PRODUCTION OF GALACTO-OLIGOSACCHARIDES FROM LACTOSE BY IMMOBILIZED β-GALACTOSIDASE AND POSTERIOR CHROMATOGRAPHIC SEPARATION

DISSERTATION

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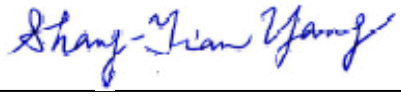

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ABSTRACT

Galacto-oligosaccharides (GOS) are non-digestible sugars containing two to five molecules of galactose and one molecule of glucose connected through glycosidic bonds. They are classified as prebiotic food because they can selectively stimulate the growth of bifidobacteria and lactobacilli in the lower intestine. The addition of GOS as a functional food ingredient has great potential to improve the quality of many foods. GOS can be produced from lactose, which is abundant in cheese whey, by enzymatic transgalactosylation with ß-galactosidase present in either free or immobilized form. The goal of this research was to evaluate the feasibility of using various microbial lactases immobilized on cotton cloth for GOS production from lactose and posterior purification by chromatographic technique using a commercial cation exchange resin Dowex 50W.

The production of GOS from lactose was first studied with lactases from *Aspergillus oryzae*, *Bacillus circulans*, and *Kluveromyces lactis.* The total amount, types, and size of GOS produced were affected by the enzyme type and the initial lactose concentration in the reaction media. In general, more GOS can be produced when the initial lactose concentration was higher. With 400 g/L of lactose solution, a maximum GOS content of 40% (w/w) was achieved with *B. circulans* lactase, followed by *K. lactis* with 31% GOS and *A. oryzae* with 27% GOS. The addition of galactose inhibited the enzyme, reducing both GOS yield and the reaction rate.

The method of immobilizing enzyme on cotton cloth via adsorption with the binding support of polyethyleneimine (PEI) and cross linking with glutaraldehyde (GA) was developed for ß-galactosidase from *B. circulans* and *A. oryzae*. A high enzyme loading of 250 mg/g support with 35% and 90% activity yield was achieved for *B. circulans* and *A. oryzae* lactase, respectively. Thermal stability of *B. circulans* and *A. oryzae* lactase increased by 12 and 25-fold, respectively, upon immobilization on the cotton cloth. The immobilized enzyme showed the same GOS formation kinetics as that of the free enzyme, indicating that there was no diffusion limitation in the catalytic cotton cloth for the reaction.

Continuous production of GOS with immobilized enzyme in packed-bed reactors was studied. A high volumetric productivity of 4300 g/L/h was attained at 50% conversion with *A. oryzae* lactase and 180 g/L/h at 60% conversion with *B. circulans* lactase. Higher overall GOS productivity and conversion can be achieved with two enzyme reactors in sequence, with the first one containing *A. oryzae* lactase and second one containing *B. circulans* lactase.

Commercial gel-type cation exchange resins (Dowex 50W) with different salt forms (Na^+, K^+, and Ca^{++}) and degrees of cross-linking (2%, 4%, and 8%) were evaluated for the separation of GOS from the multicomponent sugar mixture. Among all the factors studied, the degree of cross linking had the most significant effect on sugar adsorption capacity and selectivity, with 4% cross linking being the optimal. Other factors such as temperature and counter ion showed minimal effect. A high degree of removal of

monosaccharides (95%) from the GOS mixture was achieved using an elution chromatographic column packed with Dowex 50W resins of 4% cross linking and in the Na^+ form at room temperature.

Dedicated to my family

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CHAPTER 1

INTRODUCTION

1.1 Background

During the last 10 years, galacto-oligosaccharides (GOS) were reported to be beneficial for human health, and they are now recognized as prebiotics. GOS, non-digestible oligosaccharides, are not hydrolyzed or absorbed in the upper intestinal tract; they pass on the colon where they are fermented selectively by beneficial intestinal bacteria. Besides the prebiotic effects, these GOS have low cariogenecity, low caloric values and low sweetness (Mischberger et al., 2008; Sako et al., 1999). Furthermore, it has been estimated that foods containing functional additives will grow to represent 5.5% of the total food market to reach $34 billions by 2010 in the U.S.

GOS occur naturally in breast and cow milk, honey and a variety of fruits and vegetables, but only in trace amounts (Angus et al., 2005)**.** As a result, the development of chemical or enzymatic production of GOS is necessary. GOS can be readily produced by enzymatic transgalactosylation of ß-galactosidase from whey lactose, which is an abundant by-product in the cheese industry. Many uses have been found for lactose, including uses in infant formula, bakery, dairy and confectionary products. However, industrial use of lactose is somewhat limited because of its low solubility, indigestibility

by some individuals and tendency to crystallize in water at low temperature. Furthermore, it has been estimated that cheese production increases more than 3% annually and almost half of the entire production of cheese whey is disposed off (Novalin et al., 2005; Lodez-leiva et al., 2000; Yang et al., 1995). In consequence, finding a technology to transform lactose into more profitable product such as GOS will be of high interest to the food industry. Compared to other oligosaccharides such as fructo-oligosaccharides(FOS), GOS have shown to be very stable under conditions of high temperatures and low pH. Therefore, GOS can be used in a variety of products including fermented milk products, breads, jams, confectionery, and beverages (Sako et al., 1999; Playne et al., 1996; Crittenden et al., 1996).

Currently, GOS are commercially produced from transgalactosylation reaction of ß-galactosidase from lactose. Also, the price of ß-galactosidase is rather high and, due to the low value of the substrate, the direct addition of the enzyme is economically unacceptable. Compared with free enzyme in solution, immobilized ß-galactosidase provides many advantages in the production of GOS, including, high reusability, high yield, improvement of thermal stability, continuous operation, controlled product formation and high reactor productivity. Cotton cloth is particularly convenient support since it is an inexpensive and widely available material. Moreover, it has large surface area, high mechanical strength, and high porosity.

Figure 1.1 shows a process flowsheet for lactose production from whey permeate in the U.S. dairy industry where GOS can be produced using the same facility. By adding a small enzyme reactor (40 liters) at the end of the production chain to a plant processing 1 million lbs. of liquid whey per day, a production of 2000 tons GOS can be achieved.

1.2 Research objectives

The goal of this research was to develop a process for GOS production from lactose involving a sequential immobilized enzyme reactors and a posterior chromatographic process for purification of GOS. Figure 1.2 shows the overall design to produce GOS. Simultaneously to GOS production, hydrolysis reaction occurred to break lactose into monosaccharides: glucose and galactose. The concentrated lactose (40% w/v) was fed into two sequential immobilized enzyme reactors, with β-galactosidase from *Aspergillus oryzae* and *Bacillus circulans*, respectively. The order of the enzymes was due to the fact that *B. circulans* lactase provided higher GOS amount at higher lactose conversion. The product stream from the second reactor was sent to a chromatographic separation unit where GOS, lactose, galactose, and glucose could be separated to yield a product with desirable GOS composition. The unreacted lactose, after separating from monosaccharides, can be recycled back to the first enzyme reactor to further improve GOS yield. Purification of GOS is important because by removing monosaccharide and lactose from GOS, there is a decrease in sweetness and calorie value. This relatively low sweetness is useful, as a bulking agent with reduced sweetness and to enhance other food flavors. Compared with mono- and disaccharides, the higher molecular weight of galacto-oligosaccharides provides increased viscosity, leading to improved body and mouth feel (Crittedent et al., 1996). Also, there is an increase in enzymatic activity and/or yield resulting from the removal of byproducts (monosaccharides) in the reactor for GOS production (Boon et al., 2000). Compared to other techniques, liquid chromatography offers high selectivity, efficiency and loading capacity of the stationary phase and speed of process (Schulte et al., 2000). To achieve the overall goal of this research, the

following objectives and main tasks were studied and the results are presented in Chapters 3-6 in this dissertation.

Objective 1. Immobilization of lactase on cotton cloth

The immobilization of bacterial lactase enzyme on cotton cloth was studied. The enzyme was immobilized by adsorption onto cotton cloth activated with polyethyleneimine (PEI), a poly-cationic polymer, followed by cross linking with glutaraldehyde (GA). The optimal conditions and the efficiency of immobilization on cotton cloth were also determined. The stability of immobilized enzyme on cotton cloth was also examined. Finally, the kinetics of GOS production by the catalytic cotton was also studied and the results are reported in Chapter 4.

Objective 2. GOS production from different lactase enzymes

The amount and nature of GOS formed depend on the enzyme source. GOS production was studied by using 3 different lactases: fungal (*Aspergillus oryzae*), bacterial (*Bacillus circulans*) and yeast (*Kluveromyces lactis*). Factors affecting GOS production, including, lactose concentration, pH, presence of galactose, and enzyme loading were also studied and the results are presented in Chapter 3

Objective 3. Continuous GOS production by using two-step immobilized enzyme reactor

Continuous GOS production was studied by using a single and two sequential packed-bed reactors with immobilized ß-galactosidase from *Aspergillus oryzae* and *Bacillus circulans*. The results are presented in Chapter 5.

Objective 4. Chromatographic separation of GOS from lactose and monosaccharides

The feasibility and performance of using a commercial available gel type poly(styrene-co-divinylbenzene) (PS-DVB) strong acid cation exchange resin (Dowex 50) for purifying GOS from the sugar mixture produced from enzymatic reactions of lactose were studied. Factors affecting GOS separation including temperature, cross linking, and counter-ion were also investigated and the results are presented in Chapter 6.

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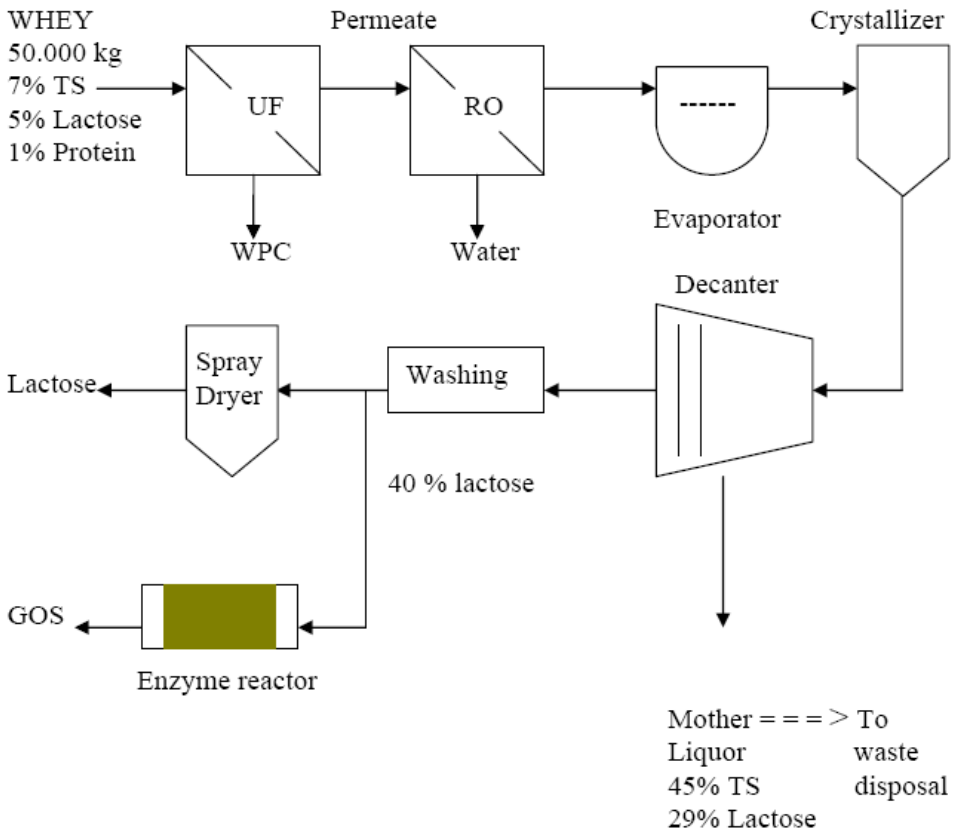

Figure 1.1: A conceptual flowsheet for lactose and GOS production from whey permeate.

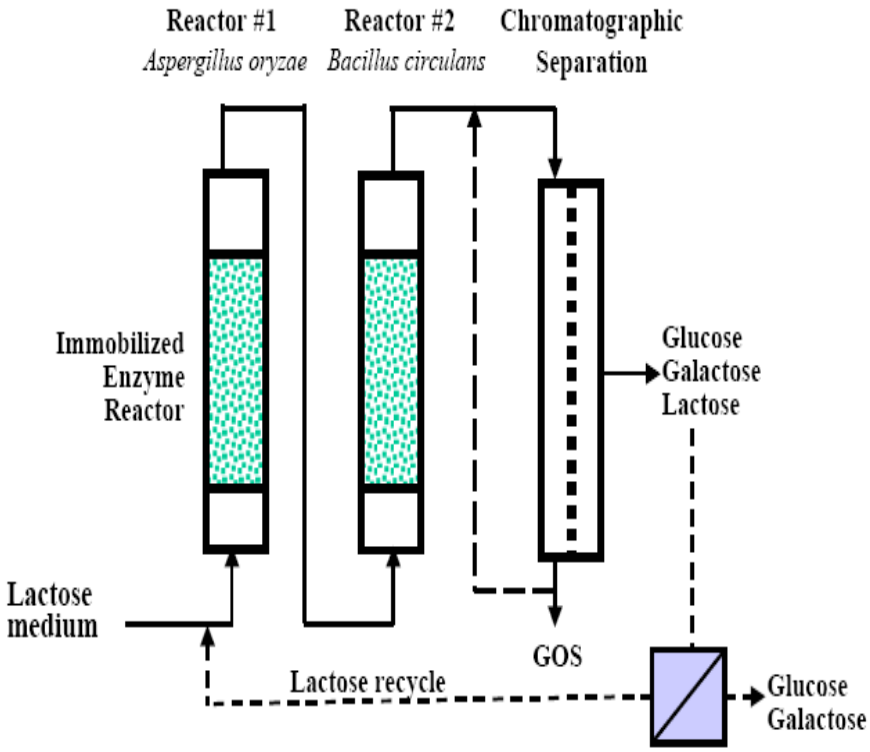

Figure 1.2: A process for GOS production from lactose with immobilized lactases and chromatographic separation.

CHAPTER 2

LITERATURE REVIEW

2.1 Prebiotics: Non- digestible oligosaccharides

The gastrointestinal tract is formed of 10^{14} colony forming units (cfu) g^{-1} of bacteria, consisting of over 400 different cultivatable species belonging to 190 genera, and known as the gastrointestinal flora. The anaerobes *Bacteroides* spp., *Bifidobacterium* spp. and *Eubacterium* spp. represent greater than 99% of those species present in the colon. The growth and metabolic activity of the flora have a tremendous influence on our physiological and nutritional well-being. A balanced gastrointestinal flora is one in which the health-promoting or beneficial bacteria predominate over the potentially harmful bacteria (Cummings et al., 2004). Many factors such as stress, poor diet, antibiotic therapy, infections, food poisoning and natural ageing process may upset this balance (Holzapfel et al., 1998). The gastrointestinal flora plays an important role in digestion and in maintaining gastrointestinal health by stimulating the immune system, preventing harmful bacteria from establishing a home on the gastrointestinal wall and promoting acidification. Foods or ingredients such as prebiotics that encourage the growth and activity of beneficial bacteria in the gastrointestinal tract help maintain a balanced gastrointestinal flora. (Angus et al., 2005).

Gibson and Roberfroid first described a prebiotic as a "non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson et al., 1995). Currently for food ingredients to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial bacteria in the colon such as the bifidobacteria, (3) fermentation of the substrate should induce beneficial effects within the host. (Manning et al., 2004)

A range of food additives such as non-digestible oligosaccharides (NDOs) that have these characteristics are considered prebiotics. During the past few years, it has been an increase in consumer demand for foods with certain beneficial effect on the human body. Furthermore, by 2010, Nutritional Business Journal predicted the functional food market in the US will double, to $ 34 billion, representing 5.5% of the total food market. Galacto-oligosaccharides (GOS), a NDO, which in Europe are sold commercially as Elix`or (Borculo Domo Ingredients, The Netherlands) belong to this class of food ingredients (Schoterman, 2007). Today over 20 different types of NDOs are on the world market. NDOs are large sugar molecules containing three to twenty monosaccharide units, joined together by glycosidic bonds (Crittenden et al., 1996). They are commonly classified according to their main monosaccharide components. Fructo-oligosaccharides (FOS) composed of fructose and glucose, or GOS composed of galactose and glucose. Table 2.1 list major groups of NDOs with their chemical structures. The chemical differences between these NDOs include chain length, monosaccharide composition, degree or branching, and purity (Mussatto et. al., 2007). Carbohydrates are extracted from natural sources (soybean oligosaccharides and lactulose) by partial enzymatic hydrolysis of polysaccharides (e.g. xylo-

oligosaccharides and isomalto-oligosaccharides) or by enzymatic transglycosylation (e.g. galacto-oligosaccharides and fructo-oligosaccharides). (Figure 2.1).

2.2 Galacto-oligosaccharides (GOS)

GOS are natural constituents in human milk as well as various types of common foods including onions, garlic, banana, soybeans, and chicory. However, the levels of GOS in these foods are too low to exert any significant effect. For this reason, the best delivery system may be through dietary supplements or through inclusion in foods. Their chemical formula is $(Galactose)_n$ - Glucose, with n ranging from 1 to 4, as can be seen in Figure 2.2. The galactose-galactose linkage is a β-(1-3), β-(1-4), β-(1-6) linkage, with the β-(1-4) being predominant: the galactose-glucose linkage is mainly β-(1-4). Some disaccharides are also present in GOS (e.g. allolactose and galactobiose) (Oku, 1996).

Galacto-oligosaccharides belong to the group of non-digestible oligosaccharides (NDO), which can be regarded as soluble dietary fibres (Tomomatsu, 1994). Authorities in several countries permit galacto-oligosaccharides to be labelled on foods as dietary fibre, although there is, as yet, no unequivocal definition of dietary fibre in Europe. Dietary fibres can be classified into two categories: (1) soluble, viscous and fermentable; and (2) insoluble, non-viscous and non-fermentable. Galacto-oligosaccharides belong to the first category, because they are completely soluble and are fermented by specific bacteria present in the colon, resulting in the production of short-chain fatty acids (propionate, acetate and butyrate) (Schoterman, 2007).

Galacto- oligosaccharides have a high solubility and a relative sweetness about 35 % that of sucrose. They are more viscous than high-fructose corn syrups, decrease

the water activity and freezing point, and show good moisture retention capacities. They also have remarkable stability at high temperatures and variable pH levels. In particular, the stability of GOS in acidic and high-temperature conditions enables them to be applied without decomposition in a wider variety of foods. GOS such as Oligomate 55 (Anonymous, 1999) , remain unchanged after treatment at 160ºC for 10 min at pH 2, where about a half or more of the sucrose is degraded. Even in acidic conditions at room temperature, GOS tend to be stable during long-term storage. (Angus et al., 2005; Sako et al., 1999). It is suggested that stability of GOS is better than that of fructo-oligosaccharides (FOS) (Voragen, 1998).

According to Roberfroid and Slavin the evaluation of an acceptable dose is difficult because each individual has his own feeling about acceptable and non-acceptable intestinal discomfort. However, excessive consumption doses of GOS may cause intestinal discomfort, flatulence or even diarrhoea because of their osmotic effect, which may transfer water into the large bowel, and because of their high fermentation rate and production of gases (Roberfroid et al., 2000). For example, galactooligosaccharides consumption higher than 20g/day, and fructooligosaccharides consumption higher than 40g/day are reported to cause diarrhoea (Sako et al., 1999; Spiegel et al. 1994). The effective bifidogenic does appear to vary among the different oligosaccharide types. Nevertheless, most oligosaccharides have been demonstrated to increase bifidobacteria numbers in the colon at doses of < 15 g/day (Crittenden et. al., 1996). Some authors have suggested that an intake of 10g/day of galactooligosaccharides is sufficient to cause a bifidogenic effect. However, when the subjects´ initial number of indigenous bifidobacteria is low, which is often the case in middle-aged and elderly people, daily intake of 2.5g/day is enough to lead to an increase in fecal bifidobacteria population (Mussatto et al., 2007; Gibson, 2004; Sako

et al. 1999). A study in humans showed that, compared with fructo-oligosaccharides (FOS) the consumption of galacto-oligosaccharides (GOS) resulted in less breath hydrogen excretion. There was also a tendency towards fewer complaints of flatulence after consumption of galacto-oligosaccharides (Schoterman, 2007).

The energy value of non digestible oligosaccharides, such as galacto-oligosaccharides, is relatively low because they are hardly hydrolyzed and absorbed in the upper gastrointestinal tract. Part of the energy of non-digestible oligosaccharides is salvaged during fermentation and absorption of short-chain fatty acids in the colon. The energy value of galacto-oligosaccharides has been estimated as 1.0-2.0 kcal/g (Schoterman, 2007; Sako et al., 1999; Salminen et al., 1998).

2.2.1 GOS health benefits

GOS provide several health benefits, which make their use as food ingredients particularly attractive. Due to their β-configuration, GOS are resistant to hydrolysis by human saliva and gastric enzymes (Mussatto et al., 2007). In the large intestine GOS are substrates that can only be consumed by a limited number of bacteria, stimulating their growth. Among the group of bacteria present in the gastrointestinal tract, the bifidobacteria and lactobacilli are those that most utilize GOS being considered as the only microorganisms able to beneficially affect the host's health. For this reason, GOS has been termed "Bifidus growth factor" (Bielecka et al., 2002).

The proliferation of bifidobacteria in the lower intestine brings about most of the health benefits attributed to oligosaccharide ingestion. This includes:

a) Reduction of Detrimental Bacteria

Upon GOS consumption by bifidobacteria, the microorganisms grow and multiply. Byproducts of GOS produced by bifidobacteria include short chain fatty

acids (such as acetic and lactic acids), CO_2, CH_4, H_2, and a few antibiotic materials. The short-chain fatty acids effectively lower the pH of the large intestine such that it is slightly acidic. This change in pH then alters the composition of the intestinal microflora: the amount of beneficial bacteria (*Bifidobacterium* and *Lactobacillus*) further increase and the amount of harmful (putrefactive) bacteria (*Clostridium, E. coli*) decrease, until equilibrium is reached. This reduction in the amount of detrimental bacteria includes pathogenic bacteria (*Salmonella, Shigella, Staphylococcus* and others), helping the body to prevent infections.

b) Production of Nutrients

Bifidobacteria produce many B vitamins, including B-1, B-2, B-6, and B-12, along with biotin, nicotinic acid and folic acid (Tomomatsu, 1994; Hoover, 1993)

c) Increase in absorption of different minerals in the intestine

The decrease in intestinal pH promotes the absorption of certain minerals such as iron and calcium. The increase on calcium absorption, in particular, reduces the risk of osteoporosis since this mineral promotes an increase in the bone density and bone mass (Rivero-Urgell et al., 2001; Takahara, 2000; Van den Heuvel, 1999; Chonan et al., 1995a; Chonan, et. al., 1995b).

d) Prevention of pathogenic and autogenic diarrhoea

This may be directly related to the possible inhibitory effect of bifidobacteria both on gram+ and gram- bacteria (Mussatto et al., 2007).

e) Prevention of constipation

The indigestibility quality of GOS means that they have effects similar to diarrhoea. The production of short chain fatty acids by bifidobacteria stimulates intestinal peristalsis and increases the humidity of the faecal bolus through osmotic pressure (Cherbut, 1995; Ito et al., 1990; Deguchi, 1997).

f) Reduction in serum cholesterol

Changes in the concentration of serum cholesterol have been related with changes in intestinal microflora. Some strains of *Lactobacillus acidophillus* assimilate the cholesterol present in the medium (Chonan et al., 1995a; Gilliland et al., 1990), while others appear to inhibit the absorption of cholesterol micelles through the intestinal wall (Suzuki et al., 1990).

g) Reduction of blood pressure

There appears to be a negative correlation between diastolic blood pressure and the percentage of bifidogenic bacteria in the total colon bacteria count (Masai et al., 1987).

h) Anticarcinogenic effect, mainly the gut cancer

The anticarcinogenic effect appears to be related to an increase in cellular immunity, the components of the cellular wall and the extra-cellular components of bifidobacteria (Mussatto et al., 2007; Kanbe, 1992; Hirota, 1990).

2.2.2 GOS applications and market

Currently, there are mainly 4 companies producing GOS, three located in Japan (e.g. Yakult producing Oligomate 55) and one located in Netherlands (Borculo Domo Ingredients producing Elix´or) (Table 2.2). Also, there are already more than 450 foods that contain OS, including GOS, in Japan. In U.S. there is no company making GOS at present time, but they should have many marketable areas since Americans are demanding natural foods with beneficial health effects. (Hughes et al., 1991) Moreover, it has been estimated that foods containing functional additives will grow to represent 5.5% annually to reach $34 billions by 2010 in U.S., being GOS one of the most favorable candidates for this market (Klont, 2000) Also, the fact that

GOS at current market price are more than $17 per Kg makes it a high-value product for the dairy industry.

Galacto-oligosaccharides are especially suitable in food products for special target groups, such as infant nutrition, clinical nutrition and foods for elderly people. Certain groups of people may gain additional benefit from the consumption of galacto-oligosaccharides. Bottle-fed infants for example, may benefit from the bifidogenic effect because their microflora contains a low amount of bifidobacteria compared with breast-fed infants (Sako et al., 1999). Consumption of galacto-oligosaccharides can also benefit the elderly in several ways, as they have lower counts of bifidobacteria in their microflora than do younger adults. They often suffer from constipation, and their capacity to absorb calcium is lower than that of younger adults (Schoterman, 2007; Mitsuoka, 1990).

In addition to the application of GOS to food products for special target groups, these materials may also be added to traditional foods. Besides their prebiotic effects, low cariogenecity, low caloric values and low sweetness, were the most important characteristics of GOS (Sako et al., 1999). Furthermore, GOS can be added to a wider range of foods than probiotics, where culture viability needs to be maintained (Manning et al., 2004). Products that are made under high temperature and low pH conditions are especially suitable for inclusion of heat- and acid-stable galacto-oligosaccharides. In general GOS can be applied to the following areas:

- Addition to processed foods, including beverages, frozen foods, snacks such as cookies and chips.
- Use as dietary supplement.
- Inclusion to infant formulas, to make them more closely resembles mother's breast milk.

- Addition to animal feeds.
- Low calorie sweetener. This is especially useful for diabetics and obese patients, since there sugars are not digested but taste and sweetness are not sacrificed.
- New drug development, including cell adhesion or transplantation based drugs.
- Other non-food applications including cosmetics use and for mouthwashes.

2.2.3 β-galactosidase

The enzyme β-galactosidase occurs widely in nature and has been isolated from animal and plant sources as well as microorganism. However, compared with animal and plant sources, the microbial is produced at higher yield and is more technologically important. (Table 2.3) (Grosova et al., 2008). Not all lactase sources are acceptable or generally recognized as safe (GRAS) when the enzyme is going to be used in food systems. The properties of the enzyme depend on its source. The largest molecular size is possessed by *E. coli* lactase (520000 up to 850000 Dalton are reported) while for *S. fragilis* and *A. oryzae* enzymes the molecular weights are 201000 and 90000 Dalton respectively. Temperature and pH optima differ also according to the source and even according to the particular commercial preparation. The enzyme "lactase" has long been used to hydrolyze lactose in the manufactures of some dairy products (Axelsson et al., 1990; Bakken et al., 1990; Gekas et al., 1985).

2.2.3.1 Reactions of β-galactosidase

The enzyme β- galactosidase (lactase) is classified as a hydrolytic enzyme specifically as a glycosidase for the hydrolysis of glycosidic bonds. The first observed

function of β-galactosidase was the hydrolysis of the disaccharide lactose into the monosaccharides glucose and galactose. This led to the extensive use of the enzyme by the dairy industry, mainly for reducing lactose content or improving storage and processing characteristics. Later, it was discovered that the enzyme could also make glycosidic bonds between two saccharides by removing water in a reaction called transgalactosylation. This allows the enzyme to synthesize oligosaccharides, specifically GOS, from lactose. β-galactosidase does this by adding galactose units to lactose, forming a multi-unit chain (Figure 2.3).

The reaction kinetics has been well documented using enzymes from a variety of sources. Due to the dual catalytic activities of β- galactosidase, the enzyme is involved in competing reactions when incubated with lactose. In general, transgalactosylation dominates early on in the reaction, producing GOS with a high yield. As the lactose conversion increases, the enzyme's hydrolytic activity then takes over; the final products after 100% lactose conversion are glucose and galactose. The hydrolysis reaction follows Michaelis-Menten kinetics. Galactose is a competitive inhibitor, causing the enzyme's actions to greatly slow down at high lactose conversions, reducing the rate of the reaction (Prenosil et al., 1987a).

Enzymatic hydrolysis of the glycosidic bond of lactose takes place via general acid catalysis that requires two critical residues, i.e., a proton donor and a nucleophile/base. The mechanism of lactose hydrolysis was first described by Wallenfels (Wallenfels et al., 1960), who used the lactase from *E. coli.* The reaction mechanism proposed was that the cystine and histidine residues acted as proton donor and nucleophile site, respectively, during the enzymatic hydrolysis procedure. This mechanism has been described in many review articles (Prenosil et al., 1987a; Nijipels, 1981). It has become clear recently, however, that the β-galactosidase from a

variety of microbial origins has two glutamic acid residues (Glu^{482} and Glu^{551}) as the proton donor and the nucleophile/base at the same time in the enzymatic reaction. The reaction mechanism is shown in Figure 2.4. The first step is the enzyme-galactosyl complex formation and simultaneous glucose liberation. In the second step, the enzyme-galactosyl complex is transferred to an acceptor containing a hydroxyl group. While in a diluted lactose solution, water rather than other sugars such as glucose, lactose can be more competitive to be an acceptor, therefore galactose is formed and released from the active site. On the other hand, in a high lactose content solution, lactose molecule has more chances to act as the acceptor, binding with the enzyme-galactose complex to form oligosaccharides (Zhou et al., 2001b).

2.2.4 Factors affecting GOS production

2.2.4.1 Enzyme origin

Table 2.4 lists the characteristics of some microbial β-galactosidases and the reaction conditions used for GOS production. Enzymes from different sources have different characteristics, such as: pH optima, temperature optima, Km for lactose and type and distribution of GOS. Amount and structures of GOS produced are dependent on the source of the enzyme (Mahoney, 1998). Regardless of the enzyme source, the maximum GOS content of 20%-40% is usually obtained at a lactose conversion higher than 50%. The higher GOS yields (>50%) are usually those also include the disaccharides. Due to the different processing conditions, a wide variety of enzyme characteristics can be advantageous. For example, β-galactosidases from *Thermus aquaticus* YT-1 and *Sterigmatomyces elviae* CBS8119 are highly thermostable, with temperature optima of 70 ºC (Berger et al., 1995 a, b) and 85 ºC (Onishi et al., 1998) respectively. In general, some bacterial (Shin et al., 1998) and yeast enzymes (Lopez-

Leiva et al., 1995) maximize GOS at higher conversion of lactose (70-80%), thus achieving higher processing yields of GOS (above 50%).

However, some of these lactases are not approved for food used and are expensive. Furthermore, others were from sources not commercially available or not available in sufficient quantities for industrial applications. The lactases used in this study are from yeast *(Kluveromyces lactis),* fungal (*Aspergillus oryzae*) and bacterial (*Bacillus circulans*) sources. These 3 lactases are commercially available and have been recognized as safe for food production (Ladero et al., 2001; Di Serio et al., 2003). Furthermore, the maximum amount of GOS from these enzyme sources is obtained at different lactose conversion. In consequence, GOS production by using these lactases in a system of sequential plug-flow reactors could lead to increase GOS yield.

2.2.4.2 Lactose concentration

Initial concentration is an important factor affecting GOS formation (Boon et al., 2000). Higher GOS amount is obtained at higher lactose concentration since in a diluted lactose solution, water can be more competitive to be an acceptor for the β-galactosyl groups, binding with the enzyme-galactose complex forming GOS (Zhou et al., 2001). Zhou et al. reported an increase in GOS production from 2% to 32% as the initial lactose concentration increased from 14% to 40% (w/v). Compared to lactose concentration and lactose conversion, other process parameters, such as enzyme concentration, pH and temperature, have minimal effects on GOS production (Iwasaki et al., 1996), although they affect the reaction rates.

2.3 Immobilization of β-galactosidase

The immobilized enzyme is defined as "the enzyme physically confined or localised in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously" (Chibata, 1978). The choice of suitable enzymatic preparation depends on its properties and the purpose of its application. The majority of the immobilized systems of β-galactosidases have been developed for the purpose of lactose hydrolysis; there have been only a few reports for production of GOS. It is important to note that what is favored for the hydrolysis of lactose is quite different from that for GOS production. Yeast β-galactosidases are habitually used for the hydrolysis of lactose in milk and sweet whey, whereas fungal β-galactosidases are more suited for acidic whey hydrolysis. Compared to yeast enzymes, fungal β-galactosidases are more thermostable, but they are more sensitive to product inhibition, mainly by galactose (Boon et al., 2000a). Immobilization of β-galactosidases can dramatically affect enzyme's properties; e.g. pH and temperature stability, kinetic parameters, etc. (Rossi et al., 1999; Sun et al., 1999; Ladero et al., 2000). If an adequate technique is applied, immobilization can improve properties of β-galactosidases such as stability of the enzyme at high or low pH and temperatures (Zhou et al., 2001b).

Many of the carriers used for immobilization of β-galactosidases applied in GOS production were some types of microparticles, such as ion-exchange (Matsumoto et al., 1989), chitosan beads (Sheu et al., 1998; Shin et al., 1998), cellulose beads (Kmínková et al., 1988) and agarose beads (Berger et al., 1995b). It was observed that the immobilized enzyme in these particle carriers often resulted in 20-30% reduction in GOS yield due to introduction of mass transfer resistance in the system (Grosova et al., 2008; Sheu et al., 1998; Shin et al., 1998). Furthermore, their

industrial applications would be quite difficult since these resins and beads are highly expensive considering industrial scale support needs (Ichijo et al., 1985). Further these supports may cause some problems when used in a packed-bed reactor, notably a high-pressure drop and ease of fouling and plugging due to structural instability, and aggregation in fluidized beds (Howlett et al., 1991). Increasing pressure across the reactor in packed-beds could not be afforded because beads have limited mechanical stability. Thus, being compressed at relatively low pressures makes them unsuited for reactor conditions (Elsner et al., 1999)

2.3.1 Methods of immobilization

β-galactosidases were immobilized by several methods to a variety of matrices, including entrapment, cross-linking, adsorption, covalent binding or the combination of these methods (Table 2.5). Since each method has its own advantages and drawbacks, the selection of suitable method depends on the enzyme, matrix, reaction, conditions, reactor, etc. (Tanaka et al., 1999).

