-line control sensors (continuous and discontinuous), which are used to control biochemical processes in the bioreactor. The first type involves sending the continuous signal feedback to the controller, whereas the second one generates a signal according to the pre-set time interval program. Jørgensen *et al.* (2006) explained a concept of multivariate process monitoring by the use of multiple measurements. The authors suggested using principal component analysis to evaluate measured variables captured from the bioreactor. In addition, they offered an equation which explains the principles of temperature control that is applicable to the continuous bioreactor:

$$V \rho C_p \, dT(t)/dt = v \rho C_p(T_{in}(t) - T(t)) + Q(t) \qquad (18)$$

where: V is the tank volume, ρ is the liquid density, C_p is the constant liquid heat capacity, $dT(t)$ is an increase in the liquid temperature in the bioreactor tank within time interval t, v is the volumetric flow rate, T_{in} is "the disturbance" inlet liquid temperature in the tank, and $Q(t)$ is the heat input to the stirred tank.

Similarly, they indicated that the knowledge about monitoring systems in bioreactor is required to properly optimize the kinetic reaction rates and the flow rates that are needed for efficient use of the bioreactor.

6. <u>Design and develop a virtual bioreactor model which would be able to automatically evaluate the efficiency of the membrane bioreactor</u>.

In future research it is recommended that developing a virtual working model would enable verification of a conceptual hypothesis about the correctness of the design. It is suggested that this model will aid in the developmental process and enhance the optimization of the membrane bioreactor design (Hoeben *et al.*

2006). In their review, the authors offered guidance applicable to biotechnological process design and optimization. They described some software tools, such as the Super-Pro-Design (www.intelligen.com) and their proprietary spreadsheet program in Excel (www.microsoft.com), which they used for the evaluation of the plant development process in the biotechnological industry.

7. Develop and formulate new value-added product categories.

The developed batch nanomembrane bioreactor system could be analyzed and tested for its potential use as a fermentable adjunct material and for its prebiotic potential in *in vitro* studies. The promising adjuncts and prebiotics would then be applied in the production of new fermented foods and beverages. A scale-up pilot production and comparative analysis of these new products would be conducted to evaluate their value as liquid adjuncts, sweeteners and prebiotics in comparison to currently available liquid and solid adjuncts. Their properties would be documented and made available to research affiliates and sponsors. The final step would be the commercialization of new technology and newly developed products. Some opportunities for the bioconversion of whey lactose to value-added products were discussed in Chapter 1. Earlier in this dissertation it was noted that the demand for the carbohydrates and the price paid for their adjuncts is increasing annually (Urlich 2009). Williams (2001) described new possible trends for beverages developed with carbohydrate adjuncts. The authors indicated that there is a growing demand for alternative non-alcoholic beverages, fermented and other functional beverages which contain carbohydrates in their base. This forces manufacturers to seek alternative carbohydrate sources

as the availability from the traditional plant sources shrinks and price escalates. Another possible value-added option is the cost effective bioconversion of whey lactose to ethanol as a fuel additive. Ling (2008) described the current status of the bioconversion of whey lactose to ethanol in the USA. He indicated some existing opportunities in this market for high ethanol yielding technologies.

8. Develop a material and process cost analysis to justify economic viability of nanomembrane bioreactor when used for whey bioconversion to value added products.

There is still a need to develop a material and process cost analysis for scaled up nanomembrane bioreactor to justify the economic viability of the bioconversion of whey lactose. This analysis would demonstrate that the bioconversion of whey lactose leads to value-added carbohydrate adjunct material at competitive market price.

Overall, this research provides a platform for the development of a high volume nanomembrane bioreactor that will capture and bioconvert milk or whey lactose feedstock to simple carbohydrates and value-added adjuncts. The methods described in this dissertation are available for immediate application in the scale up membrane bioreactor. The full potential for the usage of hydrolyzed whey lactose in value-added products as a ready-to-use adjunct or prebiotic ingredient has yet to be researched. Moreover, their potential use as a fermentable substrate contributing to the various characteristics of foods in the dairy, brewing and beverage industries has still to be fully evaluated and documented.

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APPENDIX A. PERMITS OBTAINED FROM PUBLISHER

Re: **Table 1.1** shows average concentration of lactose (g/100g) in milk from various mammals, adapted from Scrimshaw and Murray (1988).

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PhD Candidate
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Edmonton, AB
Canada T6G 2P5

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Re**: Figure 1.6** shows Michaelis-Menten kinetics model for lactose hydrolysis, adapted from Mateo *et al*. (2004); **Figure 1.7** shows Mathematical equations describing Michaelis-Menten kinetics model, adapted from Mateo *et al*.(2004).

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This dissertation will be delivered both in print and in the electronic format.
Thank you for your help.

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Wojciech

Re: Figure **1.5a & 1.5b** shows the mechanism of bioconversion of lactose, adopted from Richmond e*t al.* (2004); **Figure 1.8** shows bioreactor types for plant cell, tissue and organ cultures, adapted from Sajc *et al.* (2000); **Figure 1.14** shows two types of the bioreactor systems (A and B) equipped with membranes, adapted from Charcosset (2006).