2.3.1.1. Covalent binding

This method is mostly used. Enzymes are covalently linked to the support through the functional groups in the enzymes that are not essential for the catalytic activity. The major disadvantages are high costs and low activity yield owing to exposure of the biocatalyst to toxic reagents or severe reaction conditions (Tanaka et al., 1999). There were several matrices used for β-galactosidase immobilization. Oxides materials such as alumina, silica, silicated alumina were used for covalent binding of β-galactosidase from *K. marxianus* and applied in lactose hydrolysis processes. In spite of the immobilizates showed good stability, the immobilization yields were less than 5 % (Di Serio et al., 2003).

2.3.1.2. Entrapment

The entrapment method is based on the localisation of an enzyme within the lattice of a polymer matrix or membrane. The major limitation of this technique for the enzymes immobilization is the possible leakage during repeated use due to the small molecular size compared to the cells. Next disadvantages of the method are diffusion limitations. Entrapment method is classified into five major types: lattice, microcapsule, liposome, membrane, and reverse micelle (Tanaka et al., 1999). For β-galactosidase immobilization, the lattice method is the most widely used. The enzyme is entrapped in the matrix of the various synthetic or natural polymers. Alginate, a naturally occurring polysaccharide that forms gels by ionotropic gelation is the most popular one (Mammarella et al., 2005) entrapped *K. fragilis* β-galactosidase in alginate-carrageenan gels to form beads. Also, alginate as an immobilization matrix was used in combination with gelatin to immobilized *A. oryzae* β-galactosidase in fibers. However, the enzyme only had 25% of initial activity (Tanriseven et al., 2002).

2.3.1.3 Physical adsorption

Physical adsorption is the simplest and the oldest method of immobilizing enzymes onto carriers. Immobilization by adsorption is based on the physical interactions between the biocatalyst and the carrier, such as hydrogen bonding, hydrophobic interactions, van der Waals force, and their combinations. Furthermore, adsorption is cheap, early carried out, and tends to be less disruptive to the enzymic protein than chemical means of attachment. For these reasons, this method was used during this research.

2.3.1.4 Cross-linking

The cross-linking method utilises a bi- or multifunctional compounds, which serve as the reagent for intermolecular cross-linking of the biocatalyst. In case of β-

galactosidase immobilization, cross-linking is often used in combination with other immobilization method, mainly with adsorption and entrapment. (Grosova et al., 2008).

2.3.2 Reactors of immobilized β-galactosidase for GOS production

There are many different factors that influence the optimal reactor type, including the reaction kinetics, the support structure for the immobilized enzyme, mass transfer effects, ease of catalyst replacement/regeneration, and the form of the substrate. Differential reactors, run in batch mode, are commonly used to gather a reaction's kinetic data from immobilized enzymes. Only a small amount of catalyst is needed, and the substrate is recycled through the catalyst until the reaction is completed. This reduces diffusion effects such that the kinetics gathered are for reaction-controlled systems. Continuously stirred tank reactors (CSTRs) and packed bed reactors (PBRs) are the most common reactor types used for continuous production, with hundreds of variations and combinations available, including recycle reactors and fluidized bed reactors. PFRs often have kinetic advantages over CSTRs for immobilized enzyme applications, including a higher efficiency of conversion, but suffer from high pressure drops when the packing is particulate (Roy et al., 2003). Concerning GOS production purposes, PFRs also typically have a higher yield when the enzyme is inhibited by the products (galactose).

The main reactor systems for GOS production by immobilized β-galactosidase are PBRs. Table 2.6 shows the production of GOS by using different reactors. Nakanishi et al., 1983, and Mozaffar et al., 1985 both studied continuous GOS production from *Bacillus circulans* lactase; immobilization was on Duolite ES-762 and Merkogel, respectively (Mozaffar et al., 1986, Nakanishi et al., 1983). They

both found that for the PFR, the enzyme activity gradually decreased over time, whereas in the CSTR the immobilized enzyme seemed to be more stable initially but then activity quickly dropped when the glucose concentration reached a critical level. Mozaffar suggested that the enzyme activity remained high in the CSTR since fewer oligosaccharides accumulated in the immobilized support than in a PFR (Mozaffar et al., 1986). Lopez-Leiva and Guzman designed a novel process for GOS formation, with immobilization on a thin (0.64 mm) porous film (Lopez-Leiva et al., 1995). However, their yield was low (33%) and the scale-up for an industrial process would be difficult, especially considering industrial factors such as high pressure.

Using a continuous PBR with β- galactosidase from *Bullera singularis* ATTC 24193 immobilized in chitosan beads, 55% (w/w) of GOS was produced continuously with a productivity of 4.4 g/l·h. The substrate (100 g/l of lactose solution) was fed at flow rate 80ml/h into a reactor (100 ml of bed volume), in which 970 GU/g enzyme was immobilized. The PBR (60 ml) filled with 90 g of immobilized recombinant β-galactosidase from *Aspergillus candidus* CGMCC3.2919 (on adsorptive resin D113) was used for continuous production of GOS. The maximum productivity 87 g/l·h was reached when 400 g/l lactose was fed at dilution rate of 0.8/h. The maximum GOS yield reached 37 % at dilution rate of 0.5/h. (Zheng et al., 2006). Most of these supports were particulate, such as resins or beads. However, these supports may cause some problems when used in a continuous reactor, notably a high pressure drop, ease of fouling, and possible plugging (Grosová et al., 2008).

2.3.3 Cotton cloth

The form of cellulose makes a difference in its use. It can be prepared as beads, which possess similar disadvantages of other types of beads like agarose and

dextran. The fiber form also exhibits high hydrodynamic resistance to compaction and clogging by fine units (Sharma et al., 1984). Margel and Sturchnak (1999) compared different forms of cellulose and found that the textile form resulted in higher activity and coupling yield than that of powder form. It has been shown that the fabric or cloth form of fibers (cotton flannel or terry cloth form) provides good flow rates when compiled/stacked in a column (Sharma et al., 1984). Howlett et al. (1991) indicated that cloth possesses a regularly compressed open structure, thus column packed with cloth segments permits higher flow rates than that of fibers.

As a cellulosic material, cotton has a high mechanical strength due to its crystalline structure. The strength allows the porosity associated with fibrous structure to be maintained even at a high packing density. The cellulosic nature of cotton also possesses the desirable characteristics of stability for chemical, biochemical and physical attacks. Compared with commonly used materials, cotton fiber is widely available and inexpensive ($ 0.5 per lb) which makes the material ideal for biocatalyst applications.

2.3.4 Reagents of immobilization

2.3.4.1 Polyethyleneimine

Polyethyleneimine [PEI; $(C_2H_5N)_n$] is a synthetic polyamine with a high concentration of amino groups. PEI has found acceptance as a carrier in a number of industrial immobilized biosystems (Bahulekar et al., 1991). PEI has long been recognized as a stabilizing agent for soluble enzymes (Bernath et al., 1986). Its potential in enzyme immobilization as a carrier matrix or a reagent mediating enzyme immobilization onto another carrier recently led to development of a number of novel methods. As a strongly positively charged, nitrogen containing polymer, PEI actually

has no reactive groups but forms strong electrostatic complex with negatively charged species. Many different types of carriers have been used for immobilization utilizing PEI. Depending on the support, PEI was either adsorbed (Isgrove et al., 2001) or covalently coupled to support. When adsorbed, PEI is generally cross-linked with a bi- or multi-functional cross-linking agent, usually glutaraldehyde (Isgrove et al., 2001; Bardeletti, 1997). Since PEI forms ionic complexes with macromolecules containing acidic domains, the interaction behaviour is affected by salt concentration, pH, and the concentration of precipitable components (Jendrisak, 1987). The Food Drug Administration (FDA) has permitted PEI as a secondary direct food additive in the food for human consumption (Fed Reg, 1996) for use as fixing agents for the immobilization of glucoamylase enzyme in the manufacture of beer.

2.3.4.2 Glutaraldehyde

Several studies have described various cross linking reagents used for improvement of β-galactosidase stability in immobilized state (Table 2.7). These reagents form covalent bonds using their reactive functional groups, such as carbonyl groups of glutaraldehyde (Szczodrak, 2000), imidoester groups of dimethyladipimidate (Khare et al., 1988), carbodiimide group of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Dominguez et al., 1988), etc. Among these, glutaraldehyde, which interacts with the amino groups through a base reaction, has been the most extensively used in the view of its GRAS status, low cost, high efficiency, and stability (Nakajima et al., 1993).

Beneficial effect of glutaraldehyde as a cross-linker was shown in immobilization of β-galactosidase from *A. oryzae* by entrapment in cobalt alginate beads. Relative activity of the entrapped enzyme without cross-linking was 83 %.

After the first usage the relative activity dropped to 67.5% as a result of leakage. Relative activity of the entrapped enzyme cross-linked by glutaraldehyde was unchanged (83%) and stable even after the eighth use (Grosova et al., 2008).

2.4 Separation of GOS

The separation of carbohydrates plays an important role in food production and in cosmetic and pharmaceutical industries. Also, 90% of the cost in food production is related with separation processes (Ahlgren et al., 1997). Liquid chromatography offers high selectivity, efficiency and loading capacity of the stationary phase and speed of process (Schulte et al., 2000). Purification of GOS is important because by removing monosaccharide and lactose from GOS, there is a decrease in sweetness and calorie value. This relatively low sweetness is useful, as a bulking agent with reduced sweetness and to enhance other food flavors. Compared with mono-and disaccharides, the higher molecular weight of galacto-oligosaccharides provides increased viscosity, leading to improved body and mouth feel (Crittenden et al., 1996). Also, there is an increase in enzymatic activity and/or yield resulting from the removal of byproducts (monosaccharides) in the reactor for GOS production (Boon et al., 2000b). Techniques such as membrane separation that has been used in OS separation present a variety of problems. First of all, using high concentration solutions cause membrane fouling. Sarney et al., 2000 could separate 90% OS from a sample of only 10g/L. When higher concentrations were used, the GOS separation decreased drastically. Furthermore, the separation of lactose from oligosaccharides is difficult. Iwasaki et al., 2000 separated most of monosaccharides, 92%, from a mixture. However, 80% of lactose remained in the solution. Finally, membranes have problems of pH, temperature and mechanical resistance, making

necessary to replace them after certain period of time, increasing the cost of the whole process (Iwasaki et al., 2000). Compared to membrane separation, liquid chromatography offers high selectivity, efficiency and loading capacity of the stationary phase and speed of process (Schulte et al., 2000).

2.4.1 Chromatographic separation

Chromatography is a technique that has been used for separation and purification of sugars. Some of the qualities of this technique is that is possible to achieve high selectivity, efficiency, loading capacity of the stationary phase and speed of the process. (Schulte et al., 2000). Liquid chromatography involves a sample being dissolved in a *mobile phase* (liquid). The mobile phase is then forced through an immobile, immiscible *stationary phase*. The stationary phases are chosen such that components of the sample have different solubility in each phase. A component, which is quite soluble in the stationary phase, will take longer to travel through it than a component, which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobility, sample components will become separated from each other as they travel through the stationary phase. Figure 2.5 represents a chromatogram for GOS obtained in HPLC. The peaks on the left side represent the different GOS (6OS, 5OS, 4OS and 3OS), and since these are the first components in the chromatogram represent the sugars with less affinity for the solid phase. ON the other hand, MS (glucose and galactose) are the last peaks in the chromatogram so they are the most retained components.

2.4.2 Materials of chromatographic separation

Different types of materials can be used as a solid phase for chromatographic separation. The stationary phase is the key element in a chromatography system. Separation is enhanced by using stationary phases that present the shortest possible diffusion pathways to the solutes, have low resistance to mass transfer, reasonably narrow particle size distribution, and are uniformly packed in the column. The most frequent materials used in sugar separation are:

a) active carbon

In adsorption processes one or more components of a liquid stream are adsorbed on the surface of a solid adsorbent and a separation is accomplished. The fluid is passed through the bed and the solid particles adsorb components from the fluid. When the bed is almost saturated, the flow in the bed is stopped and the bed is regenerated thermally or by other methods, so desorption occurs. Typically, the adsorbents are in the form of small pellets, beads or granules. A particle of adsorbent has a very porous structure with many fine pores. The adsorption is usually by Van der Waals forces, and occurs between the adsorbed molecules and the solid internal pore surface and is readily reversible (Geankoplis, 1993).

In sugar industry, the most used adsorbent is active carbon, because is the cheapest and does not require a difficult pretreatment. Ajisaka et. al., 1987 studied the removal of disaccharides from monosaccharides. Boon et. al., 2000 used active carbon on the separation of GOS, showing that the carbon has higher affinity for oligosaccharides and low affinity for monosaccharides (glucose and galactose). Since GOS are the compound most adsorb for the carbon, monosaccharides were removed initially, and during regeneration step, using ethanol, the GOS were recovered. It was observed that the higher the ethanol concentration used the higher recovery level of

GOS was achieved. The problem of using carbon is the difficulty of separating lactose from GOS due to the similarity in capacity factors. Also, since regeneration step it is necessary to recover GOS, the product dilution is high, being necessary an evaporation step to concentrate the sample. Also, the low loading capacity of the carbon represents a drawback. Jung-Wook Yoo and Seug Jao Kim (Yoo et al., 2003) conducted similar research for the separation of malto-oligosaccharides.

b) ion exchange materials

The stationary phase in ion exchanger comprises of three components: (1) an insoluble matrix, which may be organic or inorganic (Example: Polystyrene, PS cross linked with Divinyl Benzene, DVB; Silica based matrix) (2) ionic sites, either attached to or an integral part of the matrix (Example: Sulfonate) and (3) associated with these fixed sites, an equivalent amount of counter-ions of charge opposite to that of the fixed sites. These counter-ions are mobile throughout the ion exchanger and have the ability to exchange with others of like charge or form complexes of varying strength when placed in contact with a solution containing appropriate groups. These materials have the following properties (Small et. al., 1990):

1. The ability to exchange their ions or to form complexes
2. Good chemical stability over wide pH range
3. Good mechanical strength and resistance to osmotic shock
4. Resistance to deformation when packed in a column and subjected to high flow of the mobile phase.

Cation exchange resins have been used extensively in the sugar industry for different types of separation. The use of cation-exchange resins together with water as the mobile phase resulted in a better separation of saccharides than when anion exchangers were used. A column packed with Dowex 50W-X4 (Ag^{+}) changers was

used for the separation of glucose from fructose, with a recovery of about 90% (Lefevre, 1992). Xylose and arabinose were separated on Dowex 50W-X8 (Ca^{2+}). The successful separation has been reported of a mixture containing sucrose, raffinose and glucose on a 2 3 100 cm column of Dowex 50W-X2 (Li^{+}), with water as the eluent (Jones et al., 1999) Clean separations of mixtures containing raffinose, mellibiose and glucose and those containing maltose, lactose and galactose were achieved by this method.

Saunders (1998) introduced a method for the separation of sugars on the cation-exchange resin Dowex 50W-X4 (K^{+}), using water as the eluent. A large number of saccharides were separated by this method, including oligosaccharides, hexoses, pentoses, acetals, methyl-α-D-glycosides and other sugar derivatives, with recoveries of greater than 95%. The resin column was used continuously in the analyses for 12 months with no loss of resolution. Furthermore, Okada et al., 1992 reported the separation of glucose/fructose, to remove glucose from malto-OS mixtures, to produce high purity maltose from starch hydrolysate.

2.4.3 Factors affecting GOS separation.

2.4.3.1 Degree of cross linking in stationary phase.

The degree of crosslinking of the PS-DVB resin can be varied by the amount of the resin can be varied by the amount of DVB used during the synthesis of the resin. Crosslinking decreases the elasticity of the resin and thereby the swelling and the equilibrium water content (Tiihonen et al., 2001). Saccharides can be sorbed into resin, either by distribution of the saccharides between the liquid inside the resin and the liquid outside the resin or complexation (Adachi et al., 1997). To be able to distribute, the saccharide molecules have to be smaller than the size of the interstices

of the resin. The size of the hydrated monosaccharides is in the same order or magnitude as the size of the pore diameter of a resin. The separation of oligosaccharides (GOS) may be improved by choosing DVB content such that disaccharides and monosaccharides are able to be sorbed by the resin while the GOS are excluded. Higher water content increases the sorption of the saccharides by distribution. The amount of cations per volume unit water increases with increasing DVB content. Therefore, complexation driven sorption might improve relative to the sorption by distribution with increasing DVB content. The DVB content not only influences the equilibrium sorption properties of the resin, but also the sorption kinetics and mechanical properties such as elasticity and attrition resistance. There is a wealth of information in literature supporting the strong influence of crosslinking in sugar separation. Resins with DVB content between 3 % and 8% are suitable for the separation of two hexoses (Lefevre, 1992). The separation of glucose from maltose improves with increasing DVB content. For malto-OS and DVB contents between 2 % and 6 %, the separation improves with DVB content, but is completely lost for a DVB content of 8 % due to size exclusion of the larger OS by the resin (Adachi et al., 1999). It can be concluded from these data that crosslinking has a profound effect on the sorption and separation properties of PS-DVB resin and more importantly that these effects are dependent on the type of sugar.

2.4.3.2 Type of counter-ion.

It has been known that variation of the counter ion attached to a PS based cation exchanger affects the chromatographic behaviour in sugar separation. The separation of many mixtures is significantly improved by the correct choice of the counter ion. When sugars in the mobile phase come in contact with the counter ions in

the stationary phase, they form coordination complexes of varying stability. The stability of the complex formed with the counter ion will increase in relation to the availability for coordination (Morel-Desrosiers et al., 1989). It has been shown through nuclear magnetic resonance and electrophoresis studies that certain orientation of hydroxyl groups (the groups that form complex with the counter ions of the resin) in the sugars imparts stability to the complexes. Therefore, in a column chromatography, the retention of sugars should increase with the number of pairs of favorably oriented hydroxyl groups present. Although the materials used for the separation are ion exchangers, ions are on purpose not exchanged in the process. In fact, water molecules from the hydrated cations are exchanged for (hydrated) sugar molecules to form different coordination complex (Stefansson et al., 1996). Cation exchange resins with different cation have been used in sugar separation. For analytical applications, it is recommended to use Ca^{2+} for the separation of monosaccharides and OS up to a degree of polymerization (DP) of 4. Ag^{+} loaded resins are more selective for OS up to DP 12 (Scobell et al., 1981; Antosova et al., 1999; Van Riel et al., 1986) and resins in the Pb^{2+} form for monomeric sugars. However, (Pirisino, 1984), it is problematic to use Ag^{+} or Pb^{2+} in a food ingredient production process because they are toxic. Na^{+} loaded cation exchange resins are applied in the starch industry for industrial separation of malto-OS (Shioda, 1992). Data on the separation of malto-OS with monovalent cations including Na^{+} and K^{+} were not compared with data on divalent cations such as Ca^{2+} (Adachi et al., 1989). Ca^{2+} (Yang et al., 1995) and Na^{+} (Takahashi et al., 1994) were reported to separate fructo-OS. However no information for the separation of GOS comparing different cations was found in the literature.

2.4.3.3. Temperature

Temperature has critical effects on sugar adsorption on cation exchange resins; however, only few studies are available (Goto et al., 1993) and they were mainly reported in industrial patents (McCulloch, 1994), involving high temperatures (60 C or higher). Theoretically, using high temperatures in chromatographic separations results in less broadening of the elution curves due to an increased diffusion rate inside the resin particles. However, raising temperature indefinitely is not desirable because sugars may lose their chemical identity at high temperatures and change their configuration. Moreover, it could break complex formation between the resin and the sugars (Saska, 1992).

2.4.3.4 Flow rate

The velocity of mobile phase in the column may vary significantly across the column diameter depending on the particle shape, porosity and the whole bed structure. Figure 2.5 shows the importance of finding optimal flow rate to obtain the higher column efficiency in the separation process.

An efficient chromatography column is one in which there is a large number of theoretical plates (N) or the height of the plate (HETP: the Height Equivalent to a Theoretical Plate) is small. The theory uses the concept of theoretical plates, in which the column is considered to consist of a series of thin sections or "plates", each of which permits a solute to equilibrate between the mobile and the stationary phases (Ladisch, 2001). The movement of a solute among the column is viewed as a stepwise transfer from one plate to the next.

Van Deemter plot (Figure 2.6), which equation is $H= A+B/u + Cu$, is used to obtain the optimum mobile velocity to separate any compound and to obtain the

maximum column efficiency. In this equation u is the average velocity of the mobile phase. A, B and C are Eddy diffusion, longitudinal diffusion and resistance to mass transfer factors respectively which contribute to band broadening.

a) Eddy diffusion

The solute molecules traversing a packed chromatographic column can follow a multitude of paths through the column. Each of these paths is of a different length, so that different molecules of the same component will take slightly varying times to travel through the column. This band broadening process is independent of the flow-rate (average linear velocity) of the mobile phase. The eddy diffusion effect can be minimized if the column is packed uniformly with particles of constant size. And also with particles with smaller diameter.

b) Longitudinal diffusion

The concentration of solute is less at the edges of the band than at the center. Molecules constituting a band of sample component in a chromatographic system will tend to diffuse out of the sample band during passage through the column. This diffusion occurs both in the direction of flow of the mobile phase, and in the opposite direction. Since diffusion is time-dependent process, the longitudinal diffusion effect increases at low mobile phase flow-rates.

c) Resistance to mass transfer

In an ideal chromatographic system, the interchange of solute molecules between the mobile and stationary phases would be instantaneous. This does not occur in practice. Additionally, different molecules of the same samples component may spend different amounts of time in the stationary and mobile phases. This leads to band broadening effect, which increases as the mobile phase flow- rate increases. Band broadening due to mass transfer can be minimized through the use of packing

materials that are either of small diameter (and therefore have short diffusion paths) or lowering the flow-rate of the mobile phase.

2.4.4 Simulated moving bed

Stimulated-moving bed system (SMB) is the most sophisticate chromatographic system. In an SMB the countercurrent movement of mobile and stationary phases has some advantages, which make it a process with maximum economy. First of all, the efficiency requirement for the sorbent is lower compared to other chromatographic modes. In contrast to batch chromatography, no total resolution has to be achieved at the column outlet. Only parts of the fronts of the axial concentration profile are withdrawn in pure form from the system. In addition, the time-space yield in term of productivity is higher because of the improved utilization of the sorbent. This leads to a third advantage of SMB chromatography: the product dilution is lower, pure fractions are withdrawn with a high yield and no work-up of fractions of less purity is necessary. Also the process is continuous and can therefore be perfectly implemented into continuous production processes (Schulte et al., 2000).

Simulated-moving bed system (SMB) processes have been successfully used in the petrochemical industry over the last 30 years. For the past 25 years, SMB processes have been applied on a massive scale in the carbohydrate industry, mostly to separate fructose-glucose solutions, resulting from the enzymatic conversion of cornstarch, in order to produce "high" fructose corn syrup (HFCS). Since fructose index of sweetness is about twice as much as that of glucose, the separation of fructose from this mixture and recycling of glucose for enzymatic isomerization is of great commercial importance.

Another important application of the SMB technology in the carbohydrate field is the separation of fine sugars and amino acids from such feedstocks as molasses and biomass hydrolisates. Molasses is a by-product from the production process of sucrose, which may be obtained either from beet or sugarcane. Companies such as Amalgamated Sugar Co. (USA), Nitten (Japan) and Organo (Japan) currently make use of the principles of SMB technology to separate sugars from non-sugars (Rearick et al., 1997), malto-OS from glucose and maltose and isolate fine chemicals like raffinose (Sayama et al., 1992) and betaine (Kikuzo et al., 2000) respectively. Also the Table 2.8 summarizes some relevant patents issued in the last 20 years, which illustrate the wide-spread use of SMB technology in the field of carbohydrate separations.

Figure 2.7 shows the design of a SMB. The adsorbent (resin) is packed into single columns, normally six to twelve, which are connected in series to form a circle. The temperature and the pH are kept constant at the optimum conditions for the equilibrium between resin and the solutes in the mobile phase. Before each column head, four two-way valves are integrated, which allow the introduction of the feed mixture and of fresh eluent, or withdrawal of the less retained compound(s) (called raffinate) or the more retained compound(s) (called extract) (lactose and monosaccharides: glucose and galactose). At a given time, only one of the lines will be active at one column head. The four different streams at the mobile phase, which are circulated inside the columns, are moved by five pumps whose flow rates are determined and set individually. In a simulated-moving bed system (SMB), the adsorbent remains fixed in the column, advancing the concentration profile of the components susceptible of separation in the mobile phase direction through this adsorbent. Once the concentration profiles move through one column is necessary to

advance inlets and outlets in the same way, in order to maintain their relative position compared with it. The inlets and outlets will stand fixed as long as the concentration profile moves in the same column. This fixed time is known as "switch time", at every switch time the inlets and outlets will be moved forward one column length in the direction of the fluid flow, simulating the movement of the stationary phase in countercurrent direction to the mobile phase.

The total chromatographic bed will be divided by the position of the inlets and outlets into four different zones, each with their own properties as it is also shown in Figure 2.8:

-Zone I: between eluent and extract port, desorption of the more retained compound (extract)

-Zone II: between extract and feed port, desorption of the less retained compound (raffinate)

-Zone III: between feed and raffinate port, adsorption of the more retained compound (extract)

-Zone IV: between raffinate and eluent port, adsorption of the less retained compound (raffinate).

Between Zones II and III the feed mixture is introduced into the system and transported with the mobile phase into Zone III. Here, the compounds which have higher affinity to the sorbent (lactose, glucose and galactose) are adsorbed and transported with the stationary phase to Zone I, where they are desorbed by the fresh eluent which is introduced between Zones I and IV, plus recycled eluent coming from Zone IV. The less retained compounds (GOS) are moved with the mobile phase to the Zone IV and have to be adsorbed in that Zone. They are transported with the stationary phase to Zone II, where they become desorbed. The different adsorption

and desorption features are controlled via the flow rates of the external pumps and the switch time (Mattisson et al., 1998).

2. 5 References

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Name	Molecular structure	Physiological features
Lactulose	Gal-Fru	β-1,4
Lactosucrose	Gal-Glc-Fru	β-1,4
Raffinose	Gal-Glc-Fru	α -1,6
Galacto-oligosaccharides	$(Gal)_n$ -Glc	β-1,4, β-1,6
Fructo-oligosaccharides	$(Fru)_n$ -Glc	β-1,2
Soybean-oligosaccharides	$(Gal)_n$ -Glc-Fru	α -1,6
Isomalto-oligosaccharides	$(Glc)_n$	α -1,6
Xylo-oligosaccharides	$(Xy)_n$	β-1,4
Palatinose-oligosaccharides	$(Glc\text{-}Fru)_n$	α -1,6
Glycosylsucrose	$(Glc)_n$-Fru	α -1,4
Malto-oligosaccharides	$(Glc)_n$	α -1,4
Cyclodextrins	$(Glc)_n$	α -1,4 cyclic structure
Gentio-oligosaccharides	$(Glc)_n$	β-1,6

Table 2.1: List of non- digestible oligosaccharides with their chemical characteristics (Sako et al., 1999).

Product	Oligosaccharide content	Manufacturer
Galacto-oligosaccharides		
TOS-syrup (Syrup)	60% solids	Borculo Whey Products, Netherlands
Oligomate 55 (Syrup)	> 55% solids	Yakult Honsha Co. Ltd, Japan
Oligomate 55 P (Powder)	> 55%	
TOS- 100 (Powder)	> 99%	
Cup-Oligo H-70 (Syrup)	70% solids	Nissin Sugar Manufacturing Co.Ltd, Japan
Cup-Oligo P (Powder)	70%	
Fructo-oligosaccharides		
Raftilose L30 (Syrup)	> 30% solids	Orafti, Belgium
Raftilose L60 (Syrup)	> 60% solids	
Raftilose L85 (Syrup)	> 85% solids	
Raftilose L95 (Syrup)	> 95% solids	
Raftilose P95 (Powder)	95%	
Actilight P (Powder)	95%	Beghin-Meiji Industries, France
Actilight G (Syrup)	55% solids	
Meioligo P (Powder)	95%	Meiji Seika Kaisha, Japan
Meioligo G (Syrup)	55% solids	
NutraFlora (Powder)	95%	Golden Technologies Co., USA
Oligo-Sugar (Syrup)	60% solids	Cheil Foods and Chemicals, Korea
Oligo-Sugar (Powder)	23%	
Inulin		
Raftiline ST (Powder)	N/A	Orafti, Belgium
Soy bean oligosaccharides		
Soya-oligo	35% solids	The Calpis Food Industry Co., Japan
Isomalto-oligosaccharides		
Isomalto-500 (Syrup)	> 50% solids	Showa Sangyo Co., Japan
Isomalto-900 (Syrup)	> 80% solids	
Isomalto-900P (Powder)	> 85%	
Panorup (Syrup)	≥ 50% solids	Hayashibara Shoji Inc., Japan
Panorich (Syrup)	> 50% solids	Nihon Shokunin Kako Co., Japan
Biotose # 50 (Syrup)	> 50% solids	
Xylo-oligosaccharide		
Xylo-oligo 20P (Powder)	20%	Suntory Ltd, Japan
Xylo-oligo 35P (Powder)	35%	
Xylo-oligo 95P (Powder)	95%	
Xylo-oligo 70 (Syrup)	75% solids	
Lactosucrose		
Nyuka-Origo LS-40L (Syrup)	42% solids	Ensuiko Sugar Refining Co., Japan
Nyuka-Origo LS-55L (Syrup)	55% solids	
Pet Oligo L55 (Syrup)	57% solids	
Nyuka-Origo LS-55P (Powder)	55%	
Newka-Oligo LS-35 (Syrup)	≥ 35% solids	Hayasibara Shoji Inc., Japan
Newka-Oligo LS-55L (Syrup)	≥ 55% solids	
Newka-Oligo LS-55P (Powder)	≥ 55% solids	
Gentio-oligosaccharides		
Gentose #45 (Syrup)	45% solids	Nihon Shokunin Kako Co., Japan
Gentose #80 (Syrup)	90% solids	
Gentose #80P (Powder)	90%	

Table 2.2: Commercial oligosaccharides products (Gibson et al., 2000).

Plants	**Bacteria**	**Fungi**
Peach Apricot Almond Tips of wild roses Alfalfa seed Coffee berries	*Escheridia coli* *Bacillus megaterium* *Thermus aquaticus* *Streptococcus lactis* *Streptococcus thermopillus* *Lactobacillus bulgaricus* *Lactobacillus helveticus* *Bacillus sp.* ***Bacillus circulans*** *Bacillus stearothermopillus* *Lactobacillus sporogenes*	*Neurospora crassa* *Aspergillus foetidus* *Aspergillus niger* *Aspergillus flavus* ***Aspergillus oryzae*** *Aspergillus phoenicis* *Mucor pucillus* *Mucor miehei* *Scopuloriopsis* *Alternari palmi* *Curvularia inaegualis* *Fusarium moniliforme* *Alternaria alternara*
Animal organs		
Intestine Brain and skin tissue		
Yeast		
Kluyveromyces (Saccharomyces) lactis *Kluyveromyces (Saccharomyces) fragilis* *Candida pseudotropicalis* *Wingea roberstii*		

Table 2.3: Possible sources of β- galactosidase (Gekas et al., 1985).

Enzyme sources	Reaction Conc. (g/L)	Conditions T (ºC)	pH	Max. GOS (w%)	Reference
Aspergillus niger	200	45	4.5	18.9	Kim et al., 1990
Aspergillus oryzae	400	40	4.5	32	[Iwasaki et al., 1996
Bacillus subtilis	200	10	7.0	18	Rahim et al., 1991
Bullera singularis	100	45	4.8	55	Shin et al., 1998
Bifidobacterium longum	400	45	6.8	30.1	Hsu et al., 2007
Cadocellum saccharolyticum	700	80	6.3	42	Stevenson et al., 1996
Cryptococcus laurentii	100			47	Ohtsuka et al., 1988
Kluyveromyces fragilis	350	35	6.2	45	
Penicillium simplicissimum	600	50	6.5	30.5	Cruz et al., 1999
Pyrococcus furiosus	450	75	5.0	29	Boon et al., 1999
Rhodoturola minuta	360	60	6.0	64	Onishi and Yokozeki, 1996
Saccharomyces fragilis	350	35	6.2	45	
Saccharomyces lactis	200	35	6.2	12.5	
Saccharopolyspora rectivirgula	600	70	7.0	41	Nakao et al., 1994
Sirobasidium magnum	360	60	6.0	67	Onishi et al., 1996
Sporobolomyces singularis	100			50	
Streptococcus thermopilus	100	37	7	25	Greenberg et al., 1983
Sterigmatomyces elviae	360	60	4.5	60	Onishi and Tanaka, 1998
Thermus aquaticus	160	70	4.6	35	Berger et al., 1995
Trichoderma harzianum	150	30	7	32	Prakash et al., 1987

Table 2.4: GOS formation conditions of β-galactosidase produced by various microorganisms (adapted from Yang et al., 2001).

Source of enzyme	Immobilization method	Recovery of activity (%)	References
K. fragilis	covalent binding on corn grits	8	Siso et al. (1994)
	covalent binding on cellulose beads	82	Roy & Gupta (2003)
	covalent binding on porous silanised glass modified by glutaraldehyde	90	Szczodrak (2000)
	entrapment in alginate-carrageenan gels		Mammarella & Rubiolo (2005)
	adsorption on phenol-formaldehyde resin	23	Woudenberg-Van Oosterom et al. (1998)
	adsorption onto bone powder	83	Carpio et al. (2000)
K. lactis	covalent binding onto glutaraldehyde-agarose	36-40	Giacomini et al. (2001)
	covalent binding onto thiolsulfinate-agarose	60	
	covalent binding on graphite surface	0.01	Zhou & Chen (2001a)
K. marxianus	covalent binding on oxides supports: alumina, silica, silicated, silicated alumina	< 5	Di Serio et al. (2003)
E. Coli	covalent binding onto glutaraldehyde-agarose	39	Giacomini et al. (2001)
	covalent binding onto thiolsulfinate-agarose	75-85	
	entrapment in liposomes	28	Rodriguez-Nogales & Delgadillo-Lopez (2006)
	covalent binding onto gelatin cross-linking with chromium (III) acetate	25	Sungur & Akbulut (2006)
	covalent binding onto gelatin cross-linking with glutaraldehyde	22	
	adsorption on chromosorb-W	-	Bodalo et al. (1991)
B. circulans	adsorption onto a ribbed membrane made from polyvinylchloride and silica	-	Bakken et al. (1992)
A. oryzae	fibers composed of alginate and gelatine cross-linking with glutaraldehyde	56	Tanriseven & Dogan (2002)
	carbodiimide coupling to alginate beads	76	Dominguez et al. (1998)

Table 2.5: The immobilization of ß- galactosidase by different methods (CONTINUED).

Table 2.5: CONTINUED.

Source of enzyme	Immobilisation method	Recovery of activity (%)	References
A. oryzae	entrapment in a spongy polyvinyl alcohol cryogel	-	Rossi et al. (1999)
	entrapment in cobalt alginate beads cross-linked with glutaraldehyde	83	Ates & Mehmetoglou (1997)
	microencapsulation in alginate beads	64	Dashevsky (1998)
	encapsulation into gelatin and cross-linking with transglutaminase	8-46	Fuchsbauer et al. (1996)
	adsorption on phenol-formaldehyde resin	54	Woudenberg-Van Oosterom et al. (1998)
	adsorption on polyvinylchloride (PVC)	-	Bakken et al. (1990)
	adsorption on silica gel membrane		
	adsorption on celite	2	
	covalent binding to chitosan	18.4	Gaur et al. (2006)
	cross-linked aggregation by glutaraldehyde	13.5	
	covalent binding in polyuretane foams	-	uU et al. (1993)
A. niger	adsorption on a porous ceramic monolith	80	Papayannakos & Markas (1993)
Chicken bean	immobilized on cross-linked polyacrylamide gel	72	Sun et al. (1999)

source of enzyme	mode of process	lac-conc. [g/l]	T [°C]	pH	max. GOS [wt %]	productivity [g/l/h]	Reference
T. aquaticus	Batch (FE)	160	70	4.6	34.8	2.3	Berger et al., 1995a
K. lactis	Bath (FE, UF)	230	45	7	22.2	11.3	Foda and Lopez-Leiva, 2000
	Continuous (FE, UF)	200	45	7	31	13.7	
B. singularis	Continuous (PBR)	100	45	4.8	55	4.4	Shin et al., 1998
A. oryzae	Batch (FE)	380	40	4.5	31	24.3	Iwasaki et al., 1996
A candidus	immobilized on on resine D113	400	40	6.5	37.1	87.1	Zheng et al., 2006

FE: free enzyme, IE: immobilized enzyme, CSTR: continuous stirred tank reactor, PBR: packed bed reactor, UF: ultrafiltration membrane reactor.

Table 2.6: GOS production by using different β-galactosidases in bach and in continuous mode.

Cross-linking reagent	References
Glutaraldehyde	ATES&MEHMETOGLU (1997); SZCZODRAK (2000); ZHOU&CHEN (2001b); TANRISEVEN and DOGAN (2002)
Chromium (III) acetate	SUNGUR&AKBULUT (1994)
Bisimidoesters	KHARE and GUPTA (1988)
Dimethyladipimidate	
Carbodiimide	DOMINGUEZ et al. (1988)
Bis-oxirane	ROGALSKI et al. (1994)
Transglutaminase	FUCHSBAUER et al. (1996)

Table 2.7: Different cross-linking reagents used in ß-galactosidase immobilization.

Application	*Purity*	*Company/Reference*	*Date Issued*	*Patent No.*
Fructose-Glucose Separation (latest improvement in SAREX process)	95%	UOP, Inc.-USA (LeRoy, 1983)	11 Oct., 1983	US4409033
Separation of Psicose from a mixture of Monosaccharides	98%	UOP, Inc. -USA (Chin-Hsing, 1989)	14 Nov., 1989	US4880920
Separation of glucose, maltose and oligosaccharides from starch hydrolizate	97%	Organo Corp.-Japan (Takayuki et al., 1995)	21 Feb., 1995	US5391299
Separation of a water-soluble polydextrose from a glucose-based reacting mixture	98%	Shin Dong Bang Corp.-Korea (Cheon et al., 1998)	03 Nov., 1998	US5831082
Separation of L-Arabinose from Sugar Beet Pulp	98%	Cultor Corp.-Finland (Antila et al., 1998)	04 Mar., 1999	WO99/10542
Demineralization of a beet-sugar solution	97%	Organo Corp.-Japan (Kikuzo et al., 1999)	12 Aug., 1999	WO9940228
Recovery of betaine from sugar-beet molasses with a continuous SMB	96%	Organo Corp.- Japan (Kikuzo et al., 2000)	08 Aug., 2000	US6099654
Recovery of betaine from sugar-beet molasses with a sequential SMB	97%	Dänisco Finland Oy- Finland (Heikkila et al., 2000)	25 Jul., 2000	US6093326

Table 2.8: Main applications of the SMB technology in carbohydrate separation.

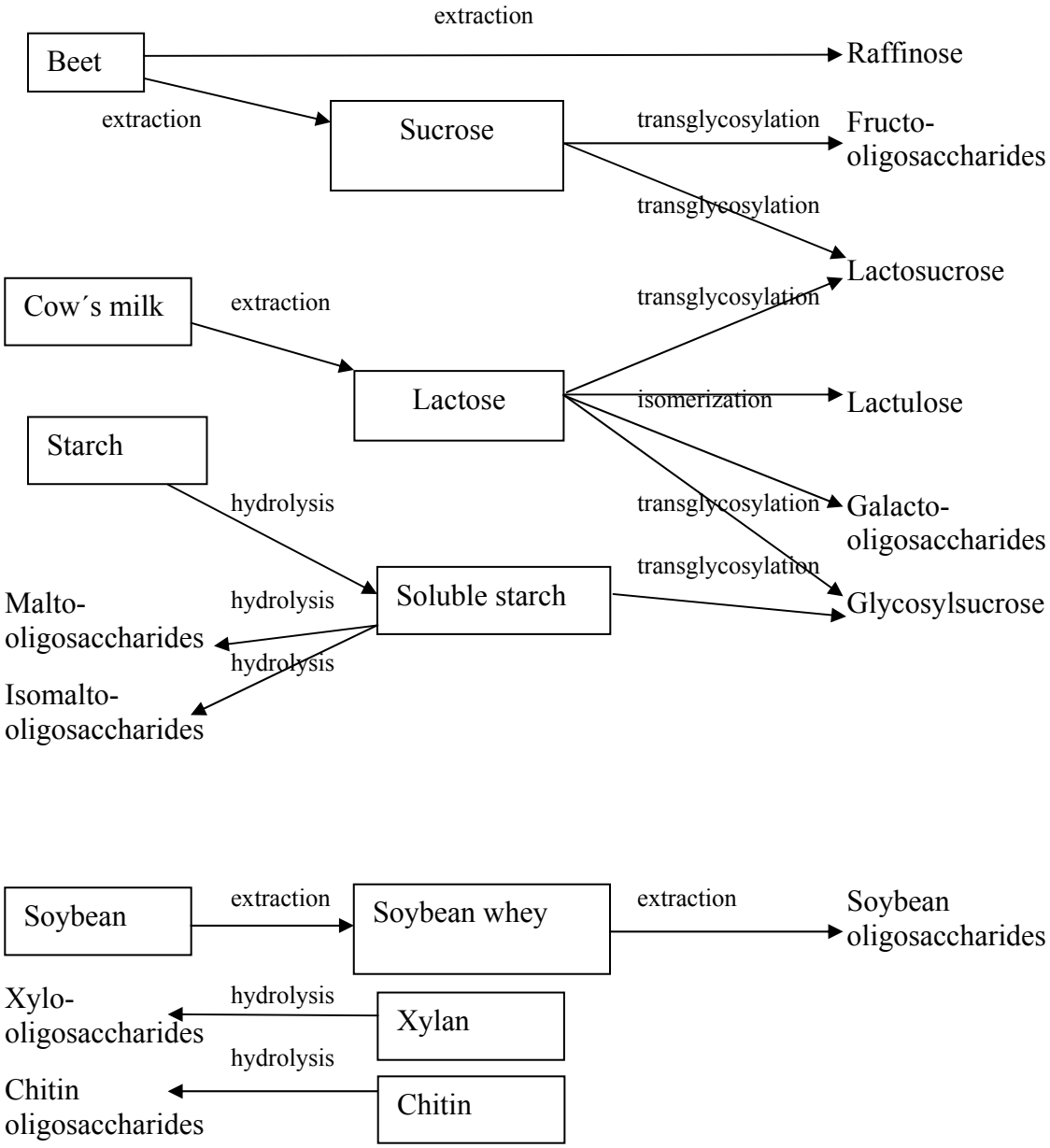

Figure 2.1: Schematic representation of production processes of non-digestible oligosaccharides.

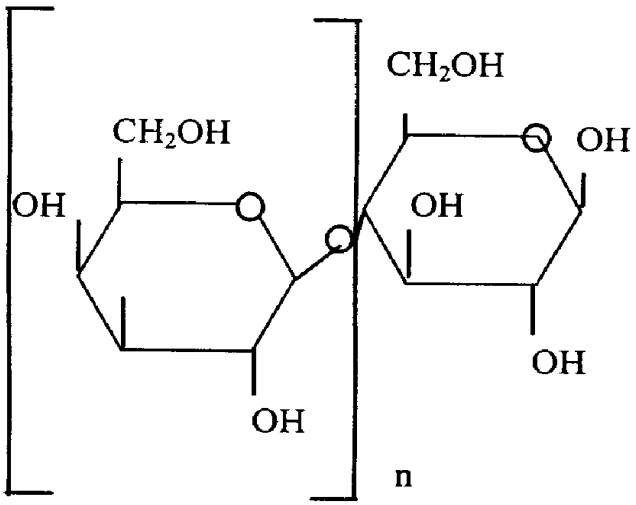

Figure 2.2: Structure of galacto-oligosaccharide

$$\mathbf{L + E \leftrightarrow (E - L) \rightarrow (E - GAL) + GLU}$$

$$(E - GAL) + H_2O \leftrightarrow E + GAL$$

$$(E - GAL) + L \leftrightarrow (E - O_1) \leftrightarrow E + O_1$$

$$(E - GAL) + O_1 \leftrightarrow (E - O_2) \leftrightarrow E + O_2$$

$$(E - GAL) + O_2 \leftrightarrow (E - O_3) \leftrightarrow E + O_3$$

$$GAL + (E - GAL) \leftrightarrow (E - GB) \leftrightarrow E + GB$$

$$GLU + (E - GAL) \leftrightarrow (E - AL) \leftrightarrow E + AL$$

Figure 2.3: General reactions for the synthesis of GOS (Prenosil et al., 1987). L: Lactose; E: β- Galactosidase; O1: Trisaccharides; O2: Tetrasaccharides; O3: Pentasaccharides; GAL: Galactose; GLU: Glucose; GB: Galactobiose; AL: Alolactose

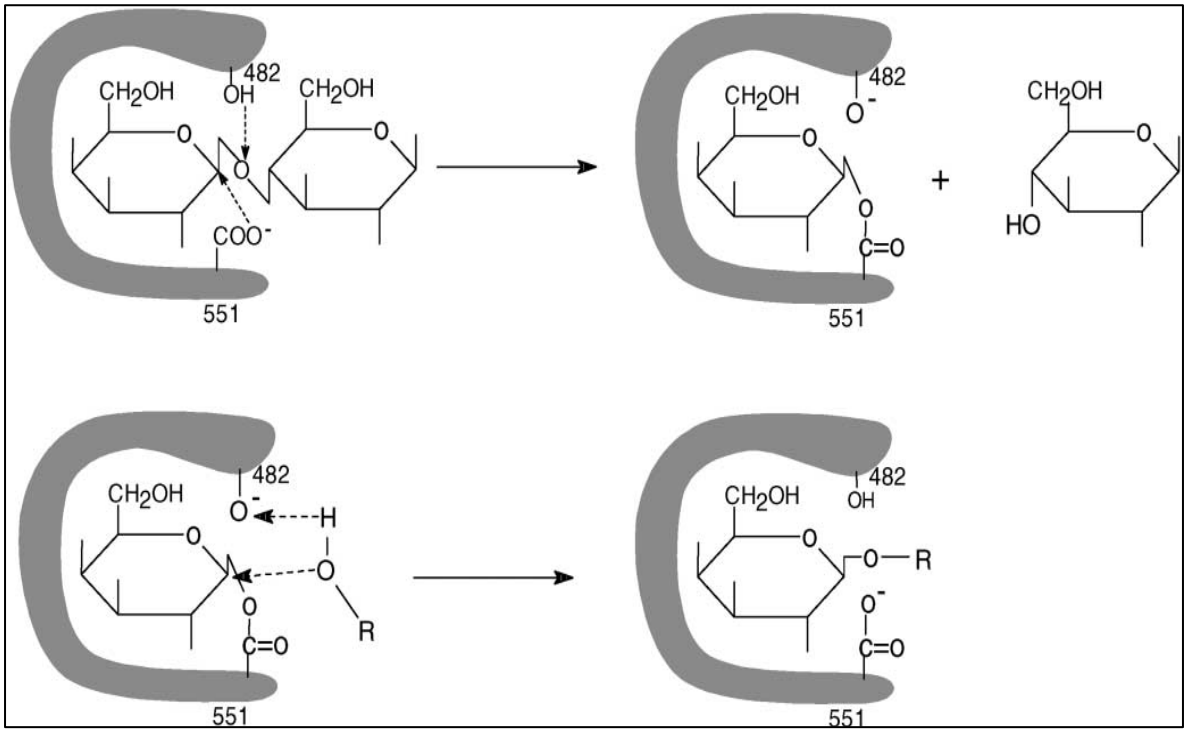

Figure 2.4: Schematic mechanism of the lactose hydrolysis by ß-galactosidase (Zhou et al., 2001b).

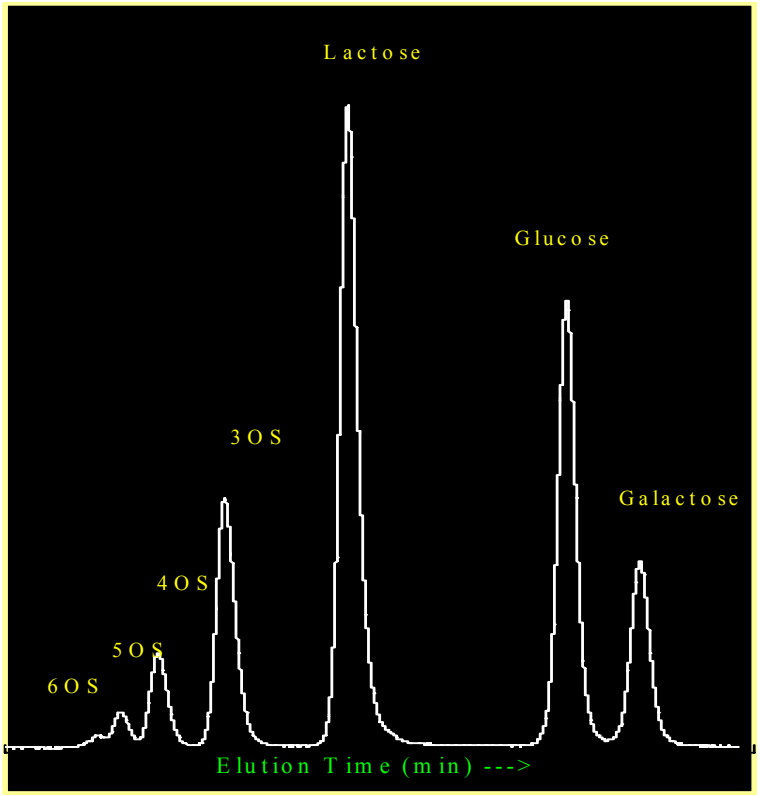

Figure 2.5: Chromatogram showing the separation of various sugars during lactose hydrolysis and transgalactosylation reaction of GOS formation.

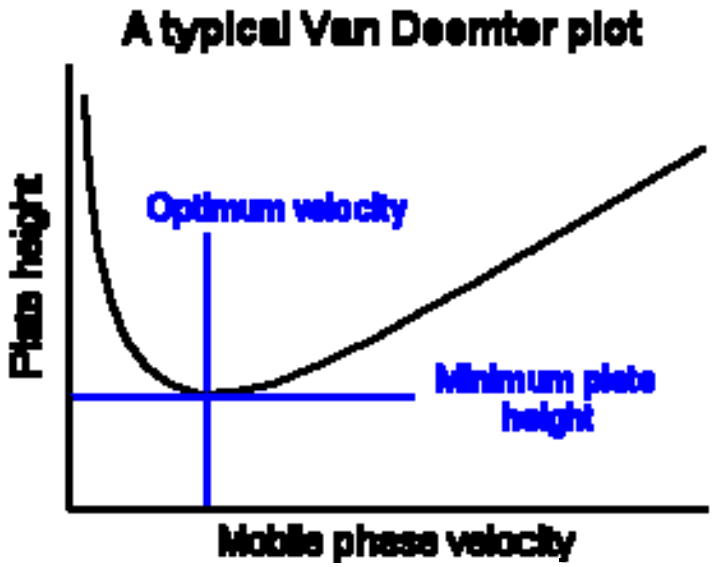

Figure 2.6: A typical Van Deemter plot.

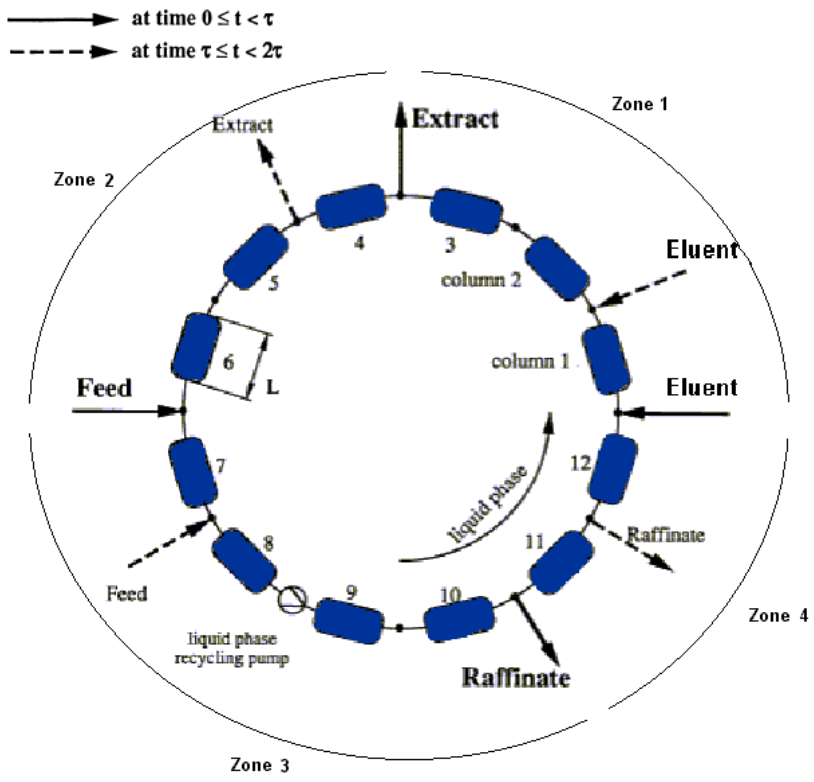

Figure 2.7: Scheme of a four section simulated moving bed system.

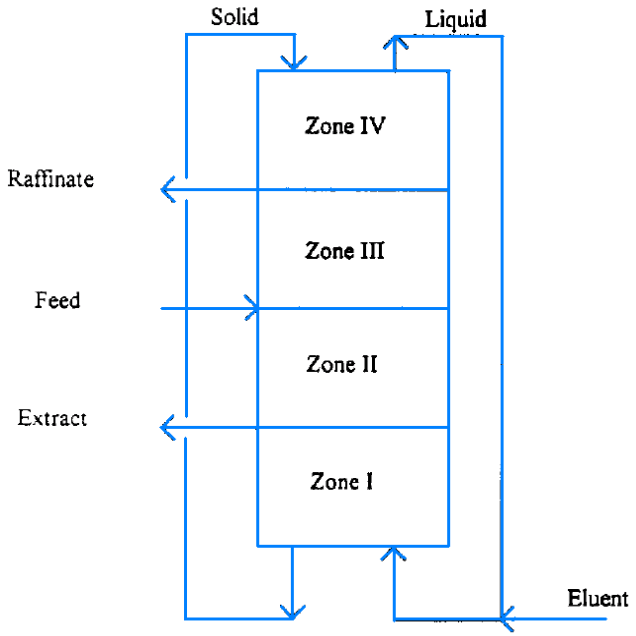

Figure 2.8: Different zones in a SMB system.

CHAPTER 3

PRODUCTION OF GALACTO-OLIGOSACCHARIDES FROM LACTOSE BY USING β-GALACTOSIDASE OF DIFFERENT ORIGINS

Abstract

The aim of this research was to quantify the effects of enzyme origin on the production of galacto-oligosaccharides (GOS) from lactose using ß-galactosidase from *Kluveromyces lactis*, *Aspergillus oryzae* and *Bacillus circulans*. The initial lactose concentration was the most important parameter affecting total amount of GOS produced. In general, more GOS were produced at higher initial lactose concentrations. Enzyme origin clearly affected the amount, types and size of GOS produced. A maximum GOS production of 40% (w/w) from 400 g/l lactose was achieved with *B. circulans* lactase, followed by *K. lactis* and *A. oryzae* with 31% and 27% GOS, respectively. Furthermore, *K. lactis* lactase produced the maximum GOS amount at a high lactose conversion of 75% as compared to the other enzymes. The pH affected the reaction rate but not the amount of GOS produced. However, at acidic pH of 4.5, *K. lactis* lactase was denatured. Also, the addition of galactose inhibited the enzyme and reduced both GOS yield and reaction rate.

3.1 Introduction

Consumers are becoming increasingly aware of the influence of diet on health and quality of life. During the past years, consumers have started to demand foods with certain beneficial effects on the human body. Furthermore, by 2010, Nutrition Business Journal predicted the functional food market in the US will double to $34 billion, representing 5.5% of the total food market. One of the ingredients used in these foods are galacto-oligosaccharides (GOS) (Schoterman, 2007). GOS are non-digestible sugars and known as *Bifidus growth factor*. They provide many health benefits, including selective stimulation of bifidobacteria in the lower intestine and suppression of potentially harmful bacteria, such as clostridia and *Bacteriodes* in the gut. These include non-cariogenicity, prevention of constipation, reduction of blood pressure and low calorific among others (Gibson, 2004; Cummings et al., 2002; Rivero-Urgell et al., 2001; Fooks et al., 1999; Tomomatsu, 1994). GOS occur naturally in breast and cow milk, honey and a variety of fruits and vegetables, but only in trace amounts **(**Angus et al., 2005; Bednarcik, 1998**).** As a result, the development of enzymatic production of GOS is necessary. GOS can be readily produced by enzymatic transgalactosylation from whey lactose, which is an abundant by-product in the cheese industry. Many uses have been found for lactose, including uses in infant formula, and bakery, dairy and confectionary products. However, its use in foods is somewhat limited because of low solubility, indigestibility by some individuals and tendency to crystallize in water at low temperature. Furthermore, it has been estimated that cheese production increases more than 3% annually and almost half of the entire production is disposed of (Novalin et al., 2005; Lodez-leiva et al., 2000; Yang et al., 1995). Consequently, finding a technology to transform lactose into more

profitable products such as GOS is of high interest to the food industry. Various oligosaccharides (OS) have been introduced as prebiotic food ingredients and dietary supplements during the last 10 years and their industrial production and applications are continuously increasing. Among these oligosaccharides, GOS have shown to be very stable at high temperatures and low pH. Therefore, GOS can be used in a variety of products, including fermented milk products, breads, jams, confectionery, beverages, etc. (Sako et al., 1999; Playne et al., 1996; Crittenden et al., 1996)

GOS are produced from lactose or whey permeate by enzymatic transgalactosylation reaction using ß-galactosidase, which occurs widely in nature and has been isolated from animal and plant sources as well as microorganisms. However, compared with animal and plant sources, microbial enzymes are usually produced at higher yields and are more technologically important. Also, the amount and nature of GOS formed depend on the enzyme source (Grosova et al., 2008; Mahoney, 1998; Zarate et al., 1990). Different microbial sources of ß-galactosidase have been used to produce GOS, including, *Escherichia coli* (Chen et al., 2003), *Lactobacillus reuteri* (Maischberguer et al., 2008), *Bulleria singularis* (Cho et al., 2003), *Thermus aquaticus YT*-1 (Berger et al., 1995), *Kluveromyces lactis* (Zhou et al., 2002*), Kluveromyces fragilis* (Boon et al., 2000), *Aspergillus niger* (Prenosil et al., 1987), *Thermotoga maritime* (Ji et al., 2005), among others. However, many of these enzymes have not been approved for food use, are expensive, or were from sources not commercially available or not available in sufficient quantities for industrial applications.

The goal of this project was to quantify and compare the performance of 3 different lactases produced from yeast (K*luveromyces lactis*), fungus (*Aspergillus oryzae*)

and bacterium (*Bacillus circulans*) in the production of GOS. These 3 enzymes are commercially available, are relatively inexpensive and have been recognized as safe for food production (Ladero et al, 2001**;** Di Serio et al., 2003). These enzymes have different optimal reaction conditions and performed differently in terms of GOS production rate and yield. Also, the maximum amount of GOS produced by these enzymes is obtained at different lactose conversion. GOS production by using these lactases in a system of sequential plug-flow reactors could lead to increased GOS yield and reactor productivity. In this chapter, effects of lactose concentration, galactose, pH, temperature and whey permeate on GOS production by different lactases were studied.

3.2 Materials and Methods

3.2.1 Materials

ß-galactosidase from *Bacillus circulans* (bacterial lactase activity 5500 LU/g) was obtained from Daiwa Kasei (Shiga, Japan). One lactase unit (LU) is defined as the amount of enzyme, which liberates 1 µmol of glucose per minute from lactose (concentration 10%) at the early stage of the reaction at 40^{o}C, pH 6. ß-galactosidase from *Kluveromyces lactis* (yeast lactase activity 3000 LU/ml) was obtained from Valley Research (South Bend, IN). One lactase unit (LU) is defined as the amount of enzyme which liberates 1 µmol of glucose per minute from lactose (concentration 4.75%) at pH 6.5 at 37 ^{o}C. ß-galactosidase from *Aspergillus oryzae* (fungal lactase activity 106,742 LU/g) was obtained from Enzyme Development Corporation (New York, NY). One lactase unit (LU) is defined as the amount of enzyme which liberates 1 µmol of glucose per minute from lactose at pH 4.5 at 37^{o}C. Lactose (99.9%) and whey permeate was

obtained from Brewster Dairy (Brewster, OH). Polyethyleneimine [PEI; $(C_2H_5N)_n$] (number average molecular weight: 60,000; average molecular weight: 750,000), as 50% (w/v) solution, and glutaraldehyde (GA), as 25% (w/v) solution, were from Sigma (St. Louis, MO). Sodium phosphate monobasic anhydrous (Fisher) and sodium phosphate dibasic anhydrous (E.M. Science) were used to prepare phosphate buffer. Glacial acetic acid (Fisher) and sodium acetate anhydrous (Fisher) were used to prepare acetic acid buffer. Cotton cloth was obtained locally. All solutions for PEI, GA, and enzyme were prepared with distilled water. The solution pH was adjusted, when necessary, using HCl or NaOH solution of sufficient concentration.

3.2.2 Reaction kinetics

GOS formation kinetics with ß-galactosidase was studied for different lactases. The reaction was carried out in 125 mL Erlenmeyer flasks in a shaker-incubator at 35, 40 and 45 ^{o}C with enzyme concentrations of 10 μL/mL for *Kluveromyces lactis* lactase and 1 mg/mL for *Aspergillus oryzae* and *Bacillus circulans* lactases. The lactose solution was prepared by dissolving lactose in distilled water at pH 6 for *B. circulans* lactase, in phosphate buffer at pH 6.5 for *K. lactis* lactase and in acetate buffer at pH 4.5 for *A.* oryzae lactase. Unless otherwise noted, the reaction kinetics was studied at 400 g/L lactose. Samples (100 μL) were drawn from the reaction mixtures at appropriate time intervals and added to 900 μL of distilled water at 95 °C to inactivate the enzyme and stop the reaction. The sugar contents were analyzed with HPLC.

3.2.3 Effect of lactose concentration on GOS production

The effect of lactose concentration on GOS production was evaluated by using lactose concentrations of 50, 100, 200, 300, 400 and 500 g/L at pH 4.5, 6 and 6.5 using 125 mL Erlenmeyer flasks in a shaker-incubator at 40, 35 and 45^{o}C for *A. oryzae*, *K. lactis* and *B. circulans* lactases, respectively.

3.2.4 Effect of pH on GOS production

The effect of pH on transgalactosylation was studied using lactose solution of 400 g/L over a pH range of 3.5 to 6 at 40^{o}C for *A. oryzae* lactase, 4.5 to 7.5 at 45^{o}C for *B. circulans* lactase and 5 to 8 at 35^{o}C for *K. lactis* lactase.

3.2.5 Effect of galactose on GOS production

The effect of galactose on GOS production was studied by adding different galactose concentrations (2.5, 5 and 10% (w/v)) in a lactose solution of 400 g/L for *K. lactis* and *B. circulans* lactases. The solutions were incubated in 125 mL Erlenmeyer flasks in a shaker-incubator at 35^{o}C for *K. lactis* lactase and 45^{o}C for *B. circulans* lactase.

3.2.6 Effect of substrate nature on GOS production

The effect of substrate nature (why permeate vs. lactose solution) on GOS production was investigated by using a lactose solution and whey permeate containing 400 g/L of lactose as the substrate for *B. circulans* lactase. The solutions were incubated in 125 mL Erlenmeyer flasks in a shaker-incubator at 45^{o}C.

3.2.7 Analytical Methods

3.2.7.1 HPLC Analysis

The concentrations of various sugars (glucose, galactose, lactose, and galactooligosaccharides) were determined by using HPLC. An HPLC system consisting of a pump (LC-10AI Shimadzu), an automatic sampler injector (Shimadzu SIL10Ai), a carbohydrate analysis column (Phenomenex, Rezek RNM carbohydrate column, 7.8 mm-150 mm), a column oven (Shimadzu CTO-10A), a refractive index detector (Shimadzu RID-10Avp), and a Shimadzu CLASS-VP chromatography data system (version 7.2 integrator) was used. The eluent was pre-degassed distilled water (at 80 °C) at a flow rate of 0.4 mL/min. Distilled water was degassed by first boiling and then sonicating for 30 min. The column temperature was maintained at 80 °C, and the detector temperature was set at 45 °C. The concentrations of these sugars (e.g., lactose, glucose, galactose, and oligosaccharides including tri-, tetra-, and pentasaccharides), presented as weight percentages of total sugars, were determined from peak heights and are reported.

3.3 Results and Discussion

3.3.1 Effects of enzyme origin on reaction kinetics

Figures 3.1-3.3 show typical reaction kinetics for lactose hydrolysis and GOS formation of three different lactase enzymes. In all cases, high GOS formation was obtained with a rapid decrease in lactose concentration until the maximum GOS percentage was reached. Then, the lactose concentration continued to decrease at a lower rate. However, the rate of GOS formation was different for different enzymes. ß-galactosidase from *K. lactis* showed the fastest initial rate of GOS formation and

produced 31 % (w/w) GOS (Figure 3.2a), followed by *A.* oryzae lactase with 27% GOS (Figure 3.1a). *B. cirulans* lactase produced the highest amount of GOS, with 40% GOS (Figure 3.3a), but had the lowest rate of GOS formation. However, it is important to note that *B. circulans* lactase was provided by the manufacturer with low protein content (10%) and with a large amount of impurities and other unknown substances. Also, ß-galactosidase from *B. circulans* is present in two different isomers: ß-galactosidase-1 and ß-galactosidase-2. This work used ß-galactosidase-2 because this isomer produces a higher amount of GOS (Mozaffar et al., 1984).

In all cases, trisaccharides were the main GOS synthesized by ß-galactosidase. Larger GOS were produced in lesser amounts and more time was necessary to obtain them. This result was expected since based on the mechanism of GOS formation in order to produce 4OS it requires the presence of 3OS, and so on. While GOS was reaching the maximum percentage and when started to decrease, glucose and galactose concentrations were increasing continuously. This demonstrated that transgalactosylation reaction dominated early in the reaction, producing GOS with a high yield, and when GOS percentages started to decrease, the hydrolysis reaction took over, producing both glucose and galactose with a slow but progressive decrease in the GOS concentration. If the reaction was allowed to continue, only hydrolytic products would be present in the final product solution. The presence of large amounts of monosaccharides in the late stage of the reaction caused a slow down in the reaction rate since glucose and galactose acted as inhibitors of the enzyme. The amount of glucose in the reaction mixture was found to be much higher than galactose, especially at the beginning of the reaction, indicating the involvement of galactose in GOS formation. However, the amount of galactose in the

reaction varied depending on the enzyme source. Figure 3.3 shows that the amount of galactose present during GOS formation in the early stages of the reaction with *B. circulans* lactase was only 2%. Clearly, most of the galactose from lactose was involved in GOS formation and consequently a higher amount of GOS was formed with *B. cirulans* lactase.

Table 3.1 shows the percentages of the different sugars obtained at maximum GOS levels for *A. oryzae*, *K. lactis* and *B. circulans* lactases, and the chromatograms are shown in Figures 3.4-3.6. The maximum GOS amount, with 40% of total sugars, was achieved by *B. circulans* lactase at 60% lactose conversion (Figure 3.3b), defined as lactose converted to other sugars: GOS and monosaccharides. Lactase from *B. circulans* produced not only the maximum amount of GOS but also the most diverse types. The GOS produced from the reaction was primarily composed of trisaccharides (23.08%). Larger GOS such as tetra- (11.56%), penta- (4.1%) and hexasaccharides (1.48%) were also produced at lower levels. Also, larger GOS reached their maximum levels at higher lactose conversions.

Figure 3.2b shows that the maximum amount of GOS produced with *K. lactis* lactase was 31% GOS at 75% lactose conversion. The composition of the different types of GOS was as follows: 25.04% trisaccharides, 3.76% tetrasaccharides and 1.29% pentasaccharides. Table 3.1 shows that *K. Lactis* lactase produced primarily trisaccharides and the production of larger GOS was minimal compared with other lactases. It is possible that the high amounts of glucose and galactose present in the reaction mixture could act as inhibitors for the formation of GOS by favoring the hydrolysis reaction. Finally, the lowest GOS formation was achieved from *A. oryzae*

lactase with 27% GOS at 50% lactose conversion (Figure 3.1b). In this case, the different types of GOS obtained were: 18.3% (w/w) trisaccharides, 5.77% tetrasaccharides, 2.35% pentasaccharides, and 1.01% hexasaccharides.

Furthermore, GOS yield, defined as the GOS formed from the amount of lactose converted to products, was obtained as function of lactose conversion for the 3 lactases (Figures 3.1-3.3c). GOS yield was relatively constant at ~70% until reaching 60% lactose conversion for *B. circulans*, ~60% until 50% lactose conversion for *A. oryzae*, and ~50% until 75% lactose conversion for *K. Lactis*. GOS yield than fell rapidly once passing the critical lactose conversion point. In all cases, the maximum GOS yield corresponds to the period in which GOS production increased and transgalactosylation reaction dominated.

The chromatograms for *B. circulans* (Figure 3.6) and *K. lactis* (Figure 3.5) lactases show a peak near to lactose that could represent some amounts of 2OS. This peak accounts for 2% of total sugars for *B. circulans* lactase and 1.7% of total sugars for *K. lactis* lactase. However, the peak did not follow a clear pattern during the reaction course in the case of *B. circulans* lactase. For this reason, 2OS produced from this enzyme was not included as part of GOS. It should be noted that the analytical system employed in this study could not differentiate different disaccharides. Since the main hydrolysis products of the reaction, glucose and galactose, could also serve as acceptors of the galactosyl moiety, it is likely that certain amounts of various non-lactose disaccharides were formed during the transgalactosylation reaction, especially during the later phase of the reaction when considerable amount of glucose and galactose were present in the reaction mixture. Also, for both enzymes, there was an imbalance between the experimental value of the mole fractions of glucose and galactose and the stoichometric

values, supporting the formation of higher amount of disaccharides. Furthermore, the formation of disaccharides other than lactose could be accounted for up to 12% of total sugars when using *B. circulans* lactase (Yanira et al., 1995**;** Mozzart et al., 1984) and the formation of 2 disaccharides was observed when using *K lactis* lactase (Prenosil et al., 1987a**;** Boon et al, 2000). The chromatogram (Figure 3.4) of *A. oryzae* lactase does not show any peak representing disaccharides, supporting previous studies with this enzyme (Albayrak et al, 2002).