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The AFNS Department
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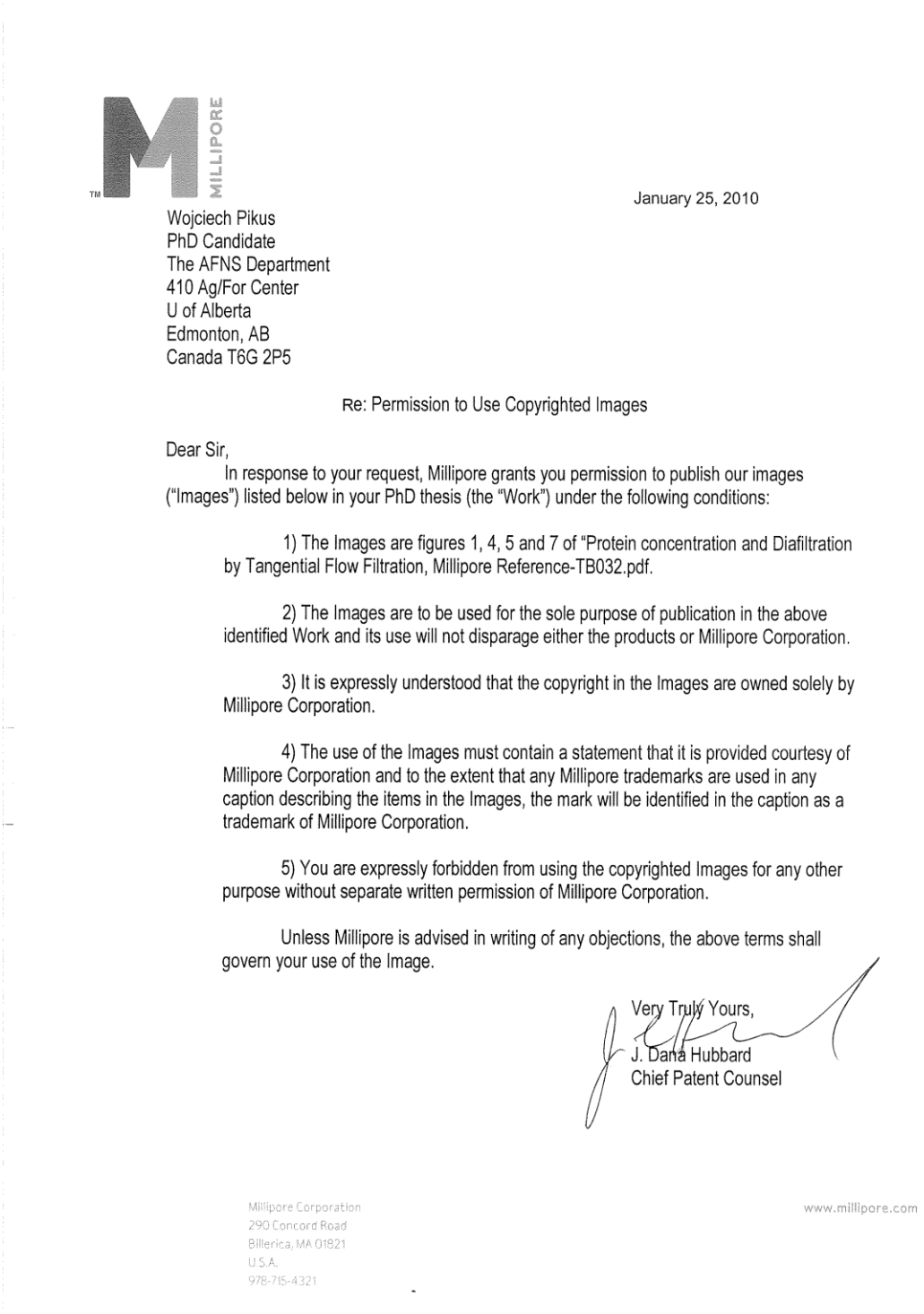
1. Richmond, M. L., Gray, J. I., and C. M. Stine. 1981. Beta-Galactosidase: Review of recent research related to technological application, Nutritional Concerns, and Immobilization. J. Dairy Sci. 64:1759-1771 (**Figure 1 & 2**)

2. Sajc, L., Grubsic, D., and G. Vunjak-Novakovic. 2000. Bioreactors for plant engineering: an outlook for further research. Biochem. Eng. J. 4, 2:89-99. (**Figure 1** shows bioreactor types for plant cell, tissue and organ cultures)

3. Charcosset, C. 2006. Membrane processes in biotechnology: Anoverview. Biotechnol. Adv. 24:482-492. (**Figure 1** shows comparison between dead end and cross-flow filtration configuration; **Figure 2** shows the membrane bioreactor configuration)

Re**: Figure 1.9** shows **c**omparison of filtration methods.**Figure 1.10** shows pressure distribution in the crossflow membrane feed channel; **Figure 1.12** shows A) Flat Plate, B) Hollow Fiber and C) Spiral-Wound crossflow filtration modules. All courtesy of Millipore Corporation, Anonymous (2003).



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Re: **Figure 1.11** shows schematic model of transport of a solute in the nanomembrane during the CFF process, adapted from Harrison *et al*. (2003).

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Best Regards,

Wojciech Pikus (PhD Candidate)
The AFNS Department
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Re: **Figure 6.1** shows Koch KMS SR3 nanofilter spiral wound element: Model # 8383808 3838 SR3-NYV, adapted from www.kochmembrane.com.

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