3.3.2 Effect of lactose concentration on GOS production

The initial lactose concentration is an important factor affecting GOS formation (Zarate et al., 1990, Boon et al., 2000). Figures 3.7-3.9 show the production of GOS at 6 different initial lactose concentrations (50, 100, 200, 300, 400 and 500 g/L) during lactose conversion by using *A. oryzae*, *B. circulans* and *K. lactis* lactases, respectively. In all cases, as the initial lactose concentration increased, the maximum production of GOS also increased from 11% GOS at 5% lactose to 27.8% GOS at 50% lactose for *A. oryzae* lactase (Figure 3.7), from 25% GOS at 5% lactose to 42% GOS at 50% lactose for *B. circulans* lactase (Figure 3.8), and from 14% GOS at 5% lactose to 32.3% GOS at 50% lactose for lactase from *K. lactis* (Figure 3.9). Consequently, the GOS yields were higher at higher initial lactose concentrations. Higher GOS amounts were obtained at higher lactose concentrations because in a diluted lactose solution, water can be more competitive to be an acceptor for the β-galactosyl groups, releasing galactose from the active site. On the other hand, in a high lactose concentration solution, lactose has more chances to act as the acceptor for the β-galactosyl groups, binding with the ezyme-

galactose complex and forming GOS (Zhou et al, 2001). Also, in all cases, when the initial lactose concentration was higher, the maximum GOS content was achieved at a higher lactose conversion level. In general, the transgalactosylation reaction dominates at low lactose conversions. Then, after reaching the maximum GOS content, the hydrolysis reaction took over at high lactose conversions. Also as the initial lactose concentration increased, higher amounts and larger GOS were obtained. The amount of 3OS produced from *A. oryzae* lactase changed from 9.2% at 5% lactose to 18.3% at 50% lactose, while 4OS and 5OS increased from 1.5% and 0.2% at 5% lactose to 5.5% and 3.1% at 50% lactose, respectively (data not shown). Moreover, some 6OS (1.1%) was found when lactose solution was about or higher than 30%. Also, the amount of 3OS produced by *B. circulans* lactase changed from 16.9% at 5% lactose to 23% at 50% lactose, while 4OS and 5OS increased from 5.8% and 1.5% at 5% lactose to 12.1% and 4.3%% at 50% lactose. Also, *B. circulans* lactase produced certain amounts of 6OS, up to 2.5%. However, 6OS were present at all lactose concentrations (Table 3.2). Finally, the amount of 3OS produced by *K. lactis* lactase changed from 10.6% at 5% lactose to 25.5% at 50% lactose, while 4OS and 5OS increased from 1.15% and 0% at 5% lactose to 4.4% and 1.1% at 50% lactose (data not shown). No 6OS was produced with this enzyme. The difference in the total GOS production by *K. lactis* lactase between using 200 g/L and 500-g/L lactose solution was no more than 5%. Furthermore, no significant amount of 5OS was produced. In contrast, clear increments on GOS production by the other two lactases were obtained as the lactose concentration increased. In particular, *B. circulans* lactase produced the most abundant amount, the most different and largest-sized GOS. In general, the amount of larger GOS increased more proportionally than the smaller GOS

with increasing the lactose concentration. Only a small difference on GOS production was observed between 40% and 50% lactose solutions. For this reason, in all subsequent experiments, 40% lactose solution was used since it was hard to work with a high lactose concentration of 50% due to the high solution viscosity and low solubility of lactose in water.

3.3.3. Effect of pH

The reaction rate is usually affected by the solution pH, but pH showed minimal effect on GOS production (Shin et al., 1998). Since the most common substrate for industrial GOS production is sweet and acid whey and whey permeate (pH 4.5), the effect of pH was investigated. Figures 3.10-3.12 show the kinetics of GOS formation for *B. circulans*, *A. oryzae* and *K. lactis* lactases, respectively, at 3 different pHs ranging from 3.5 to 8. GOS production was unchanged at all levels of lactose conversion, achieving the same maximum GOS content with the same amount of different GOS types for *B. circulans* and *A. oryzae* lactases. However, *K. lactis* lactase did not produce any GOS at acidic pH 5, suggesting that the enzyme was inactivated at low pH. Furthermore, the pH affected the reaction rate of GOS formation. The reaction rate was fastest at pH 6 for *B. ciruclans* lactase, pH 4.5 for *A. oryzae* lactase, and pH 6.5 for *K. lactis* lactase. In general, *B. ciculans* and *A. oryzae* lactase were more stable in a wider pH range than *K. lactis* lactase for GOS production.

3.3.4 Effect of galactose

Galactose is known as a competitive inhibitor of the ß-galactosidase, competing for the enzyme active site with lactose (Hsu et al., 2007). It has been shown that allolactose, a disaccharide of galactose and glucose, can be synthesized from galactose and glucose. Thus, the possible effects of galactose on GOS production and reaction rate were investigated by adding various amounts of galactose (2.5, 5 and 10% (w/v)) into the initial lactose solution (40% (w/v)) for *B. circulans* and *K. lactis* lactases. It was expected that by adding galactose to the initial solution would favor the transgalactosylation reaction since more galactose was available for the formation of GOS, allolactose and galactobiose, increasing GOS yield. However, Figures 3.13 and 3.14 show that not only the reaction rate decreased but also the percentage of GOS also decreased for both enzymes. Galactose inhibited the enzyme the most at low lactose conversion where the transgalactosylation reaction dominated; at higher lactose conversion, the GOS yield decreased less than at low lactose conversion, specially for *K. lactis* lactase (Figure 3.14b). In general, the inhibitory effect of galactose on GOS formation was more prominent for *B. circulans*. It seems that the higher the amount of galactose added into the initial solution, the more likely to favor the hydrolysis reaction instead of transgalactosylation. Similar results have also been reported for *A. oryzae* lactase (Albayrak et al, 2002).

3.3.5 Effect of substrate nature

The starting material could affect the amount of GOS produced during enzymatic hydrolysis of lactose (Mahoney, 1998). To determine the influence of substrate on the

GOS production, *B.circulans* lactase was reacted with two different 400 g/L lactose solutions: buffered lactose solution and concentrated whey permeate. Figure 3.15 shows the GOS formation of both substrates during the enzymatic reaction. Initially, the transgalactosylation reaction dominated early in the reaction since there was a fast production of GOS for both substrates. However, the rate of GOS formation was slower for whey permeate. Furthermore, the maximum GOS produced was also different. Immobilized *B. circulans* lactase produced 40% GOS at 60% lactose conversion with the buffered lactose solution. Under the same condition, 36% GOS at 50% lactose conversion was obtained when whey permeate was the substrate. The difference in GOS formation between these two substrates was due to the lack of production of 6OS and smaller amount of 5OS with whey permeate. It can be concluded that ß-galactosidase maintained higher hydrolytic activity in whey permeate than in the buffered lactose solution. It is possible that some of the proteins present in the whey permeate interacted with the lactase enzyme by favoring the hydrolysis reaction. Different authors have also reported lower GOS production when whey permeate was used as the substrate with different lactase enzymes (Zarate et al., 1990;Mozaffar, 1985).

3.3.6 Comparison of different lactase sources for GOS production

Table 3.2 compares the optimal conditions of 3 different lactases: *A. oryzae*, *B. circulans* and *K. lactis* for GOS production. The conditions and the amount and size of GOS produced were different depending on the enzyme origin. Lactase from *B. circulans* produced 40% GOS at 60% lactose conversion, followed by *K. lactis* lactase with 31% GOS at 75% conversion, and *A. oryzae* producing 27% GOS at 50% conversion. In all

cases, the feed concentration was 400 g/L lactose. *B. circulans* lactase presented the highest GOS yield with 68.5% and produced more GOS than the other lactases. The optimum pH was also different among the different lactases, with pH 4.5 for *A. oryzae* lactase, pH 6.5 for *B. circulans* lactase, and pH 6 for *K. lactis* lactase. However, *B. circulans* and *A. oryzae* lactases were more stable in a wider pH range (around 3 units from the optimum) for GOS production as compared with *K. lactis* that was inactivated at pH 5. Furthermore, *K. lactis* lactase presented the lowest enzyme activity. The price of the enzymes also varies depending on the origin. Lactase from *B. circulans* is the most expensive one, followed by *K. lactis* and *A. oryzae* lactases. However, relating the price to the initial enzyme activity of each enzyme, *K lactis* lactase was the most expensive one.

In conclusion, based on GOS yield, price, activity and enzyme stability at acidic pH, which is the most common pH for whey permeate, lactases from *A. oryzae* and *B. circulans* seem to be the most suitable enzymes for GOS production. Furthermore, the immobilization of these enzymes to produce GOS will be interesting since compared with free enzyme, immobilized enzyme provide many advantages.

3.4 Conclusions

Different lactase sources presented not only different optimum conditions for GOS production but also different amounts, types, and sizes of GOS. Among the different parameters studied, the initial lactose concentration was the most important parameter affecting the total amount of GOS produced for all lactases. In general, more GOS were produced at higher initial lactose concentrations. Lactase from *B. circulans*

produced the highest amount of GOS with 40% and also the most diverse types of GOS, followed by *K. lactis* lactase with 31% GOS and *A. oryzae* lactase with 27% GOS. The solution pH affected the reaction rate but not the GOS formation. However *K. lactis* lactase was inactivated when acidic pH was used. The addition of galactose into the lactose solution not only reduced the reaction rate but also the amount of GOS produced. Furthermore, ß-galactosidase maintained higher hydrolytic activity when whey permeate was used as the substrate.

3.5 References

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	Aspergillus oryzae (%)	*Bacillus circulans* (%)	*Kluveromyces lactis* (%)
Lactose	50.38	40.53	26.40
Glucose	17.22	16.10	29.68
Galactose	4.98	3.15	12.14
GOS	27.42	40.22	31.78
2OS	0.00	0.00	1.69
3OS	18.30	23.08	25.04
4OS	5.77	11.56	3.76
5OS	2.35	4.10	1.29
6OS	1.01	1.48	0.00

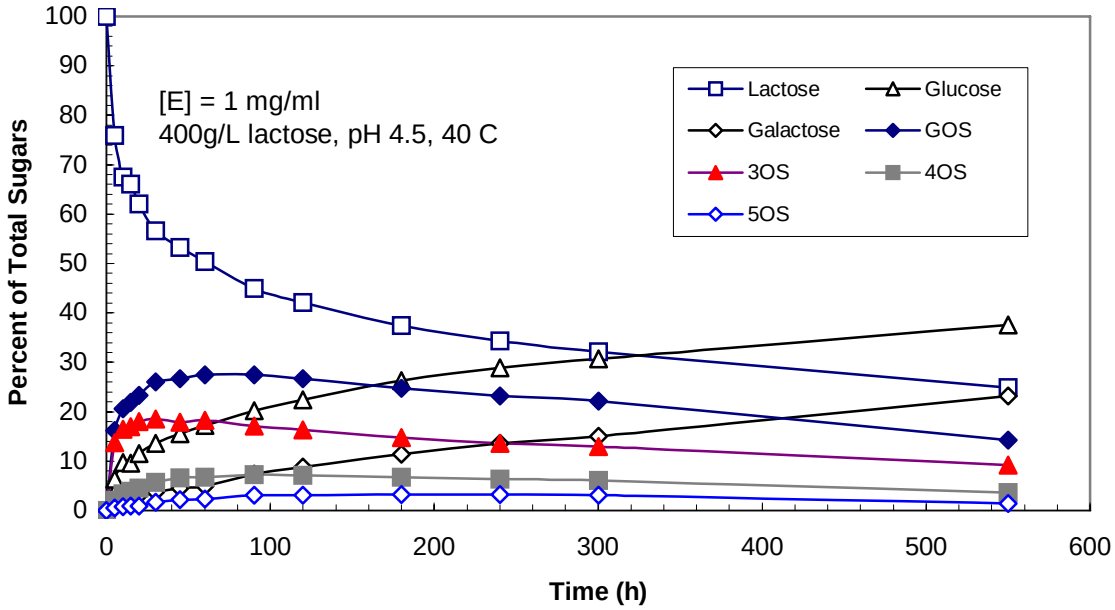
Table 3.1: Comparison of different sugar types at maximum GOS level from 3 sources of ß-galactosidase at 400g/L lactose

lactose concentration (g/L)	6OS (%)	5OS (%)	4OS (%)	3OS (%)	total GOS (%)
50	0.68	1.50	5.84	16.86	24.89
100	0.98	2.19	7.17	19.14	29.47
200	1.12	2.66	8.83	21.05	33.67
300	1.24	3.03	9.98	22.66	36.91
400	1.48	4.10	11.56	23.08	40.22
500	1.63	4.30	12.03	23.80	41.76

Table 3.2: Effect of lactose concentration on production of different types of GOS during lactose hydrolysis by using ß-galactosidase from *Bacillus circulans*.

	Aspergillus oryzae	*Bacillus circulans*	*Kluveromyces lactis*
GOS yield (%)	60.36	68.54	50.98
GOS productivity (g/L·h)	4300	180	
Optimum pH	4.5	6.	6.5
Optimum temperature (^{o}C)	40	45	35
Stability (half life, h)	399	57.7	31.5
Activity (lactase unit/g or mL)	106742	5500	3000
Enzyme cost ($ per kg)	350	800	550

Table 3.3: Optimum conditions for GOS production from 3 different lactases.



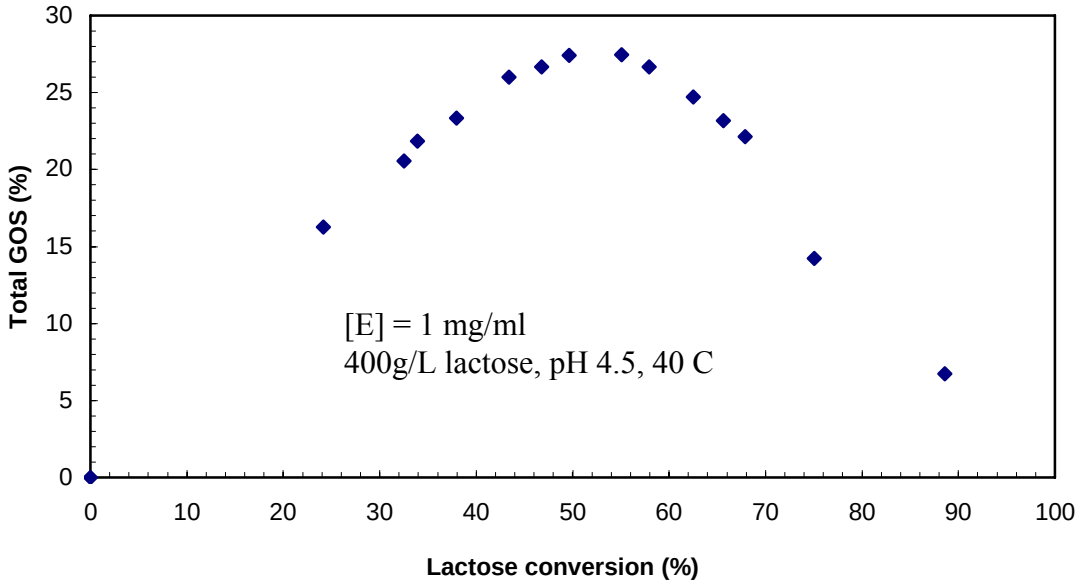


Figure 3.1: Reaction kinetics of lactose hydrolysis and GOS formation of ß-galactosidase from *Aspergillus oryzae*: a) as function of time, b) as function of lactose conversion, and c) GOS yield at 40 ^{o}C with an initial lactose concentration of 400 g/L (continued)

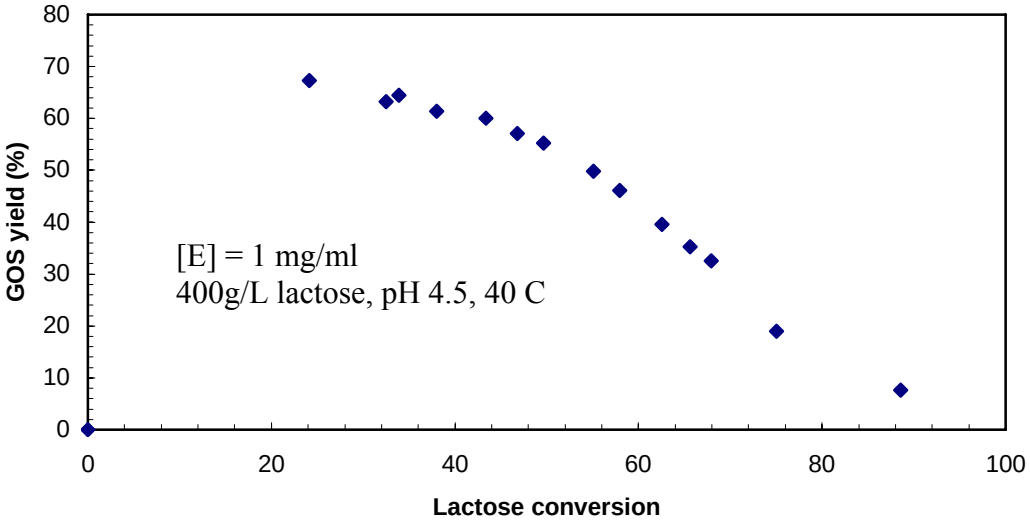
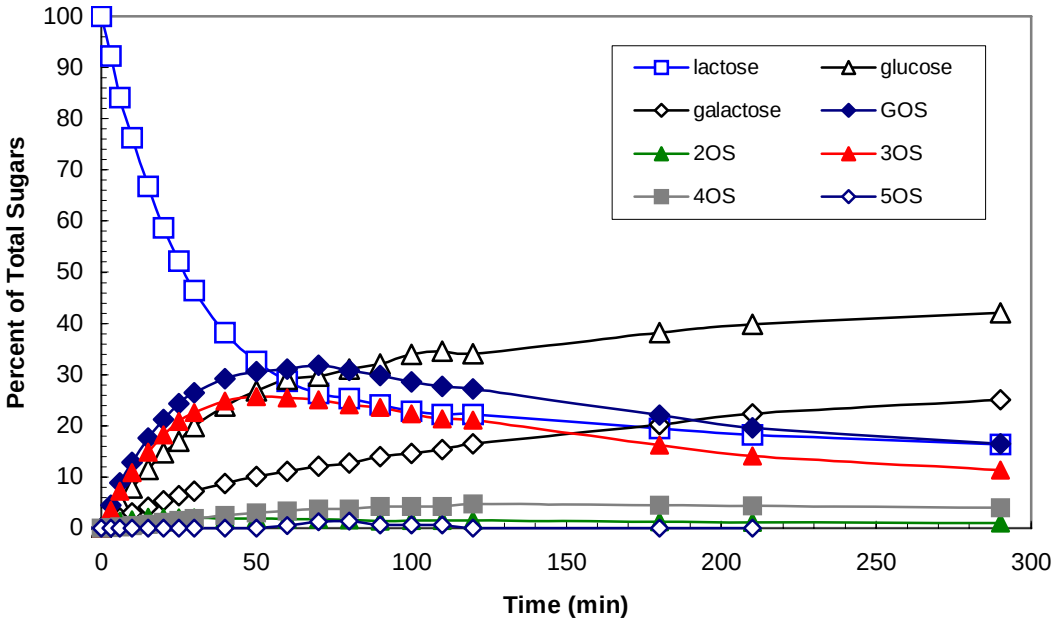


Figure 3.1: (continued).



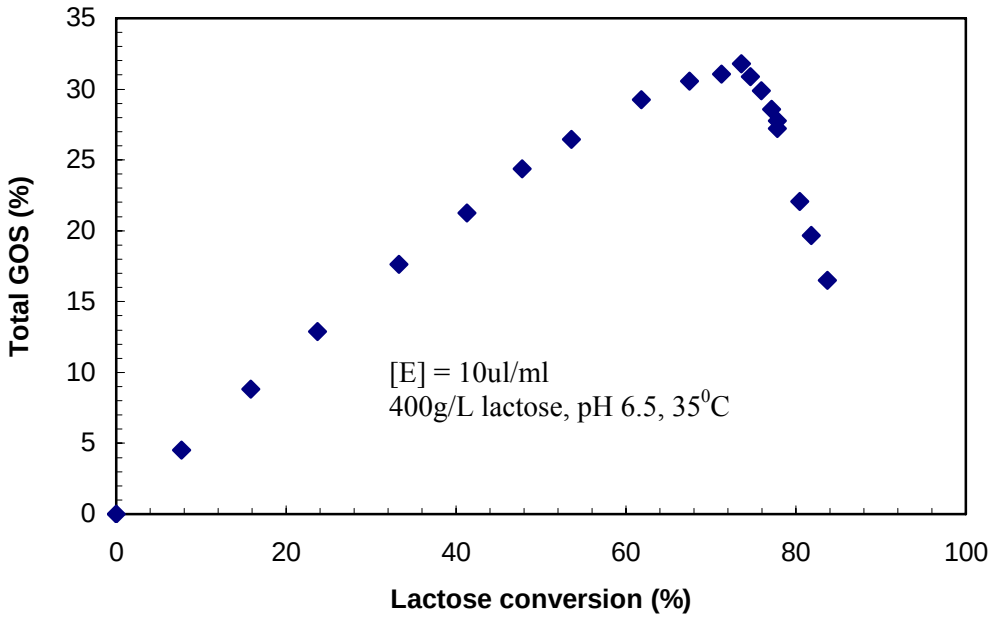
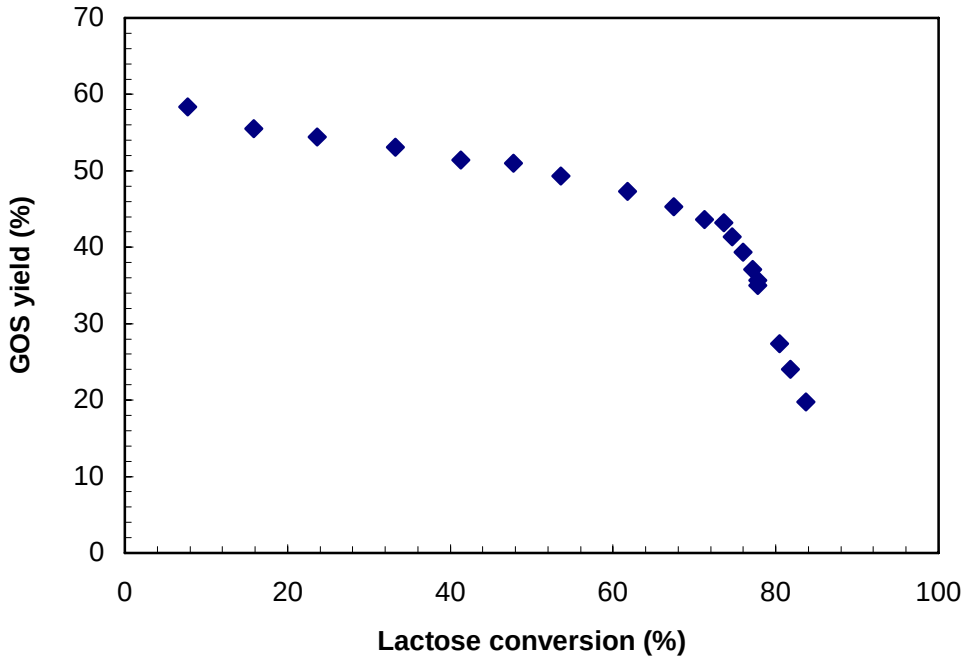


Figure 3.2: Reaction kinetics of lactose hydrolysis and GOS formation of ß-galactosidase from *Kluveromyces lactis*: a) as function of time, b) as function of lactose conversion, and c) GOS yield at 35 ^{o}C with an initial lactose concentration of 400 g/L (continued)



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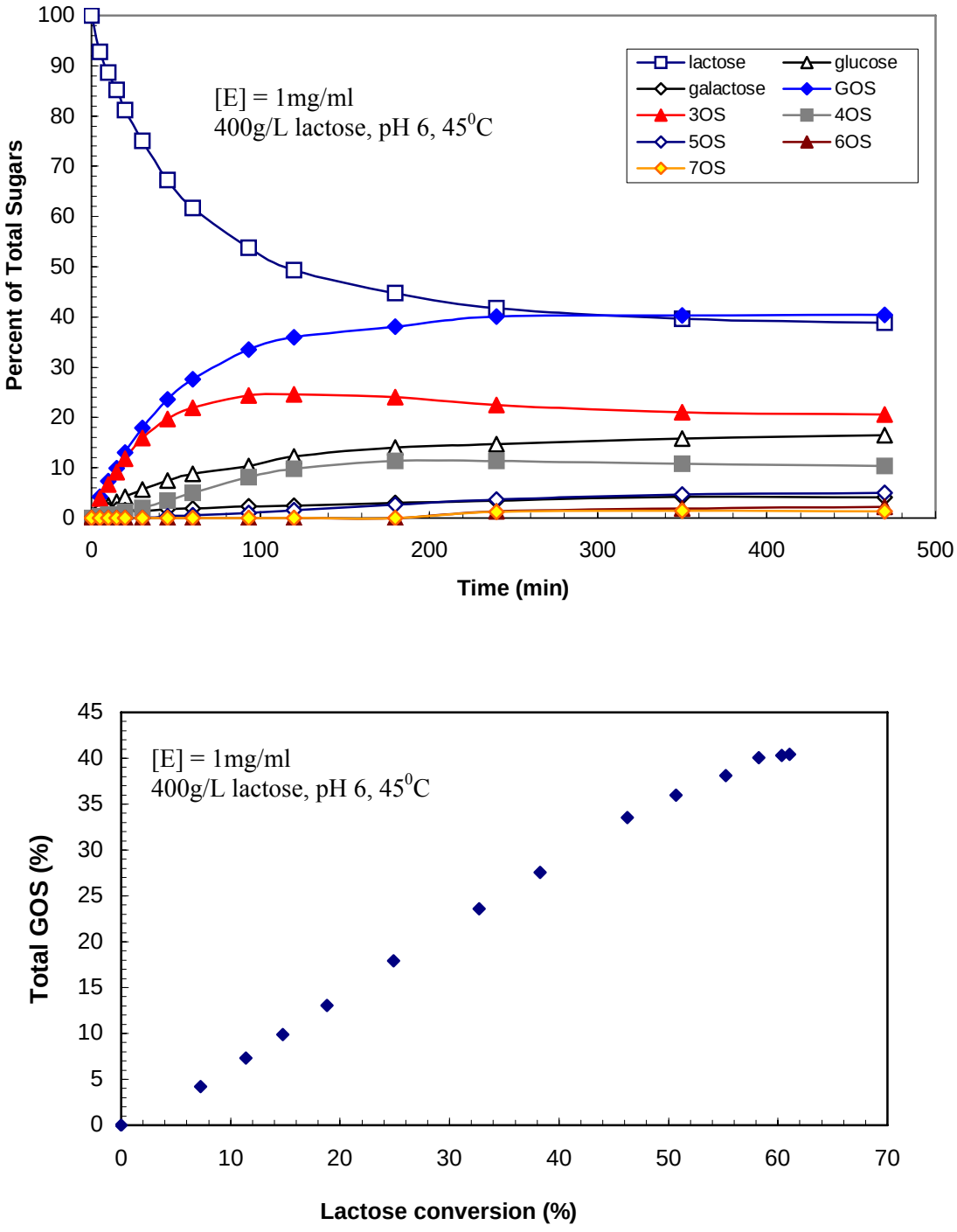
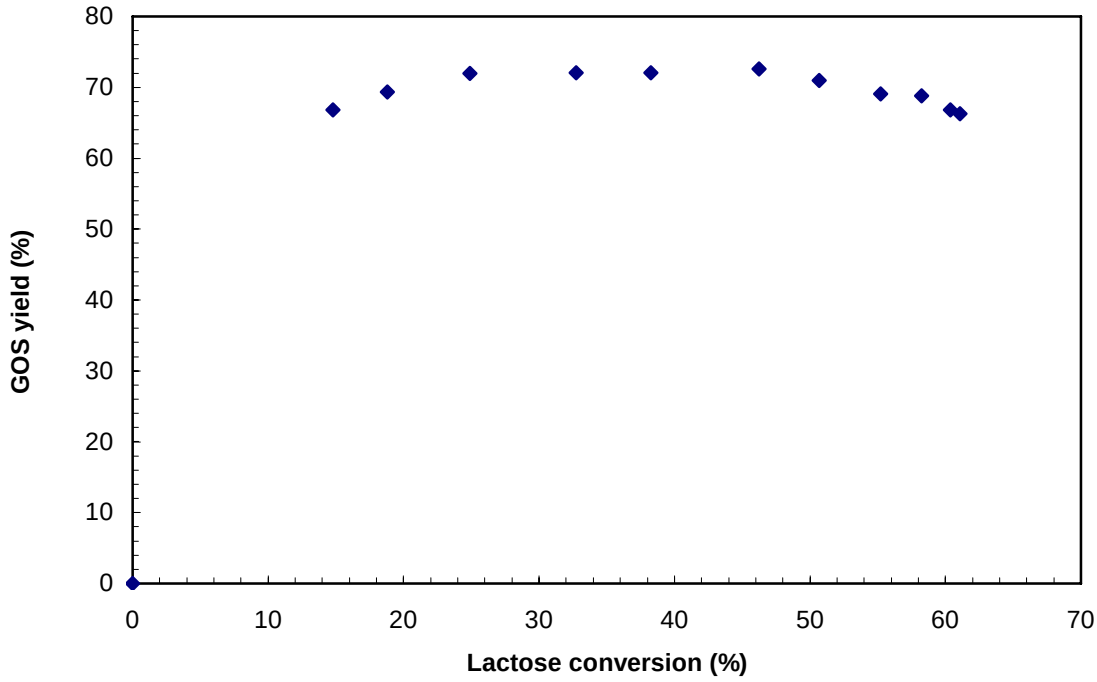


Figure 3.3: Reaction kinetics of lactose hydrolysis and GOS formation of ß-galactosidase from *Bacillus circulans*: a) as function of time, b) as function of lactose conversion, and c) GOS yield at 45 ^{o}C with an initial lactose concentration of 400 g/L (continued)



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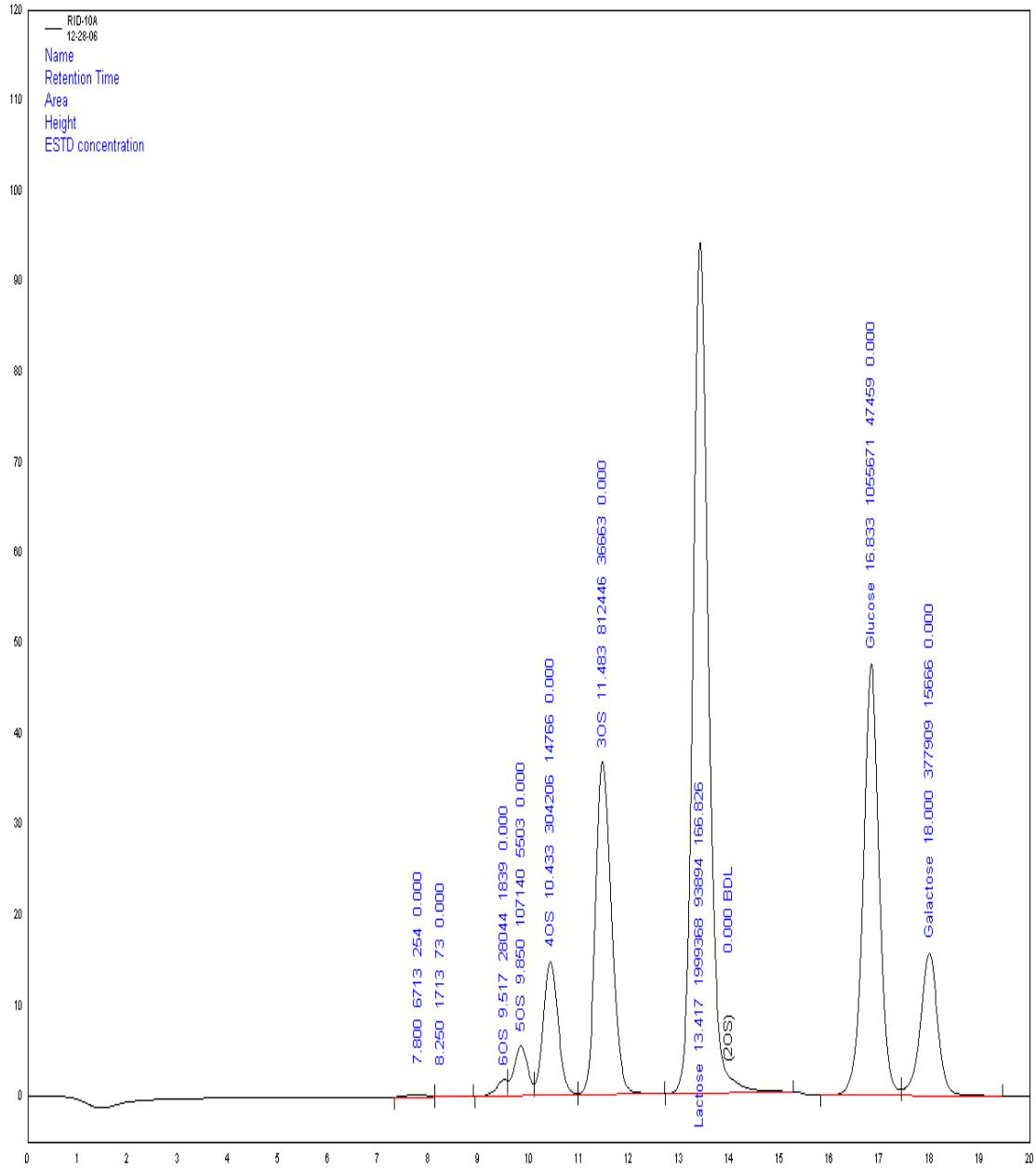


Figure 3.4: A typical HPLC chromatogram showing various sugars found in lactose hydrolysis and GOS formation catalyzed by the enzyme ß-galactosidase from *Aspergillus oryzae*

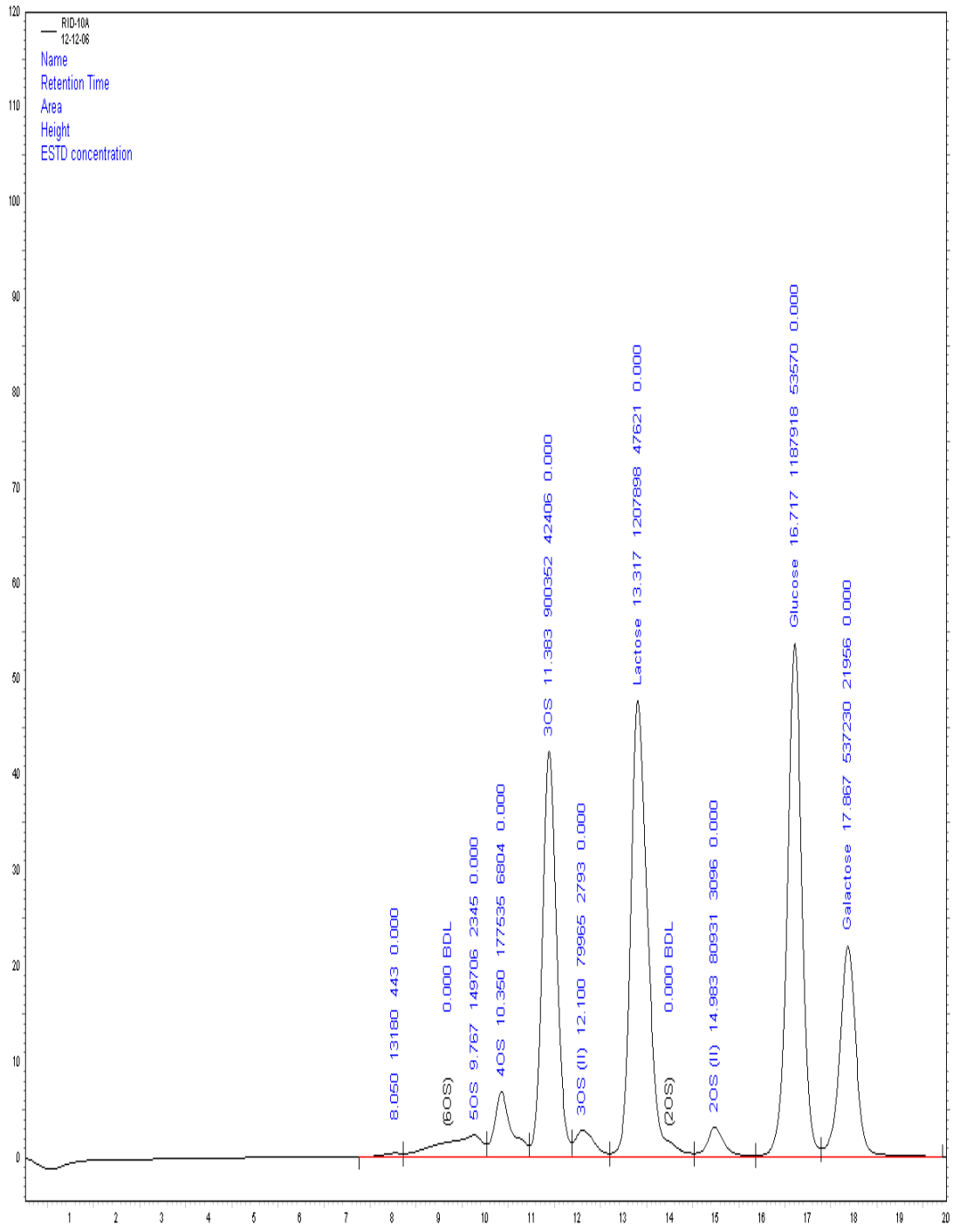


Figure 3.5: A typical HPLC chromatogram showing various sugars found in lactose hydrolysis and GOS formation catalyzed by the enzyme ß-galactosidase from *Kluveromyces lactis*

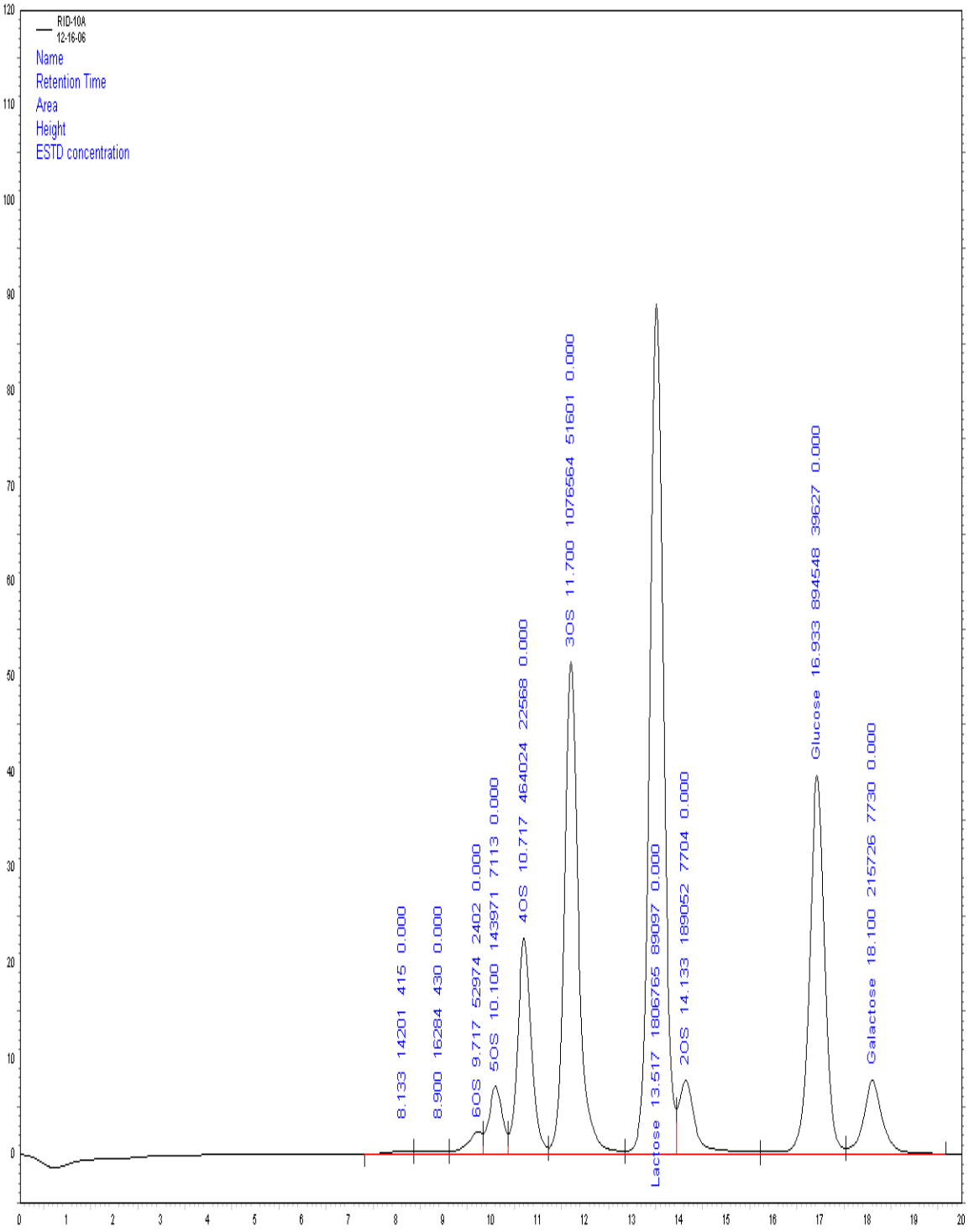


Figure 3.6: A typical HPLC chromatogram showing various sugars found in lactose hydrolysis and GOS formation catalyzed by the enzyme ß-galactosidase from *Bacillus circulans.*

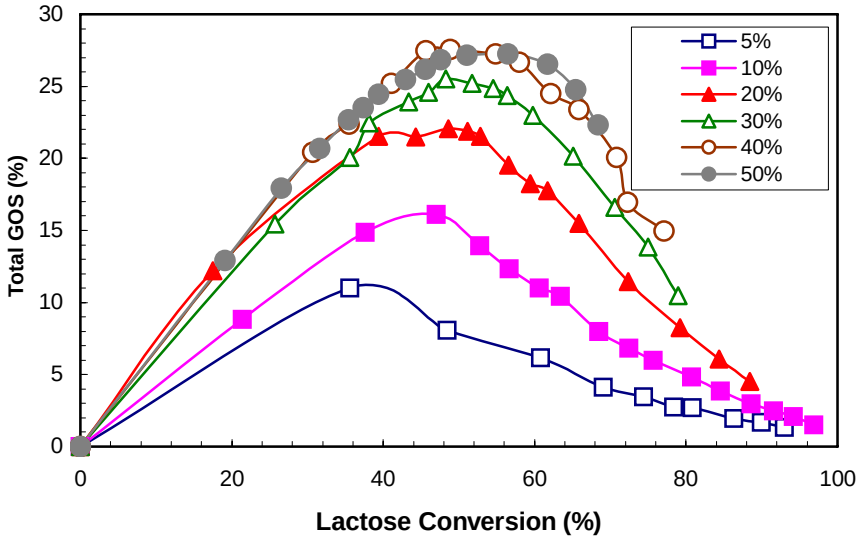


Figure 3.7: Effect of different lactose concentration on GOS production during lactose hydrolysis by using ß-galactosidase from *Aspergillus oryzae*

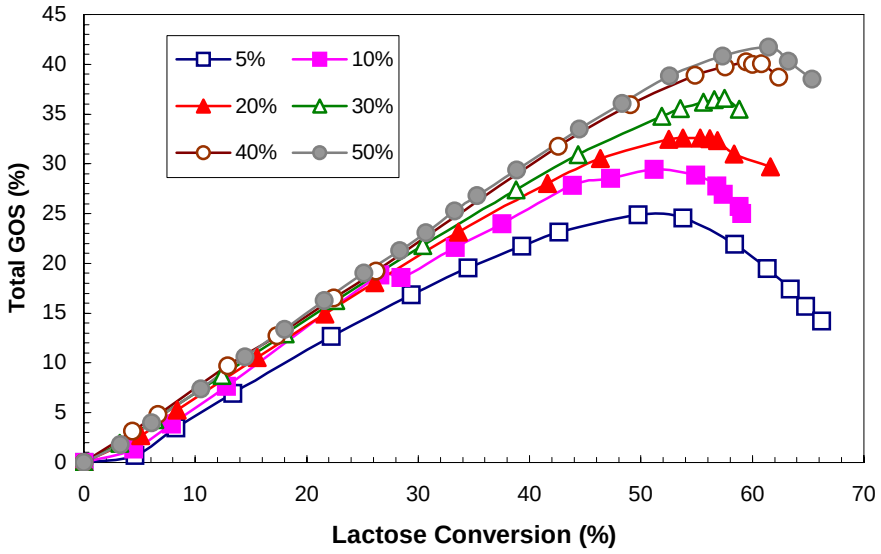


Figure 3.8: Effect of different lactose concentration on GOS production during lactose hydrolysis by ß-galactosidase from *Bacillus circulans*

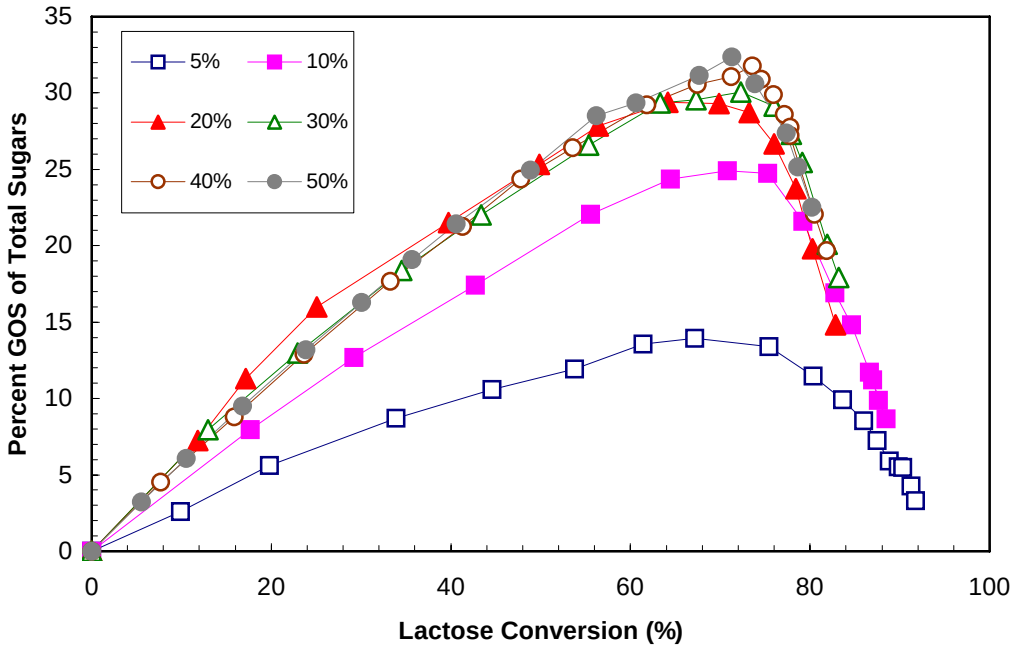
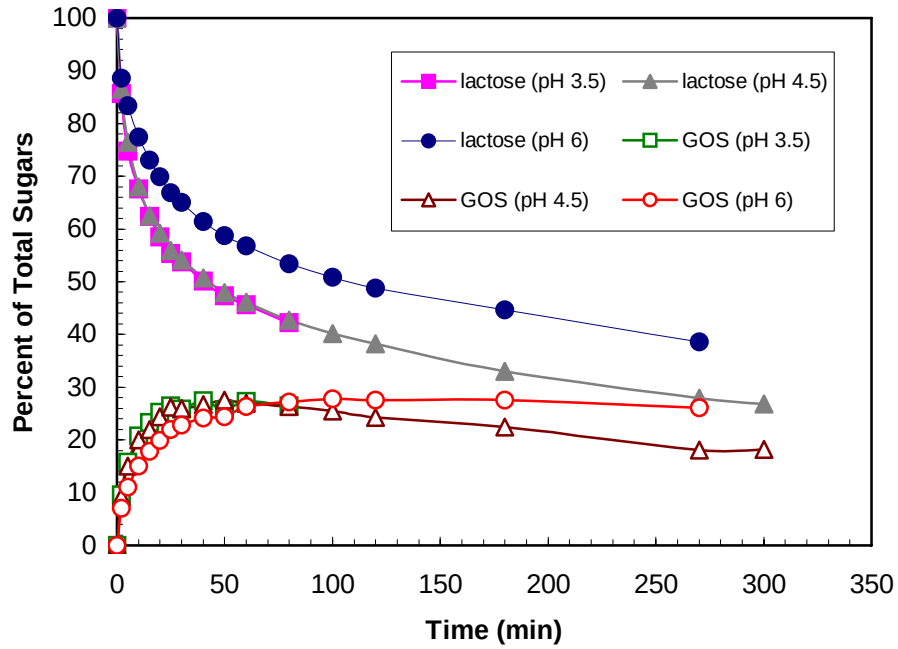


Figure 3.9: Effect of different lactose concentration on GOS production during lactose hydrolysis by using enzyme ß-galactosidase from *Kluveromyces lactis*



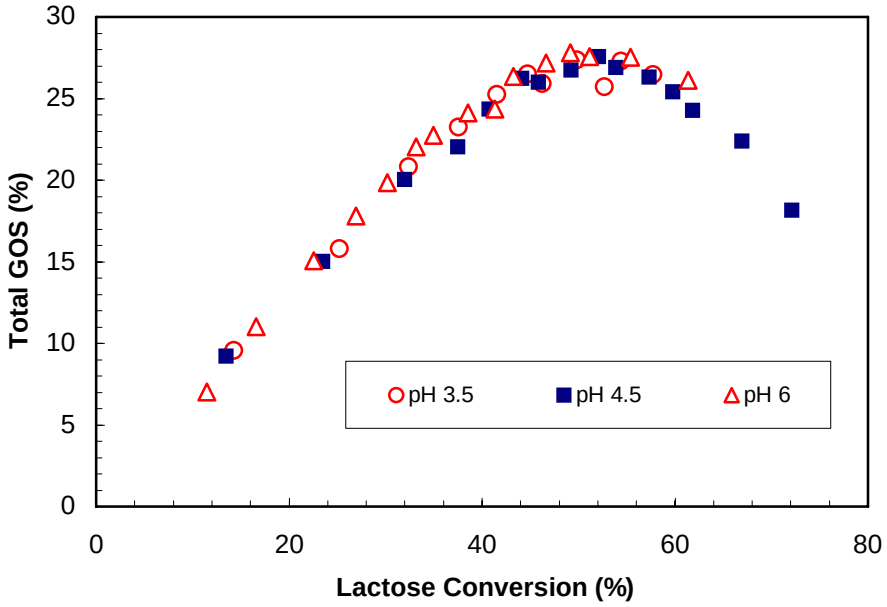
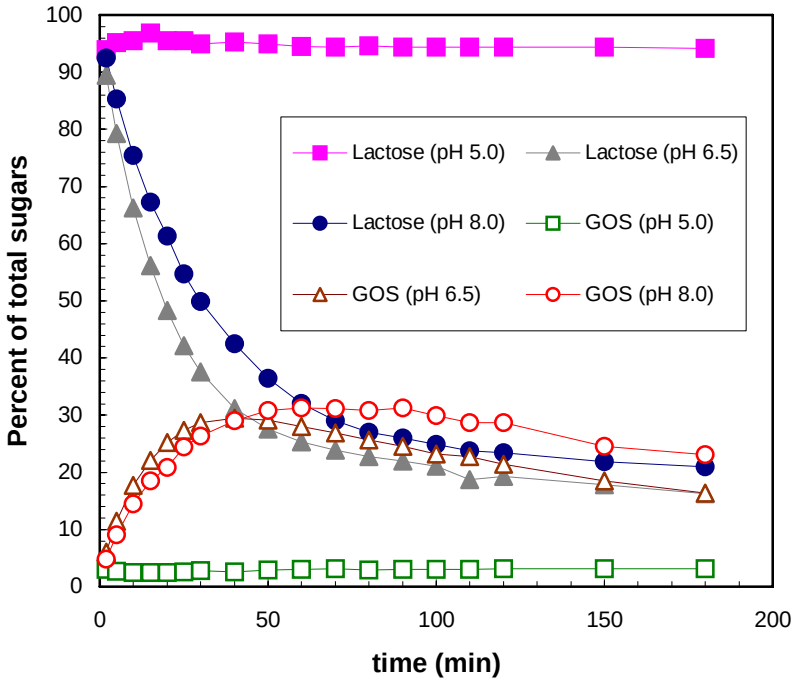


Figure 3.10: Effect of pH on GOS production during lactose hydrolysis a) as function of time and b) as function of lactose conversion by using ß-galactosidase from *Aspergillus oryzae*



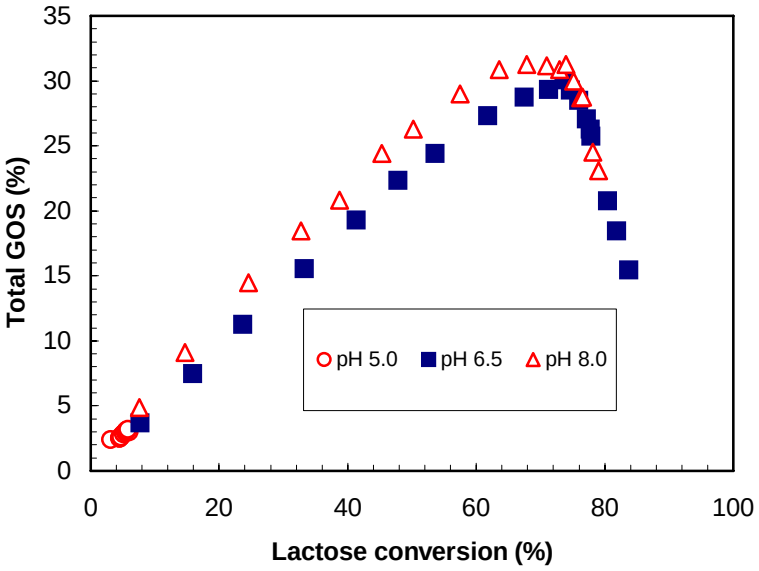
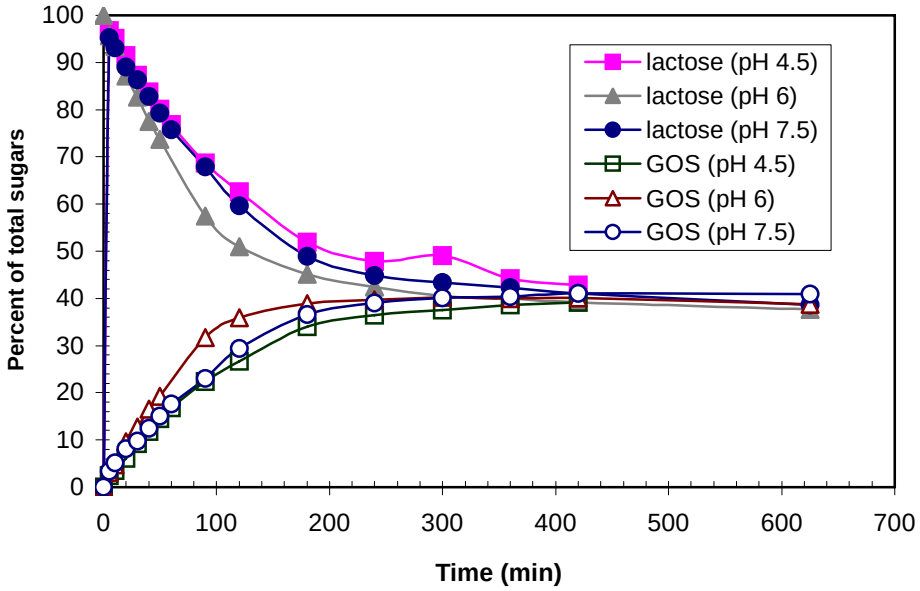


Figure 3.11: Effect of pH on GOS production during lactose hydrolysis a) as function of time and b) as function of lactose conversion by using ß-galactosidase from *Kluveromyces lactis.*



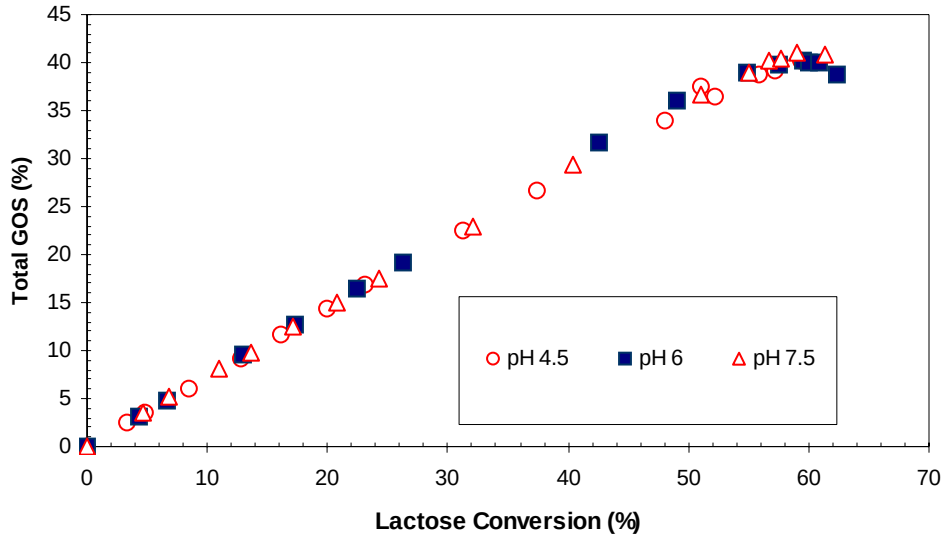


Figure 3.12: Effect of pH on GOS production during lactose hydrolysis a) as function of time and b) as function of lactose conversion by using ß-galactosidase from *Bacillus circulans.*

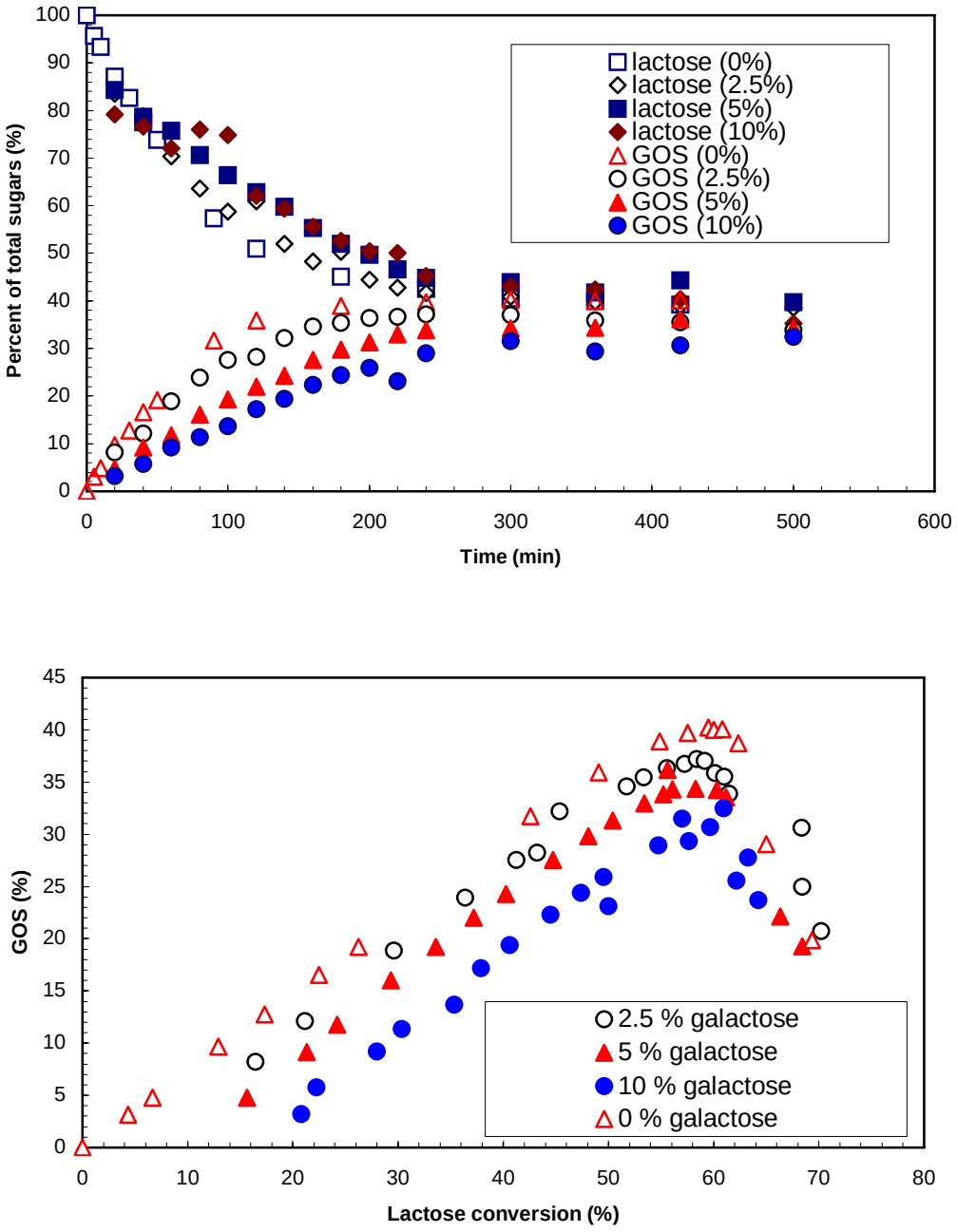
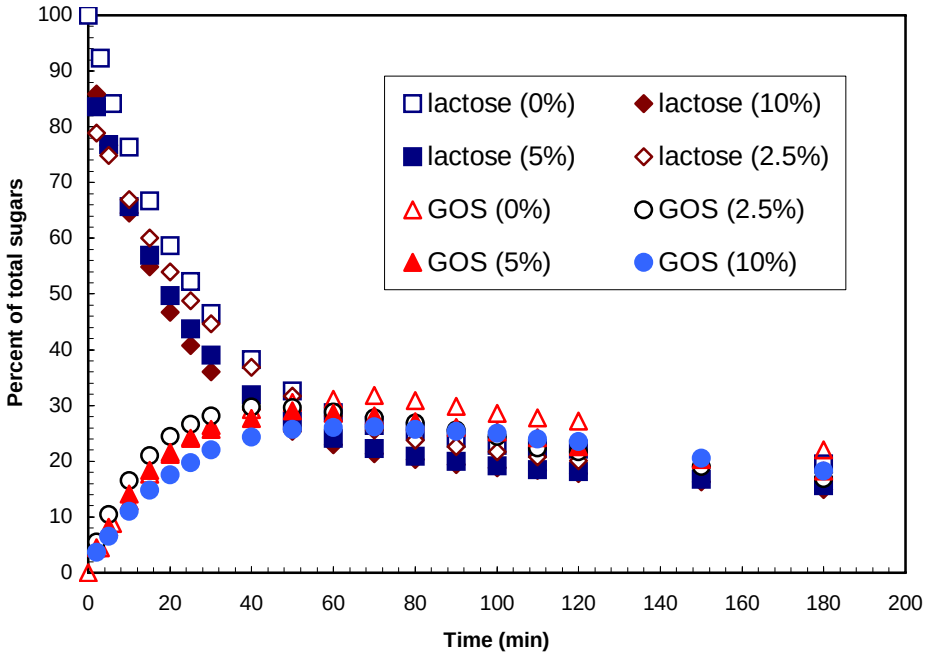


Figure 3.13: Effect of galactose on GOS production during lactose hydrolysis a) as function of time and b) as function of lactose conversion by using ß-galactosidase from *Bacillus circulans.*



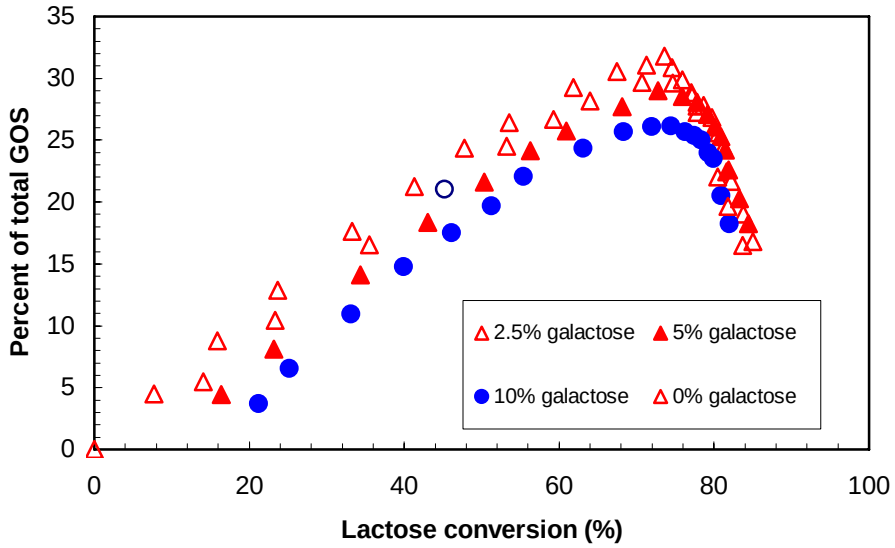
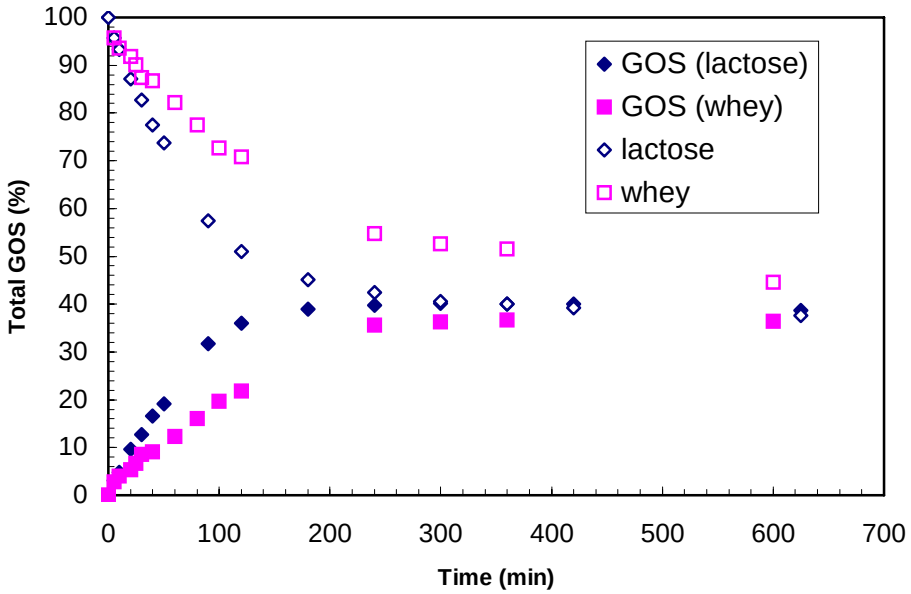


Figure 3.14: Effect of galactose on GOS production during lactose hydrolysis a) as function of time and b) as function of lactose conversion by using ß-galactosidase from *Kluveromyces lactis.*



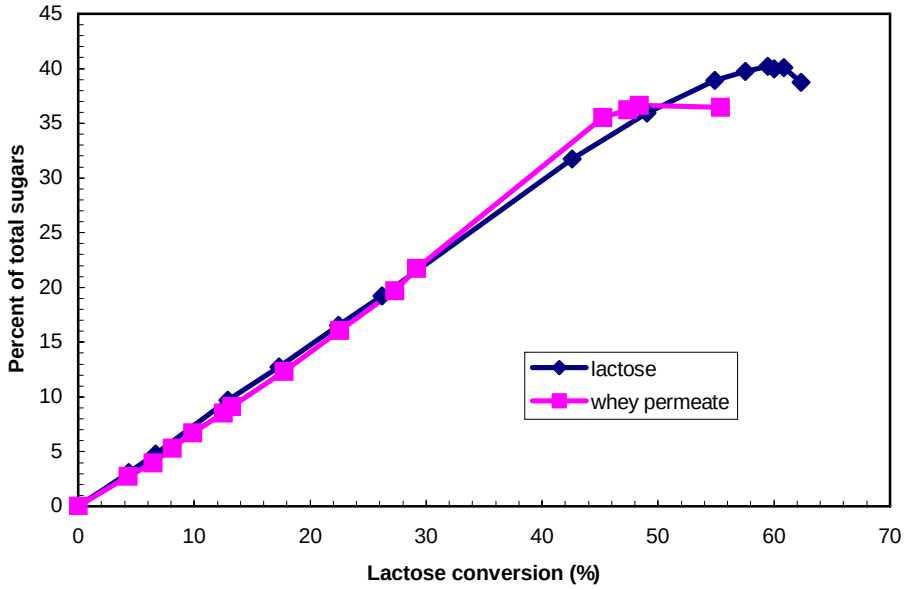


Figure 3.15: Effect of substrate nature on GOS production during lactose hydrolysis a) as function of time and b) as function of lactose conversion by using ß-galactosidase from *Bacillus circulans*

CHAPTER 4

IMMOBILIZATION OF ß-GALACTOSIDASE FROM *BACILLUS CIRCULANS* ON COTTON CLOTH FOR GALACTO-OLIGOSACCHARIDES PRODUCTION FROM LACTOSE

Abstract

The production of galacto-oligosaccharides (GOS) from lactose using *Bacillus circulans* ß-galactosidase immobilized on cotton cloth was investigated. The enzyme was immobilized on cotton cloth by adsorption with PEI (polyethyleneimine) and cross linking with glutaraldehyde (GA). A high enzyme loading (250 mg/g support) with 35% activity yield was achieved. The maximum GOS production of 40% (w/w) was obtained at 60% lactose conversion with 400 g/L lactose solution at pH 6 and 45^{o}C. The immobilized enzyme showed the same GOS formation kinetics as that of the free enzyme, indicating that there was no diffusion limitation in the catalytic cotton cloth for the reaction. Furthermore, the solution pH only affected the reaction rate of GOS formation. The half-life of the immobilized enzyme on cotton cloth was 693 h at 45^{o}C.

4.1 Introduction

In the last 20 years the immobilization of ß-galactosidase on different surfaces have gained a lot of attention in the food industry (Grosova 2008; Prenosil et al., 1986). ß-galactosidase (EC 3.2.1.23) catalyzes not only the hydrolysis of lactose but also the transgalactosylation reaction to produce GOS (Zarate et al., 1990; Mozaffar et al., 1984). In the cheese industry lactose is a waste, which causes several economical and environmental problems. Approximately 47% of the whey produced annually worldwide is disposed off (Novalin et al., 2005). Therefore, conversion of lactose into a highly valuable product such as GOS is of high interest to the food industry (Yang et al., 1995). GOS, known as *Bifidus growth factor*, because of the selective stimulation of bifidobacteria in the low intestine, have many health benefits (Delzene et al., 2003; Rivero-Urgell et al., 2001; Tomomatsu et al., 1994), and have wide commercial applications as prebiotic food ingredients and dietary supplements (Sako et al., 1999; Crittenden et al., 1996; Playne et al., 1994). The price of ß-galactosidase is rather high. Due to the low value of the lactose substrate, the direct addition of the enzyme is economically unacceptable (Mahoney, 1997). Compared with enzyme in solution, immobilized enzyme ß-galactosidase provides many advantages in the production of GOS, such as, high enzyme reusability, high yield, improvement of thermal stability, continuous operation, controlled product formation, high reactor productivity, no contamination of product by the enzyme and simplified and efficient processing (Dervakos et al., 1991). Many different techniques have been reported for the immobilization of ß-galactosidase, including cross-linking (Sheu et al., 1998; Sinha et al., 1985), covalent binding (Di Serio et al., 2003; Hernaiz et al., 2000; Bódalo et al., 1991;

Olson et al., 1983; Okos et al., 1974), entrapment (Mammarella et al., 2005; Berger et al., 1994; Hinberg et al., 1984) and adsorption (Albayrak et al., 2002; Okos et al., 1978). However, most of these methods were developed for lactose hydrolysis purposes instead of GOS formation. Also, many different supports have been used related with these methods, such as, graphite (Zhou et al., 2003), resins (Woudenberg et al, 1998; Mozaffar et al., 1986; Olson et al., 1983, Nakanishi et al., 1983), chitosan beads (Sheu et al., 1998), agarose beads (Berger et al., 1994), porous glass (Bódalo et al., 1991) and sintered porous ceramic support (Hassan et al., 1995). The main drawbacks of these methods are the use of toxic reactants, expensive supports and the difficulty to scale up for industrial proposes due to high-pressure drop. Therefore, during the immobilization process, factors like enzyme carrier, chemical reactants and possible applications related to the immobilization process have to be considered, so that easy regeneration of the immobilized enzyme and low cost operation can be achieved in industrial processing.

The enzyme support is, besides the immobilization technique, one of the most important components in affecting the performance of the enzyme. Cotton cloth is an inexpensive and widely available fibrous material. It provides many advantages, including, large specific surface area, high mechanical strength due to its crystalline structure, and high porosity. Therefore, enzyme immobilized on cotton cloth placed in a packed-bed reactor provides good flow rates with low-pressure drop, being highly desirable characteristics for industrial application. The goal of this project was to evaluate the performance of immobilized ß-galactosidase from *Bacillus circulans* on cotton cloth to produce GOS from lactose. Cotton cloth has been used in cell immobilization and fermentation studies (Huang et al., 1998; Yang et al., 1995). ß-galactosidase from *B.*

circulans has a molecular weight of $1.6*10^5$ with an isoelectric point of 4.5 and optimum pH of 6 (Mozaffar et al., 1984). The main advantage of this bacterial ß-galactosidase is the high ratio of GOS that can be obtained and the subsequent high yield at high lactose conversion. Moreover, it has been recognized as safe for using in food industry. Therefore, a successful immobilized process will be interesting from an industrial point of view.

In this chapter, an immobilization technique with ß-galactosidase from *Bacillus circulans* into fibrous matrix, such as cotton cloth, using PEI and glutaraldehyde, has been addressed as well as its application on GOS production. This technique has been previously applied in lactase from A. oryzae. (Albayrak et al., 2002). However, in order to establish the technique for general use, applicability on other types of enzymes need to be investigated. PEI, a high branched cationic polymer (Zemek et al., 1982), has many applications because of its electrostatic interaction with negatively charged species (Nguyen et al., 2003; Jendrisak et al., 1987). Cotton cloth coated with PEI has been used as a support for immobilization of several enzymes, including glucose oxidase (Kumar et al., 1997), urease (Das et al., 1998; Kamath et al., 1988), invertase (Yamazaki et al., 1984), and yeast cells (D'Souza et al., 1988). Various factors affecting PEI-enzyme aggregate formation on cotton fibrils were investigated to optimize the immobilization process. The effects of enzyme loading, enzyme stability and kinetics of GOS formation were also studied.

4.2 Materials and Methods

4.2.1 Materials

ß-galactosidase from *Bacillus circulans* (bacterial lactase activity 5500 LU/g) was obtained from Daiwa Kasei (Shiga, Japan). One lactase unit (LU) is defined as the amount of enzyme, which liberates 1 μmol of glucose per minute from lactose (concentration 10%) at the early stage of the reaction at 40 ^{o}C, pH 6. Lactose (99.9%) was obtained from Brewster Dairy (Brewster, OH). Polyethyleneimine [PEI; $(C_2H_5N)_n$] as 50% (w/v) (number average molecular weight 60,000; average molecular weight 750,000) and glutaraldehyde (GA) as 25% (w/v) aqueous solutions were from Sigma (St. Louis, MO). Sodium phosphate monobasic anhydrous (Fisher) and sodium phosphate dibasic anhydrous (E.M. Science) were used to prepare phosphate buffer. Cotton cloth was obtained locally. All solutions for PEI, GA, and enzyme were prepared with distilled water. The solution pH was adjusted, when necessary, using HCl or NaOH solution of sufficient concentration.

4.2.2 Immobilization procedure

Enzyme immobilization on cotton cloth involved three main steps: adsorption of PEI solution to cotton cloth, introduction of enzyme to PEI-containing cloth, and GA cross-linking of PEI enzyme aggregates coated on the cotton (Figure 4.1). The solutions were kept cold on ice until use. Unless otherwise noted, 1 ml of PEI solution pH4 to 10 with PEI concentration of 0 to 0.33% (w/v) was added to each piece of 0.2 g cotton cloth in a 125 ml Erlenmeyer flask. The solution volume was at a sufficient level to completely wet the cloth, thereby allowing a homogeneous distribution of PEI to the matrix. After

adsorption of PEI, 50 mg of enzyme (10 mL of 5 mg/mL enzyme solution) were added to the flask. Upon the addition of the enzyme a milky turbid solution was formed. Then, the flasks were incubated in a shaker-incubator at 4 ^{o}C for 0.5 to 24 hours. Usually, the coupling was completed when the white turbidity disappeared. Afterwards, the coupling solution was slowly decanted, and the PEI-enzyme-coated cotton was transferred to cold GA solution (1-0.05% (w/v), pH 7.0) for cross-linking for a period of time between 10 and 420 minutes at room temperature. The cotton cloth developed a yellow color at the end of the immobilization, after GA cross-linking Finally, the cross-linked cotton cloth was washed extensively with distilled water. It is important to note that there was no washing step until the completion of GA cross-linking.

The first part of the immobilization, was to study a wide range of different PEI-enzyme ratio by changing the initial PEI concentration, and the initial pH of the PEI solution which are critical factors affecting the level of immobilized enzyme on cotton cloth. It is important to mention that PEI solutions were prepared with distilled water and then adjusted with NaOH to different pH. The fact of using buffer solution to prepare PEI solution will reduce substantially the immobilization yield since the negative charges of phosphate buffer will compete with the enzyme for interacting with PEI and essentially will block the formation of PEI-enzyme aggregates. Initially, 5 different PEI concentrations were used: 0.33, 0.22, 0.15, 0.11 and 0.04% (w/v) (enzyme-PEI ratios from 1/9 to 1/125) at 4 different pH of PEI solution:4, 6, 8, and 10.

4.2.3 Reaction kinetics

GOS formation kinetics with immobilized enzyme was studied in an Erlenmeyer flask. Cotton cloth (0.6 g) in pieces of 0.2 g was placed in the flask and maintained at 45 °C, unless otherwise noted, in a shaker-incubator. The lactose solution in the flask (total solution volume, 50 mL) was agitated at 250 rpm. The lactose solution was prepared by dissolving lactose in distilled water (pH 6, unless otherwise noted). Samples (100 μL) were taken from the flask at appropriate time intervals and analyzed for sugar contents by using high-performance liquid chromatography (HPLC). The reaction kinetics was studied with 400 g/L lactose solutions at 45 ^{o}C under different conditions: three different enzyme loadings (100, 150, 250 mg enzyme/g cotton. GOS formation kinetics for free enzyme was also investigated under similar conditions. Samples (100 μL) were drawn from the reaction mixtures at appropriate time intervals and added to 900 μL of distilled water at 95 °C to stop the enzyme reaction. The sugar contents were analyzed with HPLC.

4.2.4 Thermal stability

The thermal stabilities of PEI-immobilized enzyme at various temperatures (35, 45, and 60 °C) at pH 6 were studied also in 125 ml Erlenmeyer flasks at 250 rpm in a shaker-incubator for both free and immobilized enzyme. Cotton cloth immobilized enzyme with an enzyme loading of 250 mg/g was used in this experiment. Free and immobilized enzyme was kept in distilled water adjusted to pH 6 and mixed with lactose solution (100 g/L) at appropriate time intervals to calculate the activity remain in both of

both free and immobilized enzyme based in the amount of glucose liberated. Samples were collected and analyzed with HPLC.

4.2.5 Analytical Methods

4.2.5.1 Enzyme activity assay

The activity of cotton cloth immobilized enzyme was measured with 100 g/L lactose as the substrate in distilled water (pH 6) at 45 °C in a shaker-incubator at 450 rpm for about 5 min. After incubation, the cloth was removed from the reaction mixture and a volume of sample was taken and mixed at one-to-one ratio with 0.1 N NaOH or heated at 95 °C to stop the enzyme reaction to inactivate any enzyme leached from the cotton cloth. The glucose concentration in the sample was determined with a glucose analyzer (YSI 2700 Select, Yellow Springs, OH). The activity of the immobilized enzyme was determined by direct comparison of the reading with the standard curve in the plot of glucose concentration (g/L) versus enzyme activity times the reaction time [g/L vs (mg/mL)*min] obtained from free enzyme reactions and then used to estimate the amount of active enzyme (mg/g cotton) and immobilization yield (%).

4.2.5.2 Protein determination

A protein assay kit based on the Bradford method (Bio-Rad protein assay kit II) was used to determine the protein content in solution. Absorbance at 595 nm of enzyme (protein) solution was measured with a spectrophotometer (Max-250 Spectra). The enzyme (protein) concentration was determined by comparing with the standard curve constructed using enzyme solutions with known concentrations. The amount of the enzyme (protein) coupled onto the cotton was determined from the initial protein amount

present in the enzyme solution subtracting the final total amounts present in the remaining coupling solution. The coupling yield (%) of the enzyme was then calculated from the amount of enzyme coupled on the cotton cloth divided by the initial total amount present in the initial solution.

4.2.5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method provided by Mini-PROTEAN® 3 Cell Bio-Rad System (Hercules, CA). Slab gels consisted of 12% (w/v) polyacrylamide for the separating gel and 2.5% polyacrylamide for the stacking gel. The protein sample was mixed with the loading buffer containing bromophenol blue. Then, the load sample mixture was boiled for 10 min. Each well was loaded with 30 μl of the load sample mixture and the gel was run at a constant voltage of 90 V for stacking gel and 110 V for separating gel until the tracking dye reached the gel bottom. The protein bands were stained with Comassie brilliant blue for 1 h and then destained in a destaining solution containing 20% methanol and 10% acetic acid. The protein bands were viewed using an Agfa FotoLook scanner.

4.2.5.4 HPLC analysis

The concentrations of various sugars (glucose, galactose, lactose, and galactooligosaccharides) in sample solutions were determined by using HPLC. An HPLC system consisting of a pump (LC-10AI Shimadzu), an automatic sampler injector (Shimadzu SIL10Ai), a carbohydrate analysis column (Phenomenex, Rezek RNM carbohydrate column, 7.8 mm-150 mm), a column oven (Shimadzu CTO-10A), a

refractive index detector (Shimadzu RID-10Avp), and a Shimadzu CLASS-VP chromatography data system (version 7.2 integrator) was used. The eluent was pre-degassed distilled water (at 80 °C) at a flow rate of 0.4 mL/min. Distilled water was degassed by first boiling and then sonicating for 30 min. The column temperature was maintained at 80 °C, and the detector temperature was set at 45 °C. Figure 4.2 shows a typical HPLC chromatogram. The concentrations of these sugars (e.g., lactose, glucose, galactose, and oligosaccharides including tri-, tetra-, and pentasaccharides) were determined from peak heights and are reported as weight percentages of total sugars.

4.3 Results and Discussion

4.3.1 Enzyme immobilization on cotton cloth

Figures 4.3 to 4.6 show the results of using 4 different PEI solutions at pH: 4,6,8 and 10. For each figure the coupling yield and activity yield was calculated at different PEI concentrations. As it can be seen, the ratio of PEI- enzyme had more influence than the pH of PEI solution on the enzyme immobilization. The differences in coupling or immobilization yield obtained at different PEI concentrations under the same pH were in some cases more than 30% between the lowest and the highest value. All the figures follow the same pattern at different pHs, in which, increasing the PEI amount from 2 mg/g cotton (0.04% (w/v)) to 16.5 mg/g cotton (0.33% (w/v)) for an enzyme loading of 250mg/g resulted in a decrease of the immobilization yield. The optimal being the optimal PEI-enzyme ratio was 1/125 in all cases. Also, from Figures 4.3 to 4.6, it can be observed that at higher pH of PEI solution the PEI-enzyme complex formation was better since the immobilization yield obtained was higher (from 32.77% at pH 4 to 47.26% at

pH 10) at PEI concentration (0.04% (w/v)). This result is in agreement with the fact that at higher pH the enzyme is more negatively charged since the isoelectric point is 4.5. As a consequence, the enzyme forms a stronger electrostatic complex with the positive charged PEI. Moreover, the more negatively charged the enzyme is, the less amount of PEI necessary for complex formation. This last fact is extremely important since the purpose for enzyme immobilization is to produce a food additive (GOS). However, as mentioned before, PEI concentration affected the level of immobilization the most. Comparing the immobilization yield at the same pH, a change from 9.32% (at PEI concentration of 0.33%) to 32.77% (at PEI concentration of 0.04%), from 9.9% to 36.5%, from 12.6% to 39.4% and, from 27.4% to 47.2% at pH 4, 6, 8 and 10 respectively can be observed. In conclusion, the optimal conditions for immobilization were obtained at 0.04% PEI concentration at pH 10, which corresponds to a 47% immobilization yield and 21% activity yield. As can be seen in Figure 4.7, when lower PEI concentrations were used lower immobilization yield was obtained This figure shows that by using PEI concentrations of 0.02 and 0% a minimal amount of activity yield was obtained: 7.36% and 0.93% at 0.02 and 0% PEI concentration respectively. Obtaining higher coupling yield, in this case, was due to the contact time between PEI-enzyme cotton was 20 hours. Consequently, all subsequent experiments used a PEI concentration of 0.04 (w/v) (PEI – enzyme ratio 1/125) at pH 9. The reason for using a pH 9 for the PEI solution instead of pH 10 was because it was found later that when the contact time between enzyme-PEI coated cotton was increased from 30 min to several hours the effect of using a pH 10 for PEI solution reduced the enzyme activity yield greatly and better yields were obtained at

pH 9. Since the optimum pH for this enzyme is 6 contact with a solution of pH 10 for a long period of time could deactivate the enzyme.

The next step was to increase the contact time between the enzyme and the PEI-adsorbed cotton, since the immobilization method is an adsorption method based on the physical contact between the enzyme and the support and the possible reactants used. Figure 4.8 shows the results of using different contact times: 1, 5, 10, 15, 20 and 24 hours under the optimal conditions obtained from the previous experiment: PEI concentration (0.04% (w/v) at pH 9) with an enzyme loading of 250 mg/g cotton. As the figure shows, coupling yield increased from 61% to almost 90% from 1 to 24 hours of contact time. Also, it is important to mention the influence of the temperature, since during the experiment the enzyme solutions were incubated in shaker at 4 ^{0}C to avoid possible enzyme deactivation due to the prolonged time of contact between enzyme and PEI-cotton. However, almost no differences were found in coupling and activity yields between 15, 20 and 24 hours, so it was used 20 hours as hour optimal contact time between enzyme and cotton, which corresponded to an immobilization yield of 89% and activity yield of 35%.

The last step was to use GA for cross-linking. For this proposes, different GA concentrations ranging from 0.05 % to 1% (w/v) were used. It is known that GA crosslinks the enzyme to a support, increasing enzyme retention. If no cross-linking was performed, most of the enzyme would be leached out from the aggregates during enzyme activity determination. On the other hand enzyme can be deactivated depending on the amount of GA applied. As can be seen in Table 4.1, some differences were found in activity yield between different GA concentrations ranging from 27% at 1% GA to 35%

at 0.2% GA. Similar results were obtained in a range of GA concentration between 0.15% and 0.3%. Also, it is important to mention that the activity yield was 35% when the immobilization yield was 89%. A possible reason for obtaining lower activity yield than coupling yield is that at high enzyme loading different proteins besides our enzyme were attached to the cotton since the enzyme was not previously purified. Moreover, as can be seen in Figure 3.9, different contact time between GA and coated enzyme PEI were used and obtained similar results in a range of 30 min. to 1 hour. Then, the enzyme activity yield decreased slightly as the contact time increased. This result was expected, since GA besides helping to crosslink the enzyme to a support can deactivate the enzyme over an extended period of contact time. Under these circumstances, a GA solution of 0.2% at pH 7 with a contact time of one hour with coated enzyme-PEI cotton was used in order to avoid possible enzyme leakage in all the subsequent experiments. Also, different pH of GA solution was used without finding any significant difference.

4.3.2 Different enzyme loadings

Different loadings were studied in a range of 50,100, 150, 250, 350 and 500 mg enzyme/g cotton. As shown in Figure 4.10, the level of initial enzyme concentration affected the level of immobilization. As the enzyme loading increased the coupling yield decreased as well as the activity yield. The immobilization yield decreased slightly from 50 to 250mg/g cotton, 92% to 89%, but the activity yield decreased even more from 86.42% to 35.38% in the same range. Also, for higher enzyme loadings the lost of coupling and activity yield was even higher. At higher initial enzyme concentrations, the level of immobilized enzyme stayed at the same level or decreased. Consequently, the

immobilization yield decreased drastically. A possible explanation for this enzyme behavior is an interaction of PEI with different proteins that competed with β-galactosidase for immobilization. It seems that the higher was the initial enzyme concentration the higher was the interaction with different proteins affecting more the immobilization. Figure 4.11 shows a SDS-page that corroborates this hypothesis, in which the first lane represent the marker, lane 2 a free enzyme solution and the following lanes from 3 to 10 different enzyme loadings. Since the MW of ß-galactosidase is 116 KDa the other proteins present in the free enzyme samples have to be considered as impurities. As we observed at low enzyme loadings (50, 100 and 150 represented by lanes 3 to 5) most of the enzyme was immobilized along with proteins with lower molecular weight. However, as the enzyme loading increased, the amount of enzyme immobilized decreased because the proteins with lower molecular weight were always immobilized first. The amount of enzyme active at different enzyme loadings was as follow: 43, 57, 74, 87, 63 and 60 mg of enzyme at 50,100, 150, 250, 350 and 500 mg enzyme/g cotton, respectively. As can be observed from these data, the optimal immobilization loading was 250mg/g cotton.

4.3.3 Thermal stability of enzyme

Thermal stability of the enzyme was study at 3 different temperatures (35, 45 and 60 ^{0}C) for both free and immobilized enzyme. Free enzyme and immobilized enzyme on cotton cloth were kept in distilled water at pH 6 and mixed with lactose solution (100g/l) at appropriate time intervals. As shown in Figure 4.12 thermal deactivation of the enzyme followed first-order reaction kinetics. The deactivation rate constant (K_d) was calculated from the slopes of the semi-logarithmic plot. According to these calculations, the values

of K_d at 35, 45 and 60 ^{0}C were: 0.01, 0.012 and 0.03555 h^{-1} for free enzyme and 0.0008, 0.001 and 0.0297 h^{-1} for immobilized enzyme, respectively. Also the half-lives of the enzyme for the same temperatures were: 69, 57 and 1.94 h. for free enzyme and 866, 693 and 23.3 h. for immobilized enzyme. It is clear from these results that enzyme was more stable when it was immobilized than in free solution. Also comparing the 3 different temperatures it can be concluded that the enzyme was stable below 45 ^{0}C, which was also, the optimal temperature for GOS production.

4.3.4 Kinetics of immobilized enzyme

Figure 4.13a shows typical reaction kinetics for lactose hydrolysis and GOS formation. The reaction was carried with lactose solution of 400g/L at 45 C at pH 6 and with an enzyme loading of 250 mg/g cotton. The data represents the weight percentages of total sugars (w/w) instead of the actual concentration (g/L). Initially,as reported for free enzyme in chapter 3, a higher GOS formation was obtained with a fast decrease of lactose concentration. Once, the maximum GOS percentage (40% of total sugars) was reached, the lactose concentration continued decreasing but at a lower rate. Also, it was observed that larger GOS reached their maximum levels at higher lactose conversion. This result was expected since based on the mechanism of GOS formation. While GOS was reaching the maximum percentage and when started to decrease, glucose and galactose concentrations were increasing continuously. It is clear, that transgalactosylation reaction dominated early in the reaction, producing GOS with high yield, and when the hydrolysis reaction took over GOS percentages started to decrease. Also, it was observed that glucose concentration was higher than galactose during the entire reaction, especially at the beginning of the reaction, indicating that most of the

galactose was involved in GOS formation. However, the presence of galactose in the solution acted as a competitive inhibitor slowing the reaction during the last part of the reaction.

Figure 4.13b shows GOS production affected by lactose conversion, which is defined as lactose converted to other sugars: monosaccharides and GOS. Maximum GOS formation (40% of total sugars) was obtained at 60% lactose conversion. The GOS produced from the reaction was primarily composed of trisaccharides (23.08%). Larger GOS such as tetra- (11.56%), penta- (4.1%) and hexasaccharides (1.48%) were produced at lower levels since for the formation of higher GOS is necessary the linkage of lower ones with a new unit of galactose. Similar results were obtained in previous studies (Mozzafar et al, 1984; Nakanishi et al., 1983), which also reported 40% GOS production at 60% lactose conversion but in free enzyme reaction. However, when the enzyme was immobilized onto a resin Duolite ES-762, a sharp drop in GOS formation was obtained (25% of GOS formed) at a lower degree of lactose formation due to mass transfer resistance problems imposed by immobilization (Mozaffar et al 1986). In this study, GOS formation kinetics was almost identical for reactions carried out with immobilized enzyme at high enzyme loading on cotton cloth and free enzyme in solution. Thus, the ability of the enzyme to form GOS was not affected by the immobilization of the enzyme on cotton cloth.

Initially, GOS concentration increased rapidly with lactose conversion. Also, monosaccharides (glucose and galactose) were produced but at lower rate. However, from the moment that reaction reached 60% lactose conversion, the rate of formation of glucose and galactose was higher and inversely proportional to the rate of decline in GOS

formation, favoring the hydrolysis reaction. Also, similar pattern can be seen in Figure 4.13c, which represents GOS yield during lactose conversion. GOS yield, defined as the amount of GOS formed from the amount of lactose converted to product, was around 70% between 10 and 60% lactose conversion that corresponds to the period in which GOS production increased and transgalatosylation reaction dominated. Then, GOS yield started to decrease until the end of the reaction at 100% lactose conversion in which there was no more GOS.

4.3.5 Effect of enzyme loading on GOS production

Diffusion limitation plays an important role not only on the reaction rate, but also the type of the products formed in the case of competing reactions with varying substrate sizes. The adverse effect of diffusion limitation becomes more profound due to the viscosity of high lactose concentration. Under these conditions, the effect of immobilization of the enzyme can be very significant depending on the degree of mass transfer resistance and lactose concentration used (Iwasaki et al., 1996). Decreases in GOS content and enzyme deactivation upon immobilization have been reported with different enzymes, supports and methods of immobilization (Sheu et al., 1998). Figure 4.14 shows percentages of GOS formation during lactose conversion at different enzyme loadings (100,150 and 250 mg/g cotton) and with free enzyme. Compared with free enzyme, GOS formation was not affected by enzyme immobilization although the reaction rate was slightly faster at higher enzyme loading. Also, at high level of enzyme loading (250mg/g cotton), immobilized enzyme produced as much GOS as in free

solution, suggesting that the enzyme immobilization on cotton cloth does not cause any diffusion limitation on GOS formation from lactose.

4.4 Conclusions

Enzyme immobilization of *Bacillus circulans* lactase on cotton cloth by simple adsorption is inexpensive and the resulting catalytic cotton can be applied for GOS production from lactose. The optimal conditions for enzyme immobilization on cotton cloth were obtained by using 0.04% (w/v) PEI at pH 9 and 0.2% (w/v) GA at pH 7. However the factor that affected the enzyme immobilization the most was the enzyme-PEI ratio used, being optimal at 1/125. Moreover, a better immobilization or coupling yield was obtained when the contact time between the enzyme and the coated PEI-enzyme was extended. A high enzyme loading of 250 mg/g support was obtained with 35% activity yield. The thermal stability of the enzyme increased by 12-fold upon immobilization on cotton cloth. Furthermore, cotton cloth at different enzyme loadings neither imposed any significant diffusion limitation nor affected GOS formation. Therefore, high volumetric productivity can be achieved for GOS production in a Plug-Flow Reactor (PFR), even at high enzyme loading without any loss in GOS production.

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Glutaraldehyde concentration (%)	Coupling Yield (%)	Activity yield (%)
1	87.84+_ 0.41	28.1+_ 0.86
0.8	88.91+_ 1.65	28.5+_ 1.98
0.6	88.62+_ 2.89	29.1+_ 1.45
0.4	86.96 +_ 2.75	30.3+_ 0.90
0.2	89.59 +_ 1.24	35.2+_ 1.61
0.15	89.30 +_ 1.65	33.3+_ 1.87
0.1	89.59 +_ 0.41	31.7+_ 1.27
0.05	88.23 +_0.41	27.2+_ 1.16

Table 4.1: Effect of different GA concentration during immobilization procedure. Enzyme loading of 250mg/g. Contact time between of Glutaraldehyde (GA) and PIE-enzyme coated onto cotton for 30 min. at room temperature

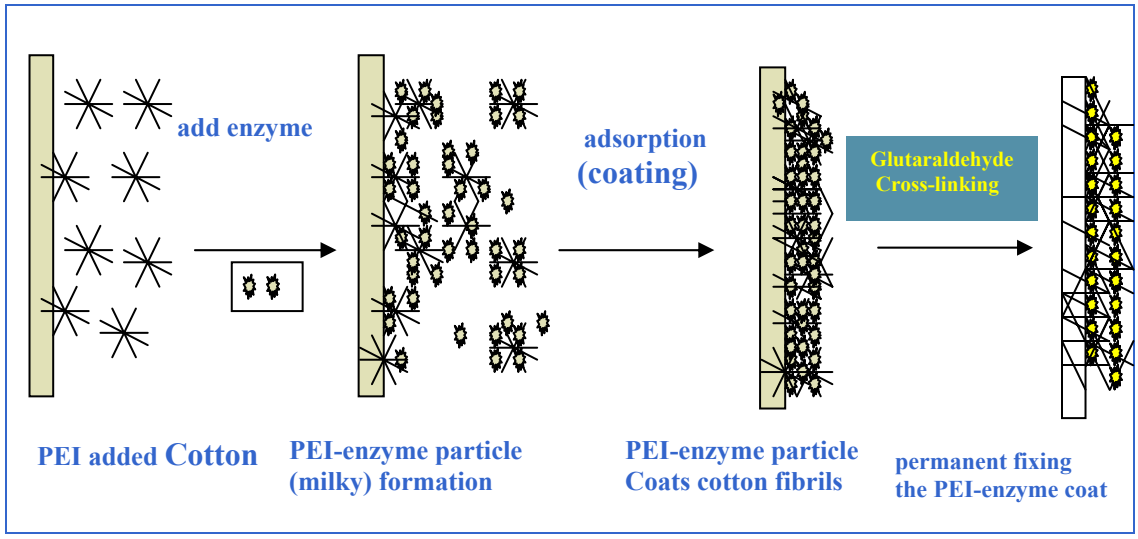

Figure 4.1: Multilayer PEI-enzyme immobilization process (Albayrak et al., 2002)

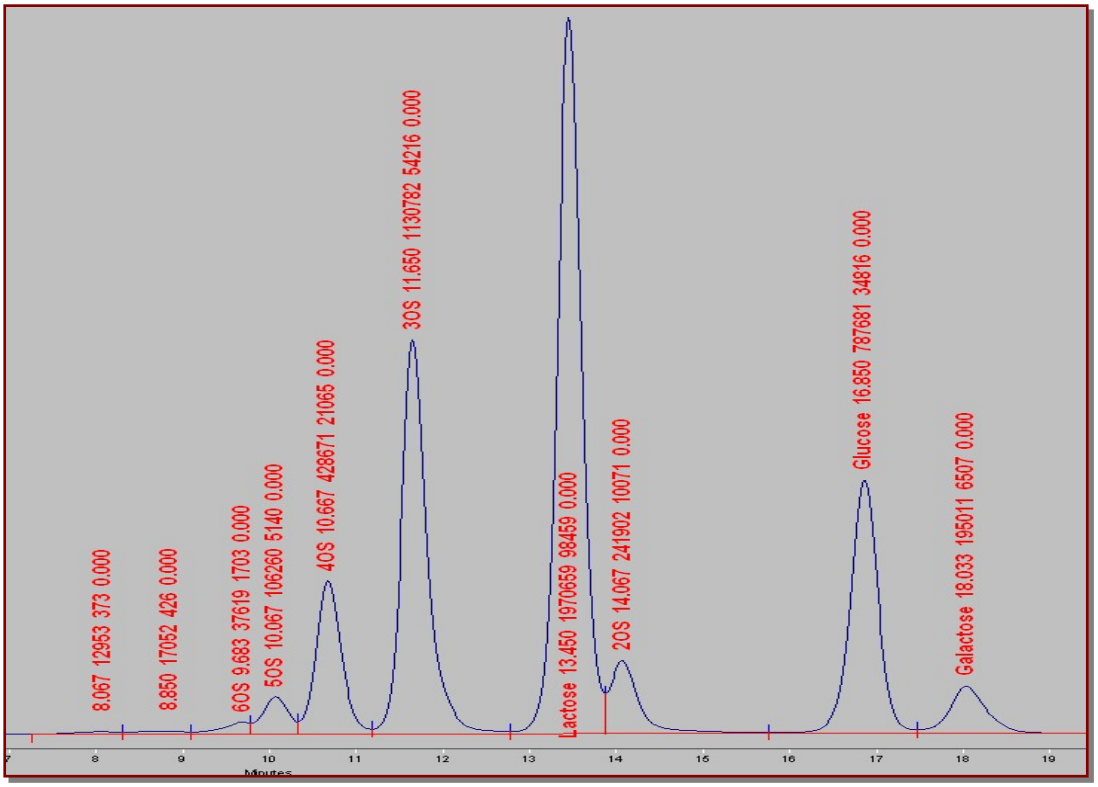

Figure 4.2: A typical HPLC chromatogram showing various sugars found in lactose hydrolysis and GOS formation catalyzed by the immobilized enzyme.

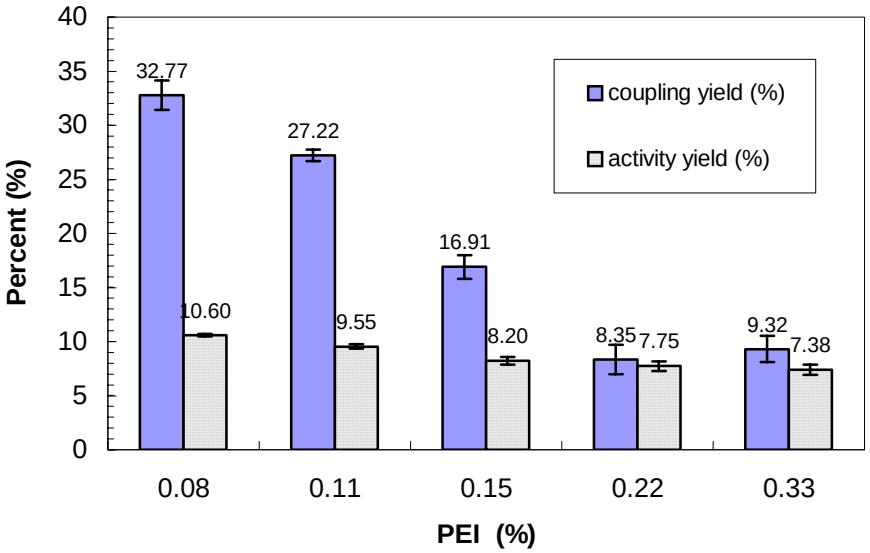

Figure 4.3: Effect of PEI concentration during enzyme immobilization with PEI solution at pH 4. Enzyme loading of 250 mg/g . Contact time PEI-enzyme 30 min.

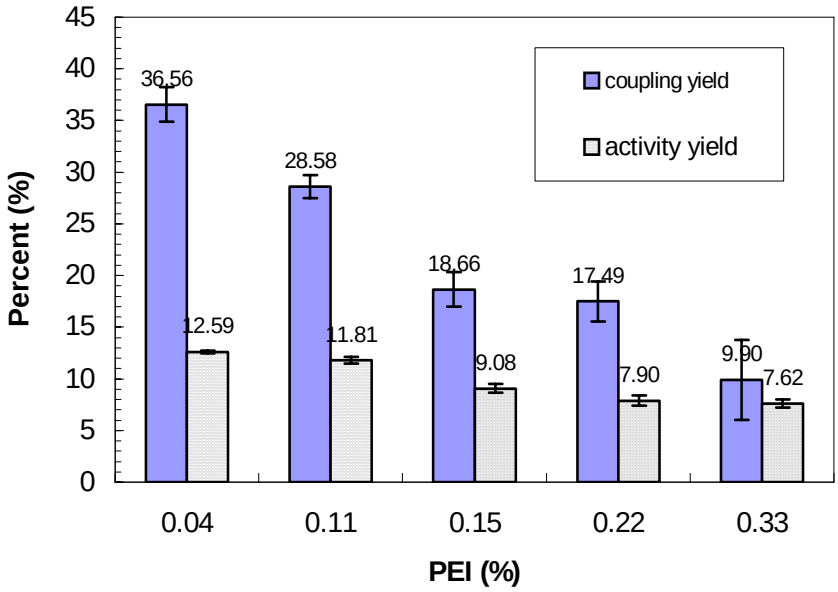

Figure 4.4: Effect of PEI concentration during enzyme immobilization with PEI solution at pH 6. Enzyme loading of 250 mg/g . Contact time PEI-enzyme 30 min.

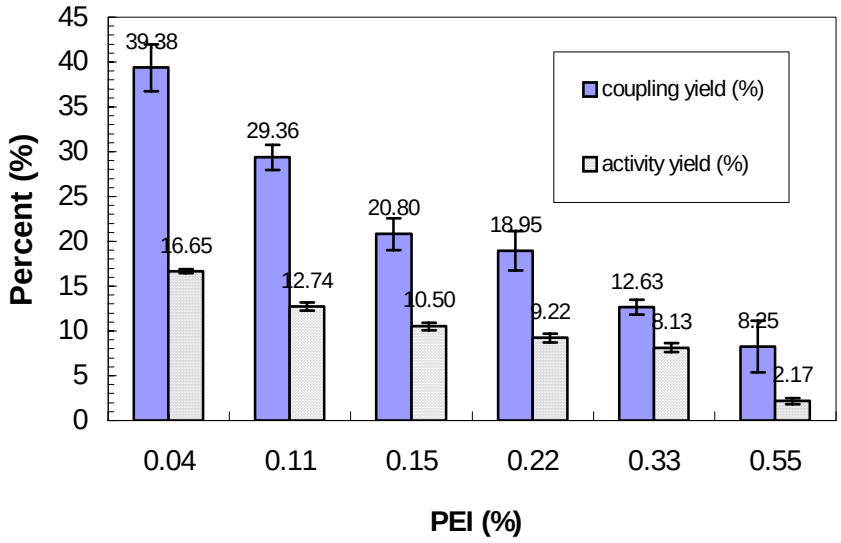

Figure 4.5: Effect of PEI concentration during enzyme immobilization with PEI solution at pH 8. Enzyme loading of 250 mg/g . Contact time PEI-enzyme 30 min.

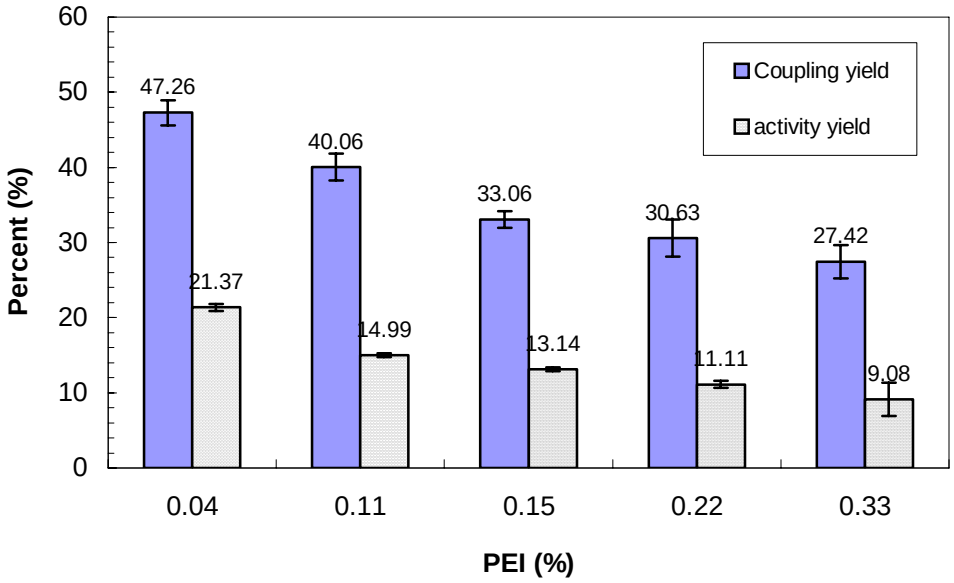

Figure 4.6: Effect of PEI concentration during enzyme immobilization with PEI solution at pH 10. Enzyme loading of 250mg/g. Contact time PEI-enzyme 30 min.

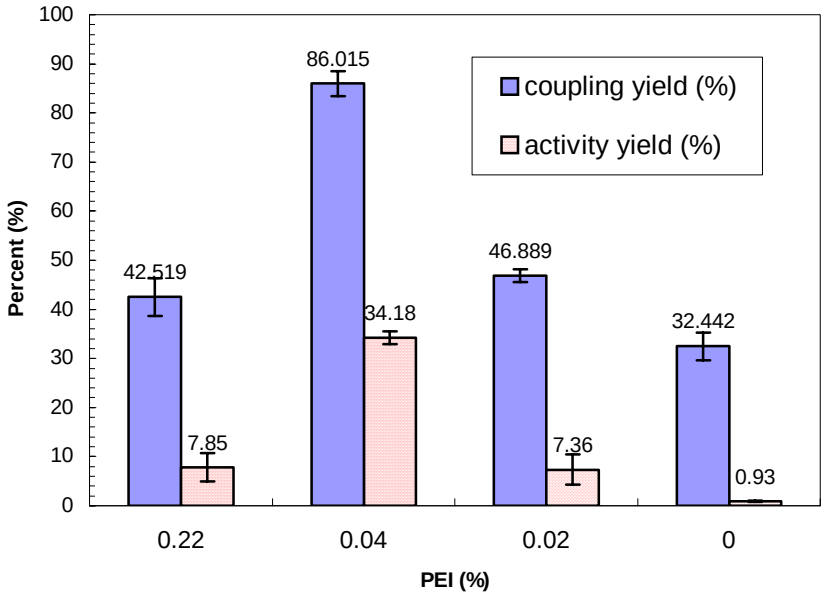

Figure 4.7: Effect of PEI concentration during enzyme immobilization with PEI solution at pH 9. Enzyme loading of 250mg/g. Contact time PEI-enzyme 20 h.

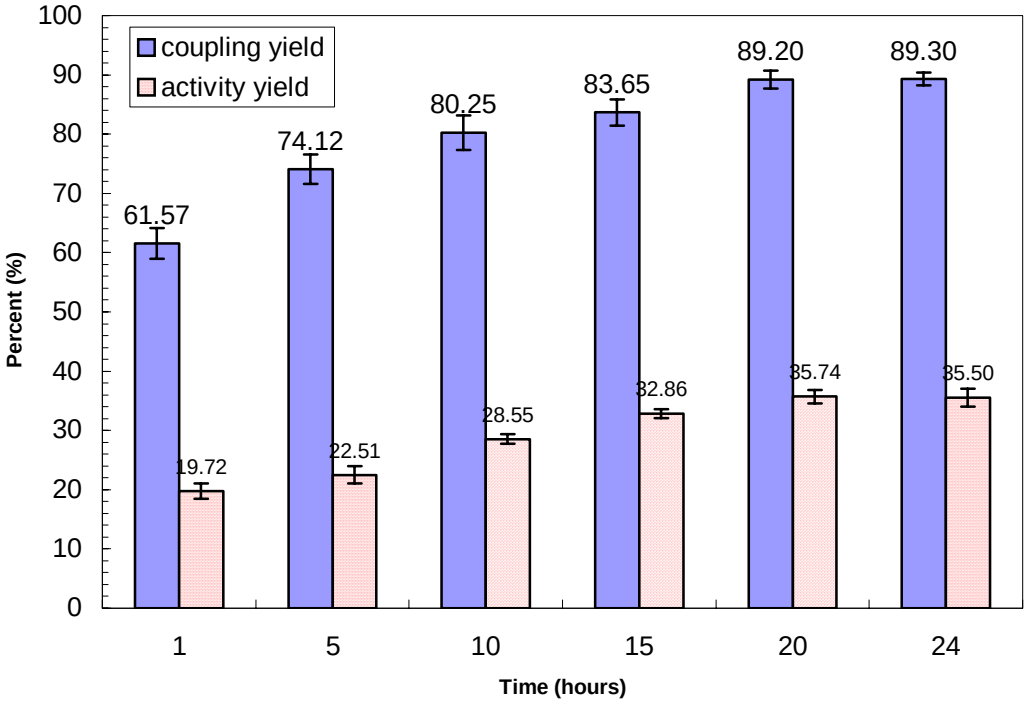

Figure 4.8: Different contact time between PEI-cotton and enzyme at 4 ^{o}C. Enzyme loading 250mg/g. PEI (0.4%, pH 9).

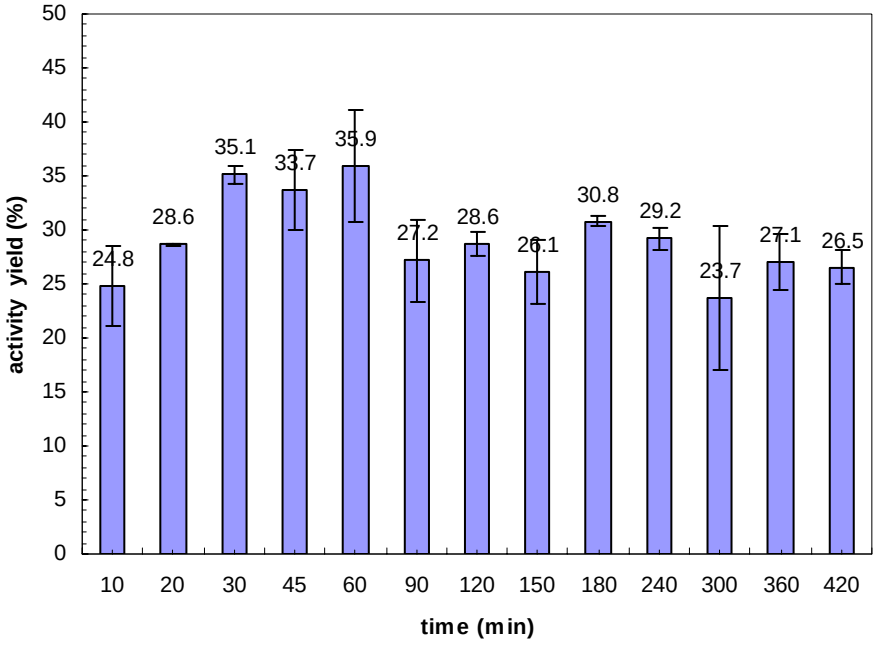

Figure 4.9: Effect of different contact time between Glutaraldehyde (GA) and PEI-enzyme coated onto cotton. Enzyme loading of 250 mg/g cotton

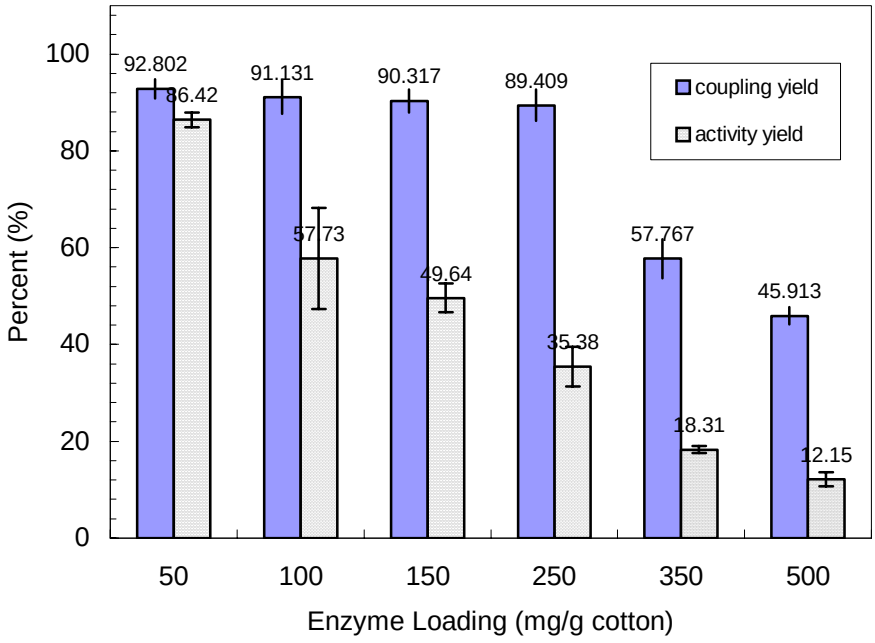

Figure 4.10: Effects of enzyme loading on enzyme coupling and activity yield. PEI (0.04%, pH 9 at 4 ^{o}C, contact time enzyme-PEI 20 h), GA (0.2%, pH 7 at room temperature, contact time 30 min).

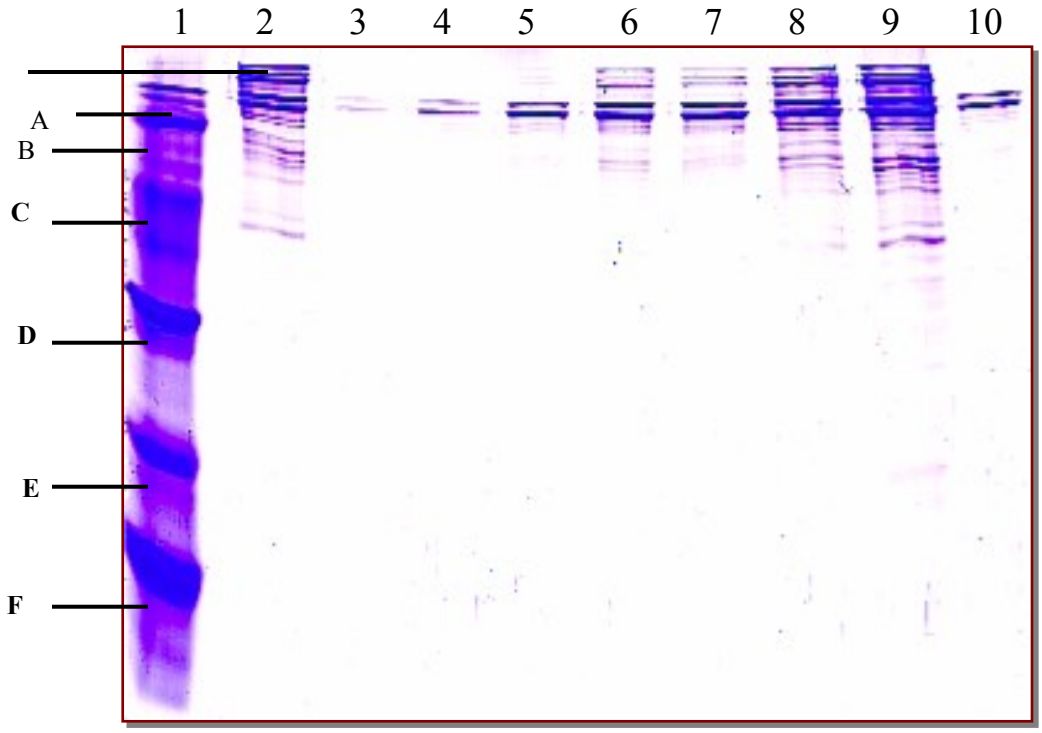

Figure 4.11: SDS page of immobilized enzyme ß-Galactosidase at different enzyme loadings compared with free enzyme in solution. (Lane 1: Molecular marker A, phophorylase b [97 KDa]; B, Albumin [66 KDa]; C, ovalbumin [45 KDa]; D, carbonic anhydrase [30 KDa]; E, trypsin inhibitor [20.1 Kda]; F, α-Lactalbulmin [14.4 Kda]; Lane 2: Free enzyme ß-Galactosidase; Lane 3: Immobilized enzyme (50mg/g cotton); Lane 4: Immobilized enzyme (100mg/g cotton); Lane 5: Immobilized enzyme (150mg/g cotton); Lane 6 and 7: Immobilized enzyme (250mg/g cotton); Lane 8: Immobilized enzyme (350mg/g cotton); Lane 9: Free enzyme; Lane 10: Immobilized enzyme (250mg/g cotton);

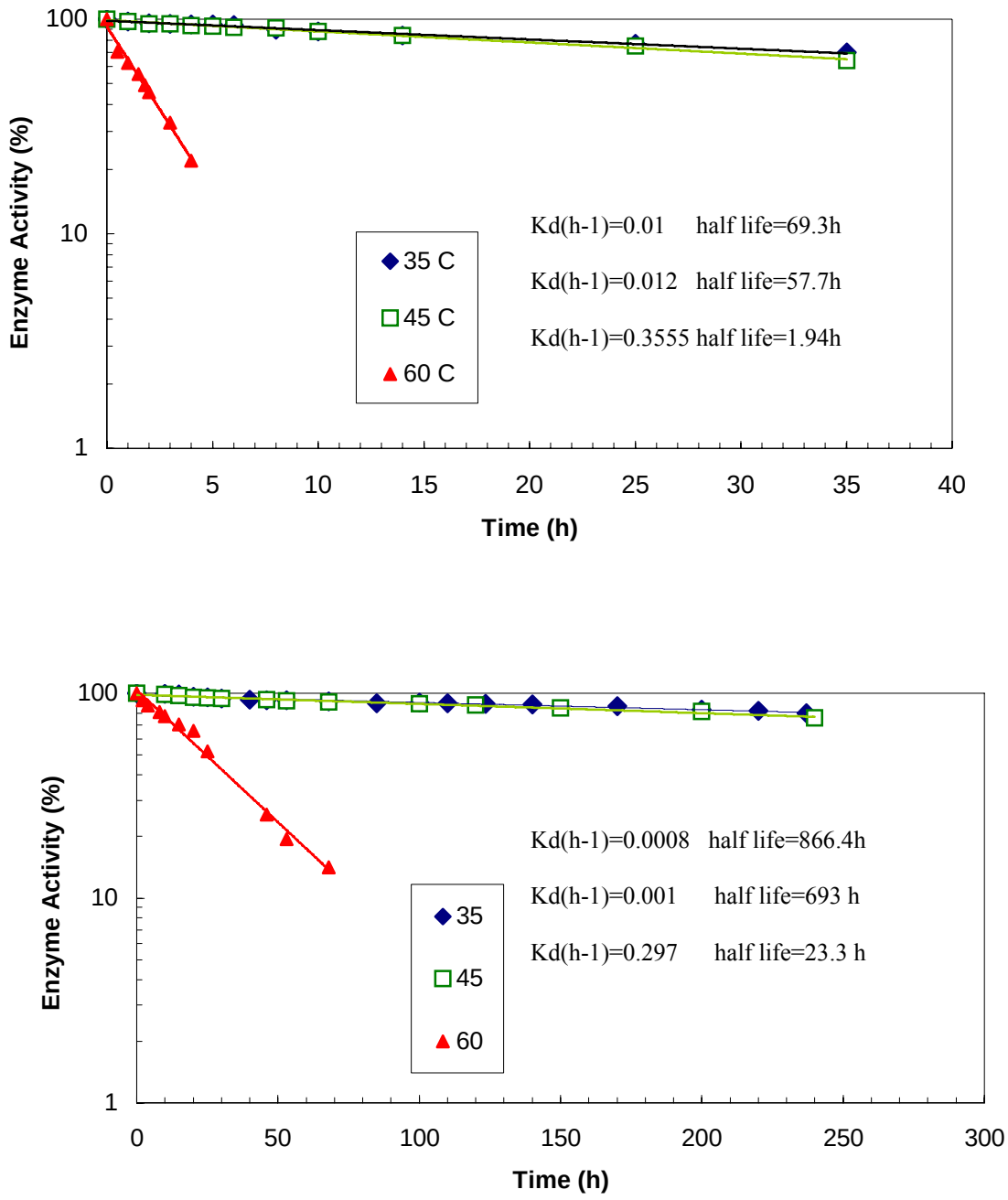

Figure 4.12: Thermal stability for a) free enzyme in solution and b) immobilized enzyme on cotton cloth at various temperatures.

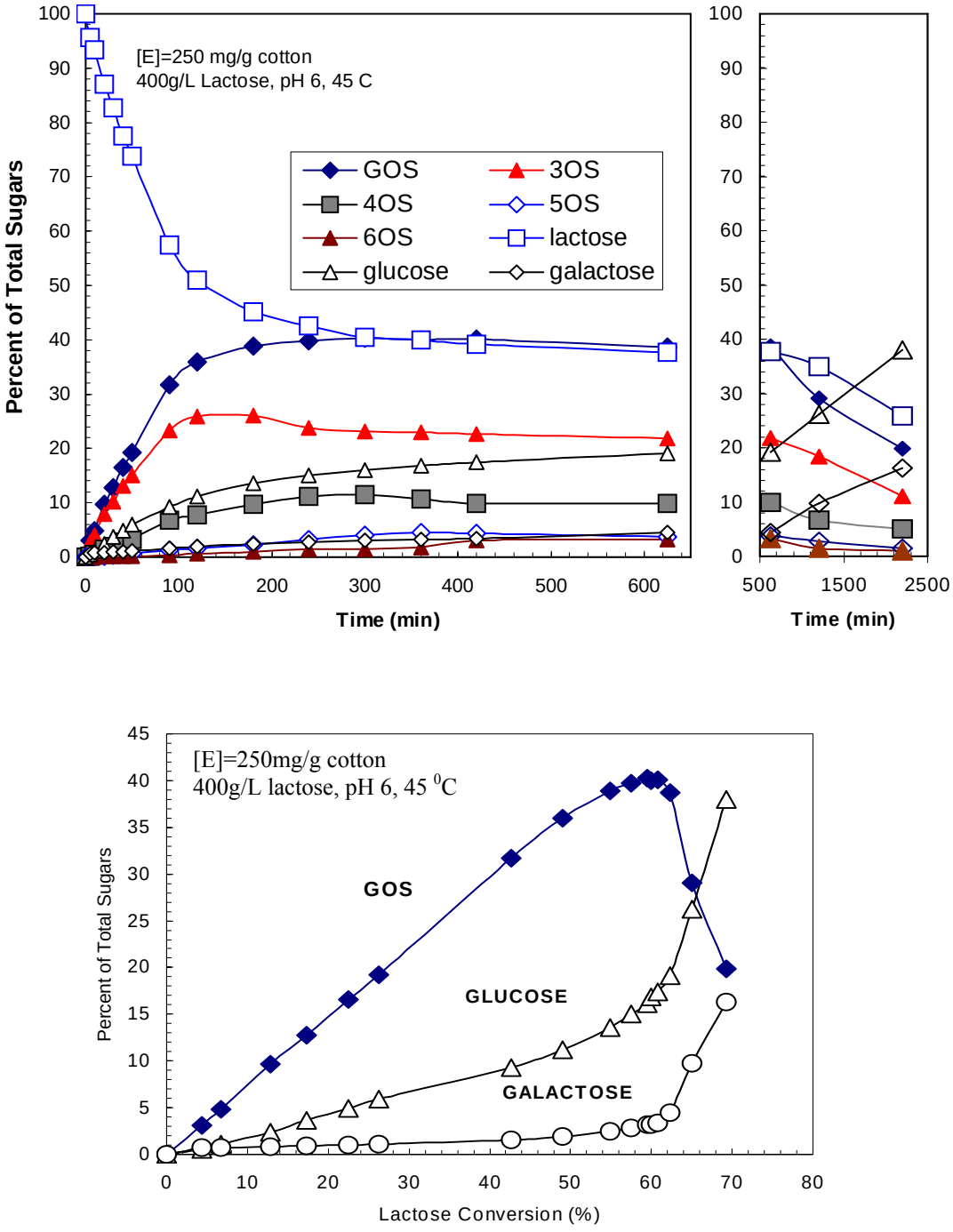

Figure 4.13: Reaction kinetics of lactose hydrolysis and GOS formation of immobilized enzyme: a) as function of time, b) as function of lactose conversion, and c) GOS yield at 45 ^{o}C with an initial lactose concentration of 400 g/L (continued)

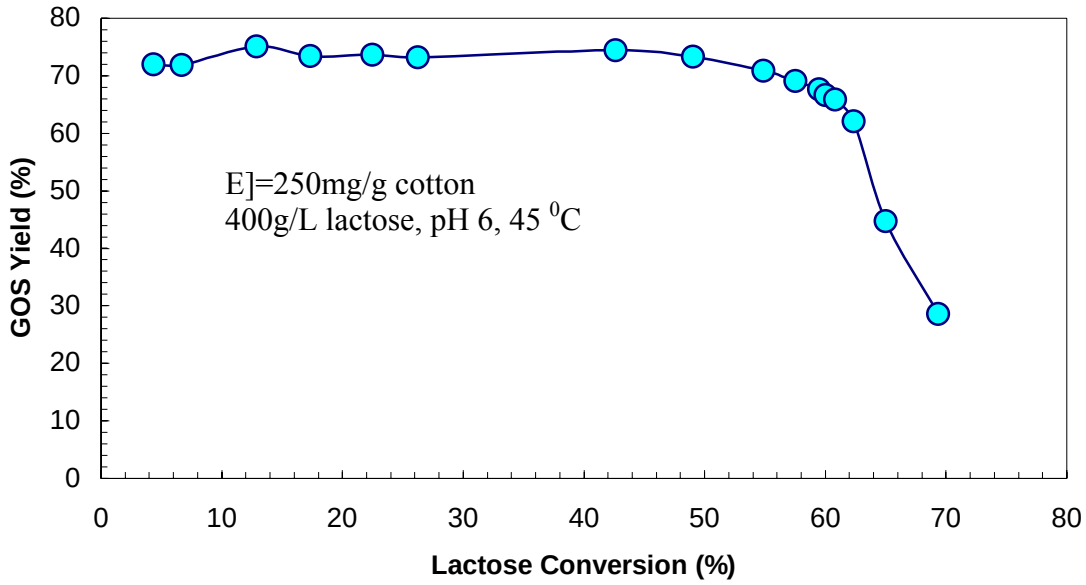

Figure 4.13: (continued)

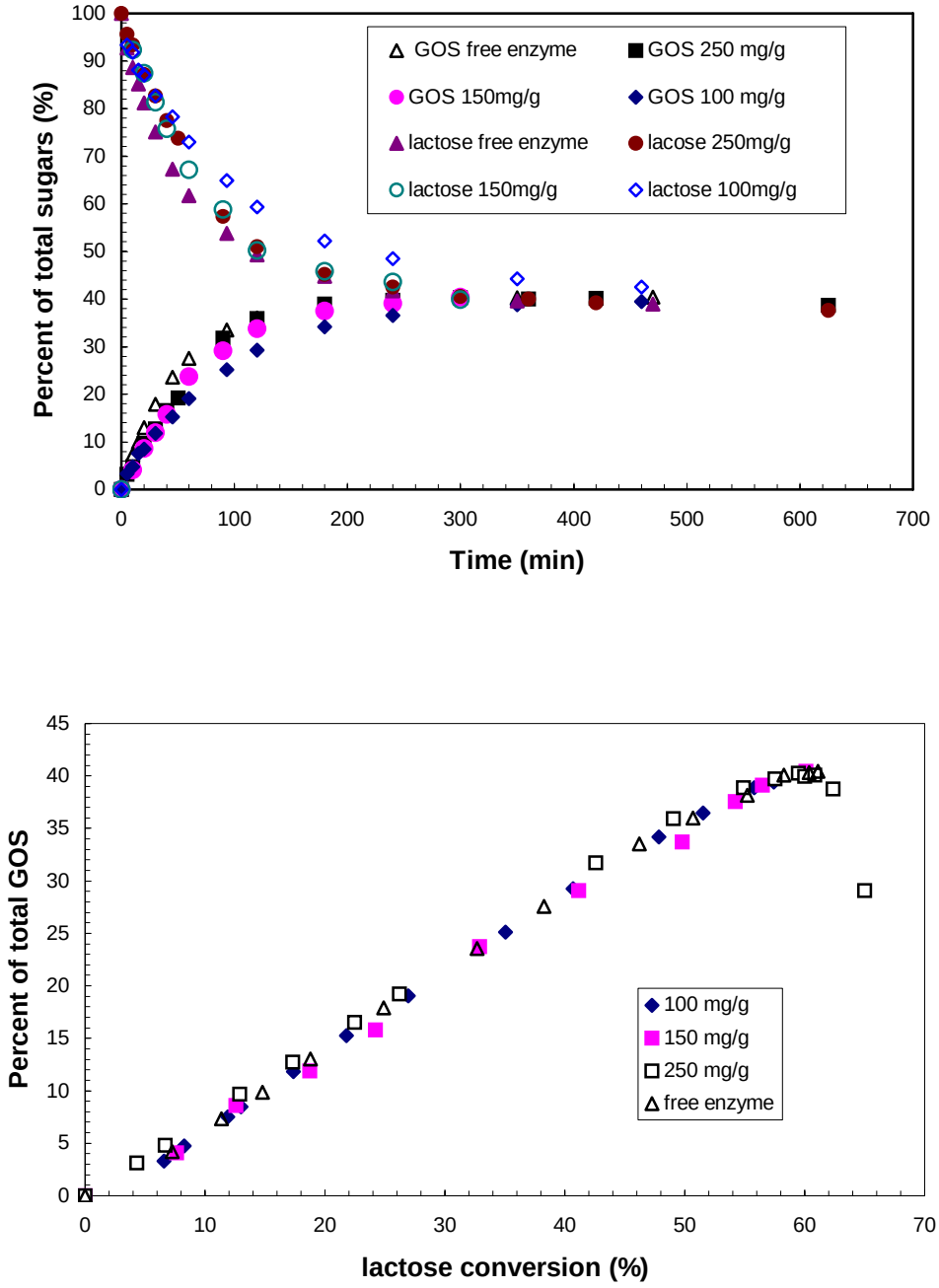

Figure 4.14: Immobilized enzyme kinetics a) as function of time and b) as function of lactose conversion at different enzyme loading.

CHAPTER 5

PRODUCTION OF GALACTO-OLIGOSACCHARIDES WITH TWO SEQUENTIAL IMMOBILIZED ENZYME REACTORS BY USING β-GALACTOSIDASES FROM *ASPERGILLUS ORYZAE* AND *BACILLUS CIRCULANS*

Abstract

In this work, a process consisting of two sequential packed-bed reactors with immobilized β-galactosidases from *Aspergillus oryzae* and *Bacillus circulans* on cotton cloth was developed for galacto-oligosacccharides (GOS) production. Continuous operation was studied for 10 days, obtaining a maximum GOS production of 25% (w/w) and 40% (w/w) with initial feed concentration of 400 g/L lactose for lactases from *A. oryzae* and *B. circulans*, respectively. The reactor productivity was 4300 g/L·h for *A. oryzae* lactase and 180 g/L·h for *B. circulans* lactase at the enzyme loading of 250 mg/g cotton. Higher overall GOS productivities can be achieved with two immobilized enzyme reactors with *A. oryzae* and *B. circulans* lactases in sequence.

5.1 Introduction

GOS, also know as *Bifidus growth factor*, are defined as prebiotic ingredients since they stimulate selectively the growth of bifidobacteria and lactobacilli (Mussatto et al., 2007; Cumming et al., 2002; Sako et al., 1999). The addition of GOS as functional food ingredient, it has great potential to improve the quality of many foods (Rivero-Urgell et al., 2001; Playne et al., 1994; Fooks et al, 1999). GOS can be produced from lactose, which is an abundant component of cheese whey, in an enzymatic transgalactosylation reaction catalyzed by microbial β-galactosidases (Mahoney, 1998; Zarate et al., 1990). Continuously stirred tank reactors (CSTRs) and plug-flow reactors (PFRs) are the most common reactors types used for continuous production, with different variations and combination available, including recycle reactors and fluidized bed reactors. However PFRs often have kinetics advantages over CSTRs, including higher efficiency conversion. Concerning GOS production purposes, PFRs also have a higher yield. Furthermore, one of the largest influences on reactor choice is the type of support the enzyme is immobilized on (Genari et al., 2003). Many of the carriers used for immobilization of ß-galactosidase applied in GOS production were some types of microparticles, such as ion-exchange resins (Woudenberg et al., 1998; Matsumoto et al., 1989), chitosan beads (Sheu et al., 1998; Shin et al., 1998) cellulose beads (Kminkova et al., 1988) and agarose beads (Berger et al., 1995). It was observed that the immobilized enzyme in these particles carriers often resulted in 20-30% reduction of GOS yield due to introduction of mass transfer resistance in the system (Sheu et al., 1998; Shin et al., 1998). Furthermore, most of these carriers are expensive and cause problems, including high pressure drop when used in a packed bed reactor (Grosova et al., 2008). In this

research, cotton cloth was used to immobilize the enzyme β-galactosidase for the purpose of continuous GOS production in a PFR. Cotton cloth is an inexpensive and widely available fibrous material. It provides many advantages, including large specific surface area, high mechanical strength due to its crystalline structure, and high porosity. Therefore, immobilized enzyme on cotton cloth placed in a plug-flow reactor provides good flow rates at low-pressure drops, being highly desirable characteristics for industrial application.

In this work, a process consisting of two sequential plug-flow reactors with immobilized enzyme β-galactosidase from *Aspergillus oryzae* (fungal) and *Bacillus circulans* (bacterial) was studied for GOS production by using lactose as the substrate The first enzyme was chosen because it is commercially available, has already been used in food products and is relatively inexpensive. The second enzyme gives high GOS yields at high lactose conversions and a high GOS content in the final product. Simultaneously to transgalactosylation reaction for GOS production, ß-galactosidase hydrolyzes lactose in both monosaccharides: glucose and galactose. This chapter focuses on the sequential production of GOS by using two plug-flow reactors. The GOS kinetics for both enzymes was studied by using a recycle plug-flow reactor with lactose as the substrate. Also, continuous production of GOS in a single-pass reactor was monitored over several days. Finally, the reaction kinetics of β-galactosidase for GOS production by using two sequential immobilized enzyme reactors were obtained as follows: a) recirculating a 25% GOS solution obtained from *Aspergillus oryzae* lactase into an immobilized enzyme reactor with the same enzyme, b) recirculating a 25% GOS solution obtained from *Aspergillus oryzae* lactase into an immobilized enzyme reactor with *Bacillus circulans*

lactase. The reaction kinetics of lactase by using two sequential reactors were studied to see if higher GOS amount could be obtained when the substrate used contained already some amounts of GOS.

5.2 Materials and Methods

5.2.1 Enzymes and chemicals

ß-galactosidase from *Bacillus circulans* (bacterial lactase activity 5500 LU/g) was obtained from Daiwa Kasei (Shiga, Japan). One lactase unit (LU) is defined as the amount of enzyme, which liberates 1 μmol of glucose per minute from lactose (concentration 10%) at the early stage of the reaction at 40 ^{o}C, pH 6. ß-galactosidase from *Aspergillus oryzae* (fungal lactase activity 106,742 LU/g) was obtained from Enzyme Development Corporation (New York, NY). One lactase unit (LU) is defined as the amount of enzyme, which liberates 1 μmol of glucose per minute from lactose at pH 4.5 at 37 ^{o}C. Lactose (99.9%) was obtained from Brewster Dairy (Brewster, OH). Polyethyleneimine [PEI; $(C_2H_5N)_n$] as 50% (w/v) (number average molecular weight 60,000; average molecular weight 750,000) and glutaraldehyde (GA) as 25% (w/v) aqueous solutions were from Sigma (St. Louis, MO). Sodium phosphate monobasic anhydrous (Fisher) and sodium phosphate dibasic anhydrous (E.M. Science) were used to prepare phosphate buffer. Glacial acetic acid (Fisher) and sodium acetate anhydrous (Fisher) were used to prepare acetic acid buffer. Cotton cloth was obtained locally. All solutions for PEI, GA, and enzyme were prepared with distilled water. The solution pH was adjusted, when necessary, using HCl or NaOH solution of sufficient concentration.

5.2.2 Enzyme immobilization of β-galactosidase from *A. oryzae* and *B. circulans* on cotton cloth

Enzyme immobilization on cotton cloth involved three main steps: adsorption of PEI solution to cotton cloth, introduction of enzyme to PEI-containing cloth, and GA cross-linking of PEI enzyme aggregates coated on the cotton. The exact conditions used during the immobilization procedure depended on the source of ß-galactosidase. After immobilization, cross-linked cotton cloth was washed extensively with distilled water. All the solutions used during immobilization were kept cold until use. Unless otherwise noted, 1 mL of PEI solution (pH 8) containing 2.2 mg (0.22%) was added to each piece of 0.2 g cotton cloth in a 125 ml Erlenmeyer flask for immobilization of ß-galactosidase from *A. oryzae*. At the same time, 1 mL of PEI solution (pH 9) containing 0.4 mg (0.04%) was also added to each piece of 0.2 g cotton cloth in a 125 ml Erlenmeyer flask for immobilization of ß-galactosidase from *B. circulans*. The solution volume was at a sufficient level to completely wet the cloth, thereby allowing a homogeneous distribution of PEI to the matrix. After adsorption of PEI, 50 mg of enzyme (10 mL of 5 mg/mL enzyme solution) was added to the flask. Upon the addition to the enzyme a milky turbid solution was formed. This turbidity was higher for *A. oryzae* lactase. Then, the flasks were incubated in a shaker-incubator at room temperature for 10-15 minutes for *A. oryzae* lactase and at 4 ^{0}C for 21 hours for *B. circulans* lactase. Usually, the coupling reaction can be assumed to be completed when the white turbidity disappear. Then, the coupling solution was slowly decanted, and the PEI-enzyme-coated cotton was transferred to a cold GA solution (0.2% (w/v)), pH 7.0 for 5 minutes for *A. oryzae* lactase and (0.25% w/v), pH 7.0) for 60 minutes for *B. circulans* lactase for cross-linking at room

temperature. Afterwards, the cross-linked cotton cloth was washed extensively with distilled water. There was no washing step until the completion of GA cross-linking. When bigger pieces of cotton were used, the contact time for coupling between the enzyme solution and PEI-adsorbed cotton was also longer.

5.2.3 Reaction kinetics of β-galactosidase from *Aspergillus oryzae*

GOS formation kinetics with immobilized ß-galactosidase from *A. oryzae* was studied in a recycle batch packed-bed reactor (Figure 5.1). Cotton cloth (1.4 g) in pieces of 0.2 g and separated by a metallic mesh, was placed in the glass column reactor (internal diameter 0.9 cm.) with a water jacket maintained at 40 ^{0}C. The total volume of the reactor was 3 mL. The lactose solution in the flask (total solution volume, 100 mL) was continuously re-circulated through the immobilized enzyme reactor at a flow rate of 10 mL/min. The lactose solution was prepared by dissolving lactose in 0.1 M acetate buffer at pH 4.5. Samples (0.5 mL) were taken from the flask at appropriate time intervals and analyzed for sugar contents with high performance liquid chromatography (HPLC). The reaction kinetics was studied by using two different substrates. The first substrate consisted of pure lactose with an initial concentration of 400 g/L. The second substrate was a product obtained from a continuous reactor with immobilized *A. oryzae* lactase. This product contained: 100 g/L of GOS, 200 g/L of lactose and 100 g/L of monosaccharides (glucose and galactose). However, to reach an initial concentration of 400 g/L of lactose, 50mL of 600 g/L of lactose were mixed with 50 mL of the original GOS solution. After mixing, the solution contained: 14% GOS (w/w), 67% (w/w) lactose and 19% (w/w) monosaccharides. The total sugar concentration was 500 g/L. Once the

reaction was completed, all samples were diluted 20 times before they were analyzed with HPLC.

5.2.4 Reaction kinetics of β-galactosidase from *Bacillus circulans*

GOS formation kinetics with immobilized ß-galactosidase from *B. circulans* was studied in a recycle batch fixed-bed reactor. Cotton cloth, in pieces of 0.2 g and separated by a metallic mesh, was placed in a glass column reactor (internal diameter 1.5 cm.) with a water jacket maintained at 45 ^{0}C. The total volume of the reactor was 30 mL. The lactose solution in the flask (total solution volume, 125 mL) was continuously re-circulated through the immobilized enzyme reactor at a flow rate of 10 mL/min. The lactose solution was prepared by dissolving lactose in distilled water (pH 6, unless otherwise notice). Samples (0.5 mL) were taken from the flask at appropriate time intervals and analyzed for sugar contents with high performance liquid chromatography (HPLC). The reaction kinetics was studied by using two different substrates. The first substrate consisted in pure lactose with an initial concentration of 400 g/L. The second substrate was a 25% GOS sample obtained from a reactor with immobilized *A. oryzae* lactase, with a total concentration of 400 g/L. Figure 5.2 shows the laboratory set up for the two sequential packed-bed reactors.

5.2.5 Continuous GOS production by using β-galactosidase from *A. oryzae*

Continuous production of GOS from lactose was studied in a single-pass reactor. Approximately 41.6 g of cotton cloth was placed in the column reactor (internal diameter of 51 mm) with a total packed bed length of 177 mm (bed volume 363 mL). Each piece

of cotton (0.7 g) was cut in circles and a layered on top of a metallic mesh to allow good contact with the substrate. Continuous production of GOS from lactose was studied at 45 °C to evaluate the reactor long-term performance. The reactor was fed with 400 g/L lactose solution (0.1 M acetic buffer, pH 4.5) for about 10 hours at 250 mL/h. The flow rate was adjusted to obtain the maximum GOS content in the product stream, which in the case of *A. oryzae* lactase was 50% lactose conversion. A smaller reactor was used to test the continuous GOS production for a longer time. A total volume of 4 mL of cotton was placed in a column reactor (internal diameter 0.9 cm). The lactose solution was fed in the reactor at a flow rate of 2.8 mL/min for 10 days. The lactose solution was kept in a 60 °C water bath to prevent crystallization of lactose. Samples from the reactor effluent were collected at proper time intervals and analyzed with HPLC

5.2.6 Continuous GOS production by using β-galactosidase from *B. circulans*

Continuous production of GOS from lactose was studied in a single-pass reactor. Approximately 5 g of cotton cloth was placed in the column reactor (internal diameter of 15 mm) with a total packed bed length of 15.2 cm (bed volume 26.8 mL). Each piece of cotton (0.08 g) was cut in circles and a metallic mesh to allow better contact with the substrate separated each cotton piece. A total of 63 pieces were used. Continuous production of GOS from lactose with the reactor was studied at 45 °C to evaluate the reactor long-term performance. The reactor was fed with two different solutions: a) 400 g/L lactose solution (distilled water at adjusted pH 6) for about 10 days at 0.5 mL/min, b) 50 g/L lactose solution for about 24 hours at 1.8 mL/min. The flow rate was adjusted to obtain maximum GOS content in the product stream. The lactose solution was kept in a

60 °C water bath to prevent crystallization of lactose. Samples from the reactor effluent were then collected at proper time intervals and analyzed with HPLC.

5.2.7 Analytical Methods

5.2.7.1 Determination of GOS productivity

The GOS productivity (g/L/h) was calculated for continuous GOS production on plug flow reactor and also for the reaction kinetics of GOS formation as follows:

a) GOS productivity for continuous run in PFR:

$$\text{Productivity} = (\text{GOS})^* (\text{FR})/ (V_{reactor}) \quad (5.1)$$

where GOS is the galacto-oligosaccharides concentration (g/L), FR is the flow rate (mL/min) and $V_{reactor}$ is the reactor volume (mL).

b) GOS productivity for GOS kinetics in a recycle PFR:

$$\text{Productivity} = (\text{GOS})^*(V_{substrate}) / (T_1)^*(V_{reactor}) \quad (5.2)$$

where GOS is the galacto-oligosaccharides concentration (g/L), $V_{substrate}$ is the substrate volume recycled in the reactor (mL), T_1 is time of reaction for GOS formation (min) and $V_{reactor}$ is the reactor volume (mL).

c) Overall GOS productivity for GOS kinetics in a two-step recycle PFR:

$$\text{Overall Productivity} = (GOS_2)^*(V_{substrate})/ ((T_{max})^* (V_{reactor})_1) + ((T_2)^* (V_{reactor})_2) \quad (5.3)$$

where GOS_2 is the galacto-oligosaccharides concentration (g/L) of the second reactor, $V_{substrate}$ is the substrate volume recycled in the reactor (mL), T_{max} is time of reaction needed to obtain maximum GOS from reactor 1 (min) and $(V_{reactor})_1$ is the volume of

reactor 1(mL), T_2 is the time of reaction for GOS formation in reactor 2 (min) and $(V_{reactor})_2$ is the volume of reactor 2(mL).

5.2.7.2 HPLC Analysis

The concentrations of various sugars (glucose, galactose, lactose, and galactooligosaccharides) in sample solutions were determined by using HPLC. An HPLC system consisting of a pump (LC-10AI Shimadzu), an automatic sampler injector (Shimadzu SIL10Ai), a carbohydrate analysis column (Phenomenex, Rezek RNM carbohydrate column, 7.8 mm-150 mm), a column oven (Shimadzu CTO-10A), a refractive index detector (Shimadzu RID-10Avp), and a Shimadzu CLASS-VP chromatography data system (version 7.2 integrator) was used. The eluent was pre-degassed distilled water (at 80 °C) at a flow rate of 0.4 mL/min. Distilled water was degassed by first boiling and then sonicating for 30 min. The column temperature was maintained at 80 °C, and the detector temperature was set at 45 °C. The concentrations (w/v) of these sugars (e.g., lactose, glucose, galactose, and oligosaccharides including tri-, tetra-, and pentasaccharides), presented as weight percentages of total sugars, were determined from peak heights and are reported in this paper.

5.3 Results and Discussion

5.3.1 GOS kinetics of immobilized β-galactosidase from *A. oryzae* and *B. circulans*

Two different recycle batch packed-bed reactors of immobilized ß-galactosidase from *A. oryzae* and *B. circulans* were employed to investigate the GOS production kinetics. Figures 5.3 and 5.4 show reaction kinetics for lactose hydrolysis and GOS

production affected by lactose conversion for lactase from *A. oryzae* and *B. circulans*, by using an initial lactose concentration of 400 g/L. As reported for free enzyme, GOS concentration increased rapidly with lactose conversion for both enzymes. At the same time, there was formation of monosaccharides: glucose and galactose, but a lower rate. It is clear, that transgalactosylation reaction dominated early in the reaction, producing GOS with a high yield. Maximum GOS formation of 25% and 40% of total sugars was obtained at 50% and 60% lactose conversion for *A. oryzae* and *B. circulans* lactase, respectively. The percentage of the different GOS obtained at 60% lactose conversion for *B. circulans* lactase was as follows: 23% 3OS, 11.6% 4OS, 4.5% 5OS and 1.7% 6OS of total sugars. For *A. oryzae* lactase the percentage of different GOS from total sugars at 50% lactose conversion was also as follows: 17.2% 3OS, 5% 4OS, and 2% 5OS. GOS formation kinetics was almost identical for reactions carried out with immobilized enzyme on cotton cloth using a packed-bed reactor and free enzyme in solution. Thus, the ability of the enzyme to form GOS, for both enzymes, was not affected by the immobilization of the enzyme on cotton cloth.

Finally, from the moment that reaction reached the maximum amount of GOS at 50% and 60% lactose conversion for *A. oryzae* and *B. circulans* lactase, the rate of formation of glucose and galactose was higher and inversely proportional to the rate of decline in GOS formation, favoring the hydrolysis reaction. Figure 5.5 shows GOS yield as affected by the lactose conversion for *A. oryzae* and *B. circulans* lactase. The GOS yield was 70% between 10 and 60% lactose conversion for *B. circulans* lactase and 55% until 50% lactose conversion for *A. oryzae* lactase, which corresponded to the period in which GOS production increased and transgalatosylation reaction dominated over

hydrolysis reaction. Then, GOS yield shifted and started to decrease when the formation of monosaccharides were higher.

In conclusion, since at high enzyme loading (250mg/g cotton) immobilized lactase from A. oryzae and B. circulans produced as much GOS as in free enzyme solution, high volumetric productivity can be achieved for continuous GOS production in packed-bed reactors for both lactases. Furthermore, since the maximum amount of GOS from these enzymes were obtained at different lactose conversion, GOS production by using these lactases in a system of sequential plug-flow reactors could lead to increase GOS yield and reactor productivity compared with a single reactor.

5.3.2 Continuous GOS production by using β-galactosidase from *A. oryzae*

Figure 5.6 shows the continuous production of GOS in a plug flow reactor with immobilized ß-galactosidase from *A. oryzae* on cotton cloth at 40 ^{0}C. A small reactor of 4 mL was used to study the operational stability during 10 days. By feeding the reactor with a lactose solution of 400 g/L at 2.8 mL/min, which in this case was the optimum to obtain maximum GOS production (25% w/w) at 50% lactose conversion, a productivity of 4300 g/L/h was obtained. The reactor productivity was calculated by multiplying the final GOS concentration (g/L) by the feed rate and divided with the reactor volume. The reactor was stable during 10 days with no apparent decrease in the level of GOS formation. The high productivity obtained was mainly because of the high enzyme loading (250mg/g cotton) achieved during immobilization, the initial lactose concentration used (400 g/L) and the lack of significant mass transfer limitation caused by the cotton, producing similar amounts of GOS both in free and immobilized enzyme.

Furthermore, a bigger reactor was set up to continuously produce GOS to observe if a scale up would affect GOS production. A reactor of 363 mL was fed with 400 g/L lactose solution at 250 mL/min during 10 consecutive hours, As it can be seen in Figure 5.7, the amount of GOS produced was 23% (w/w), which was 2% less than the maximum amount obtained previously. However, the difference in GOS production could be due to the difficulty to adjust the flow rate to obtain the optimum lactose conversion with the manual pump used. Therefore, the GOS production of 23% (w/w) was obtained at the lower than 50% lactose conversion. Higher GOS content could have been achieved if a slightly lower flow rate was used.

5.3.3 Continuous GOS production by using β-galactosidase from *B. circulans*

Continuous production of GOS by using an immobilized enzyme on cotton cloth in a plug flow reactor was examined for a possible industrial application. The continuous production was studied for 10 days by using a bed volume of 26.8 mL with a 400 g/L lactose solution that was fed continuously in the reactor at 45 ^{0}C. The flow rate was adjusted to obtain 60% lactose conversion, which is, the optimum conversion to produce the maximum amount of GOS. Initially, as can be seen in Figure 5.8, an outlet product of 40.4% (w/w) GOS was obtained at a flow rate of 0.5 mL/min with a productivity of 180 g/L/h. However, the amount of GOS declined between 3% and 8% of the initial 40% GOS during the 10 days of continuous run. Initially, it was thought that a leach of the enzyme from the support due to a poor cross linking was causing the lost of productivity since this problem has been reported previously by Shin et al., 1998 when ß-galactosidase from *B. singularis* immobilized on chitosan beads was used for GOS production at high

flow rates. They reported that to avoid the leach of the enzyme the flow rate was reduced for long-term production, loosing much of the reactor productivity. However, in this study, most of the enzyme activity was recovered after running for 14 hours a buffer solution through the reactor everyday. It seems that the inactivation of the enzyme was due to the accumulation of GOS on the cotton cloth, which blocked the enzyme from contacting with the substrate. Nakanishi et al., 1983 reported similar inactivation when they tried to run a plug flow reactor for continuous production with immobilized lactase from *B. circulans* on resin Duolite ES-762. However, they started the reaction with 5% lactose solution and the immobilized enzyme lost almost 40% of its initial activity after 20 hours of reaction. In our case, even though a clear steady state was never achieved, the enzyme inactivation was least pronounced due to the high surface area and high porosity provided by the cotton cloth, where the immobilized enzyme can be thoroughly exposed to the substrate. Furthermore, the assumption of the reversible inactivation was proven when the reactor was fed with a lower lactose concentration of 50 g/L (Figure 5.9). Initially, it was obtained an outlet product of 22% GOS at a flow rate of 18 mL/min with a productivity of 44 g/L/h. The stability of the reactor for GOS production was better since only 4% of the initial 22% GOS was lost in a continuous running during 24 hours. At initial lower concentration the maximum amount of GOS produced is also lower therefore it is necessary more time to block the enzyme.

Table 5.1 compares the productivity and maximum GOS content obtained from different lactases. As can be seen, the productivities from *A. oryzae* and *B. circulans* lactases obtained in this study were the highest among all the lactases studied. The higher

productivity obtained was mainly because of the high enzyme loading (250 mg/g cotton) achieved during immobilization and the high initial lactose concentration used

5.3.4 Continuous GOS production by using two sequential plug flow reactors

Lactases from some species, especially bacterial (Berger et al., 1995a; Shin et al., 1998) and yeast enzymes (Foda et al., 2000) usually maximize GOS content at higher lactose conversion (60% or higher), obtaining high GOS yield. For this reason, a 25% (w/w) GOS sample obtained from a continuous run in the plug flow reactor of immobilized lactase from *A. oryzae* was used as the substrate. The 25% GOS solution was recycled through an immobilized enzyme reactor with ß-galactosidase from *A. oryzae* and *B. circulans*, simulating two set-ups of two sequential reactors. It was thought that by feeding the reactor with a sample that already contained GOS a higher GOS percentage could be achieved, obtaining higher product yield. The reactor with immobilized *A. oryzae* lactase was run with a GOS sample modified by adding certain amount of lactose to obtain an initial lactose concentration of 400 g/L. For this reason, the initial percentage of GOS from total sugars dropped from 25% (w/w) to 16% (w/w). As can be seen in Figure 5.10b, the percentage of GOS obtained was slightly lower that the percentage obtained by using a single reactor and was around 23% (w/w). However, lactose shifted to a higher degree of conversion, about 58% instead of 50%, when pure lactose was the substrate. The percentage of different GOS obtained at the maximum production was as follow: 14.3% 3OS, 6.3% 4OS and 2.2% 5OS. As in a single reactor run, no amount of 2OS was observed. Also, as previously, larger GOS obtained its maximum level of production at higher lactose conversion. However, as can be seen in

Figure 5.10a, the rate of GOS production was reduced. It is possible that the presence of monosaccharides in the original substrate sample played inhibitory effect, since monosaccharides and especially galactose have been reported previously as competitive inhibitor for product formation (Boon et al, 2000). Also, figure 5.10c shows the GOS yield obtained as function of lactose conversion. As expected, since the amount of GOS produced was the same as in the single reactor but at a higher lactose conversion, the maximum GOS yield decreased from 60% to 45%. In general, transgalactosylation was the dominant reaction at the beginning, before it was reached maximum GOS production. From this point, hydrolysis reaction took over decreasing the GOS yield.

Figure 5.11b shows the GOS formation achieved in a plug flow reactor with immobilized *B. circulans* lactase by using a solution containing 25% (w/w) GOS as the substrate, obtained from a continuous run of a reactor with immobilized ß-galactosidase from *A. oryzae*. This GOS solution was used to simulate the GOS production in two sequential reactors with both lactases. The maximum amount of GOS production was around 34%, which was 6% less than the 40% (w/w) achieved when pure lactose was the substrate. However, as for immobilized *A. oryzae* lactase, the maximum amount of GOS was produced at a higher lactose conversion, around 70% compared to 60% in the case of using pure lactose as substrate. The 6% difference in GOS production was due to lower formation of higher size GOS since, for example, 6OS were not synthesized. It is possible that the presence of monosaccharides (18% glucose and 7% galactose) in the initial substrate acted as inhibitors of GOS formation, which it has been reported previously (Boon et al., 2000), not allowing formation of GOS higher than 5OS. For the same reason, as can be seen in Figure 12a, the rate of GOS formation was slower compared

with the rate of GOS production with pure lactose. The percentage of different GOS obtained at the maximum production was as follows: 20.85% 3OS, 9.75% 4OS and 3.76% (w/w) 5OS. The major difference was found in the formation of monosaccharides present in the reaction mixture at this point, with 7.2% (w/w) galactose and 26.8% (w/w) glucose, compared to 2.5% (w/w) galactose and 17.7% (w/w) glucose when pure lactose was initially used. Moreover, Figure 5.11c shows how the maximum GOS yield was around 50% at 70% lactose conversion, dropping from 70% when pure lactose was the substrate. This result was expected since the maximum GOS amount produced was 34%, instead of 40%, and it was achieved at a higher lactose conversion.

Table 5.2 compares the maximum GOS production achieved for both lactases: *A. oryzae* and *B. circulans* in a reactor when lactose and 25% (w/w) GOS solutions were used as substrate. As can be seen, higher productivities at maximum GOS production were achieved when single immobilized enzyme reactors were used. Lactase from A. oryzae produced 25% GOS with a productivity of 4300 g/L/h, followed by lactase from *B. circulans* producing 40% GOS (w/w) with a productivity of 180 g/L/h. However, maximum GOS amounts were achieved at a higher lactose conversion with two sequential reactors: 34% GOS (w/w) was obtained at 70% lactose conversion with *A. oryzae-B. circulans* lactases followed by 25% GOS at 58% lactose conversion with *A. oryzae-A. oryzae* lactases. The production of maximum GOS at a higher lactose conversion is crucial from industrial point of view since further purification removing lactose from the reaction mixture is the most difficult task in GOS separation process. On the other hand, using two consecutive reactors, it would reduce considerable the productivity of the system since GOS yield was lower at maximum GOS production.

Figure 5.12 show the productivities obtained at different lactose conversion for a single and dual reactor using immobilized *A. oryzae* lactase. As can be seen, higher productivities were obtained at lower lactose conversion in both cases. This result was expected since at higher lactose conversion the presence of monosaccharides reduced the rate of GOS formation favoring the hydrolysis reaction and consequently reducing the productivity. Furthermore, the productivity of the second reactor was lower at same lactose conversion compared with the first reactor. However similar productivities were achieved when it was compared the first reactor with the overall productivity of the system. It can be concluded that using two consecutive reactors with *A. oryzae* lactase could not obtained higher GOS yield or productivity. Figure 5.13 shows the GOS productivities obtained at different lactose conversion for single reactors using immobilized *B. circulans* lactase and dual reactor with *A. oryzae-B. circulans* lactase. Again, higher productivities were obtained at lower lactose conversion. However, at the same lactose conversion the overall productivity obtained with two-step reactor was much higher than in a single reactor. Consequently, using two-step reactor with *A. oryzae-B. circulans* lactase not only improved GOS productivity of the system but also maximized the GOS produced at a higher lactose conversion.

5.4 Conclusions

A process consisting of two sequential plug-flow reactors with immobilized β-galactosidase from *Aspergillus oryzae* and *Bacillus circulans* on cotton cloth was developed to produce GOS. Initially, single reactor with immobilized *A. oryzae* lactase produced 25% GOS at 50% lactose conversion with a productivity of 4300 g/L/h. The

reactor was stable during 10 days with no apparent decrease in the level of GOS formation. The high productivity obtained was mainly because of the high enzyme loading (250mg/g cotton) achieved during immobilization, the initial lactose concentration used (400 g/L) and the lack of significant mass transfer limitation caused by the cotton, producing similar amounts of GOS both in free and immobilized enzyme. Also, a single reactor with immobilized *B. circulans* lactase was feed continuously with 400g/L lactose during 10 days. The maximum GOS produced was 40% GOS at 60% lactose conversion with a GOS productivity of 180 g/L/h. A clear steady state was never achieved due to enzyme inactivation with a drop between 3% and 8% of the initial 40% GOS during the 10 days of continuous run. However, this enzyme inactivation was reversible since most of the enzyme activity was recovered after running buffer solution through the reactor for several hours. It seems that the inactivation of the enzyme was due to the accumulation of GOS on the cotton cloth, which blocked the enzyme from contacting with the substrate. Furthermore, when two-step reactor was run with immobilized *A. oryzae* lactase no increment on productivity or GOS yield was obtained due to the inhibitory effect of monosaccharides on the rate of GOS formation. Furthermore, two-step reactor with immobilized *A. oryzae-B. circulans* lactase produced 34% GOS at 70% lactose conversion. Again the presence of monosaccharides slowed down the rate of GOS formation. However, higher GOS productivity was obtained compared with a single reactor. In conclusion, the two-step reactor with immobilized *A. oryzae-B. circulans* lactase is suitable for GOS production since maximum GOS were produced at higher lactose conversion obtaining higher GOS productivities.

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source of enzyme	mode of process	lac-conc. [g/l]	T [°C]	pH	max. GOS [wt %]	productivity [g/l/h]	Reference
B. circulans							
	Batch (FE)	45.6	40	6	24	2.2	Mozaffar et al., 1984
	Continuous (IE, CSTR)	45.6	40	6.4	40	4.2	Mozaffar et al., 1986
	Continuous (IE)	**400**	**45**	**6.5**	**40**	**180**	**This study (Figure 5.8)**
T. aquaticus	Batch (FE)	160	70	4.6	34.8	2.3	Berger et al., 1995a
K. lactis	Bath (FE, UF)	230	45	7	22.2	11.3	Foda and Lopez-Leiva, 2000
	Continuous (FE, UF)	200	45	7	31	13.7	
B. singularis	Continuous (PBR)	100	45	4.8	55	4.4	Shin et al., 1998
A. oryzae	Batch (FE)	380	40	4.5	31	24.3	Iwasaki et al., 1996
	Continuous (IE)	**400**	**40**	**4.5**	**25**	**4300**	**This study (Figure 5.6)**

Table 5.1:GOS production by using various β-galactosidase in different modes.

Plug-flow reactor	6OS (%)	5OS (%)	4OS (%)	3OS (%)	total GOS (%)	lactose (%)	lactose conversion (%)	2OS (%)	glucose (%)	galactose (%)	Yield (%)	Productivity (g/L/h)
A. oryzae	0	2.18	5.05	18.50	25.74	50.6	59.36	0	14.81	8.80	52.1	4300
A. oryzae - A. oryzae	0	2.24	6.30	14.88	23.42	41.68	58.32	0	23.40	11.49	40.1	3141
B. circulans	0.66	3.29	12.23	24.22	40.40	39.87	60.13	0	17.84	1.89	67.1	180
A. oryzae - B. circulans	0	3.76	9.75	20.85	34.36	31.53	68.47	0	27.19	6.62	50.1	81.33

Table 5.2: GOS production by immobilized enzyme ß-galactosidase from *Aspergillus oryzae* and *Bacillus circulans* in a single and dual Plug-Flow Reactor (PFR).

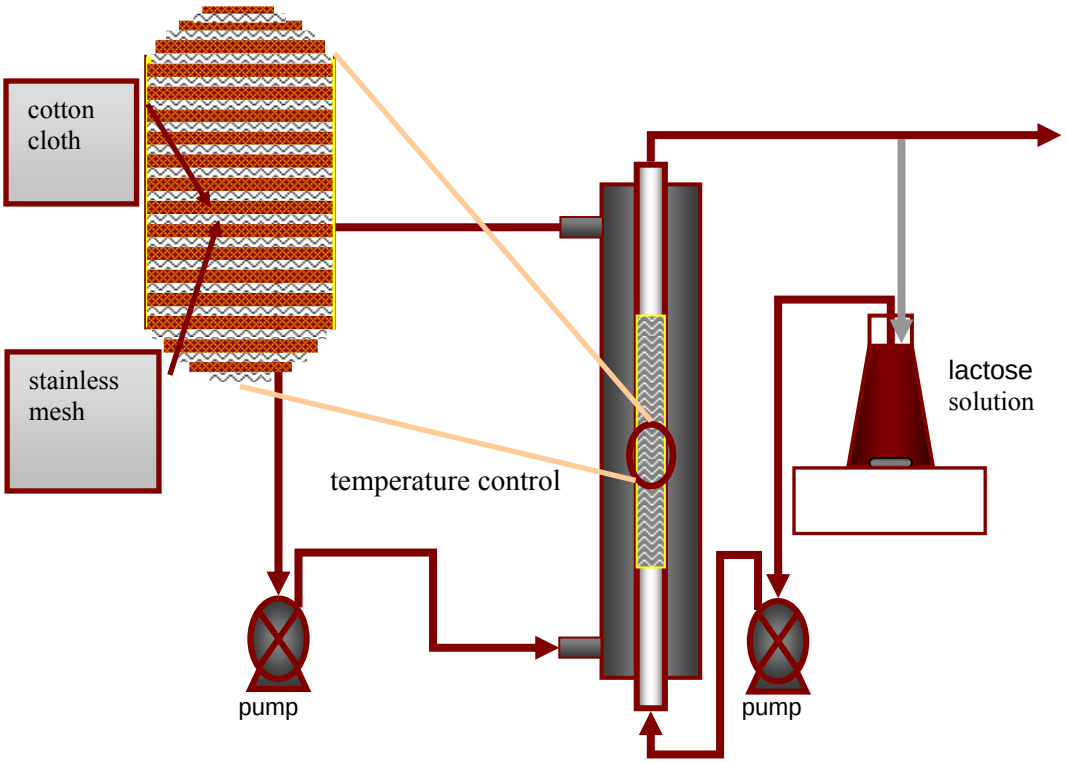


Figure 5.1: Schematic diagram of immobilized enzyme reactor on cotton-cloth for GOS production. The system was operated as a continuous and recycle single-pass reactor.

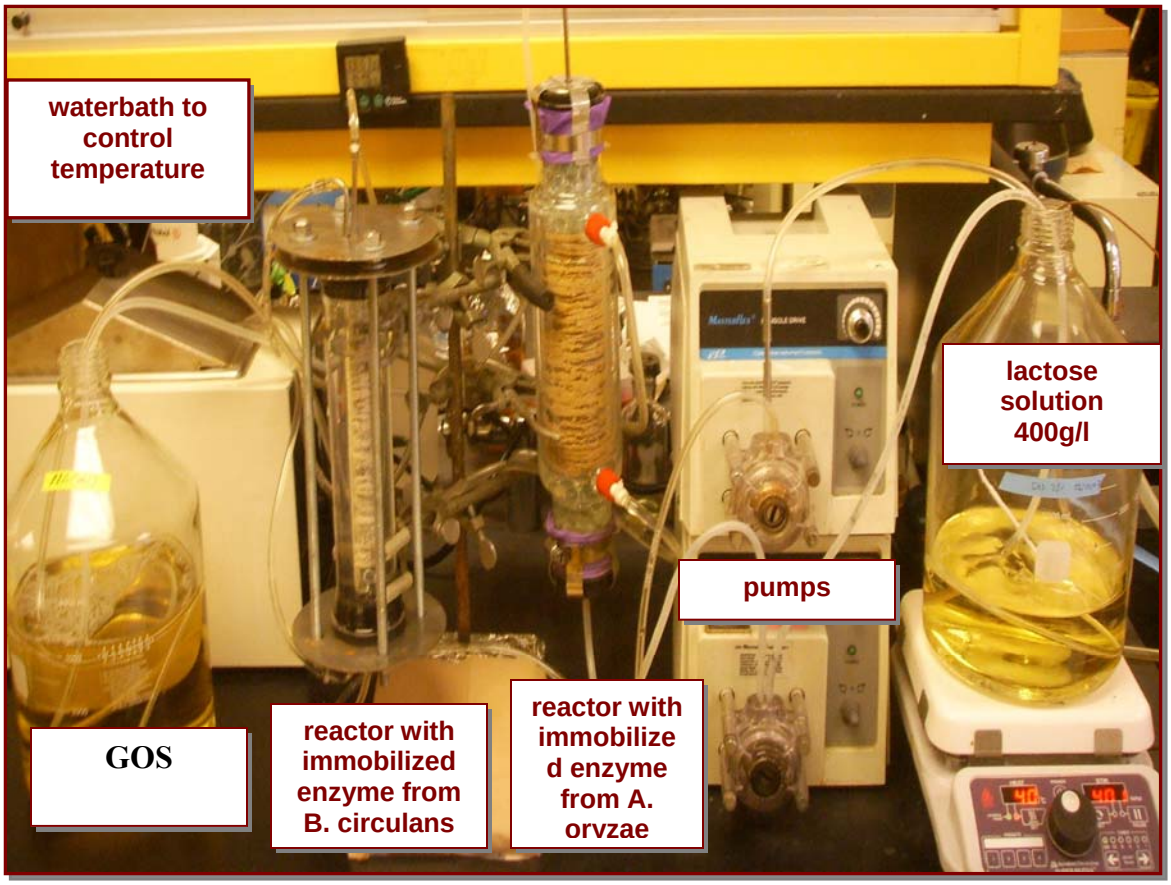
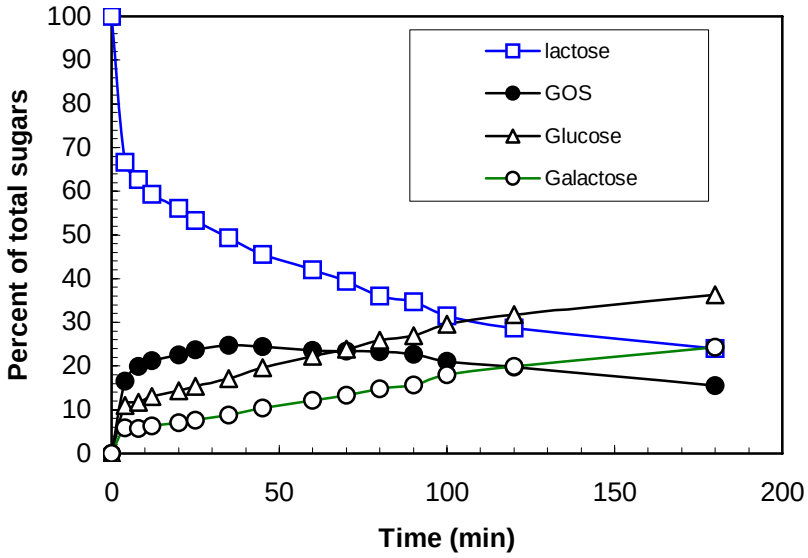


Figure 5.2: Picture of the laboratory set up for GOS production by using two sequential Plug-Flow Reactors by using the enzyme β-galactosidase from *Aspergillus oryzae* and *Bacillus circulans*.



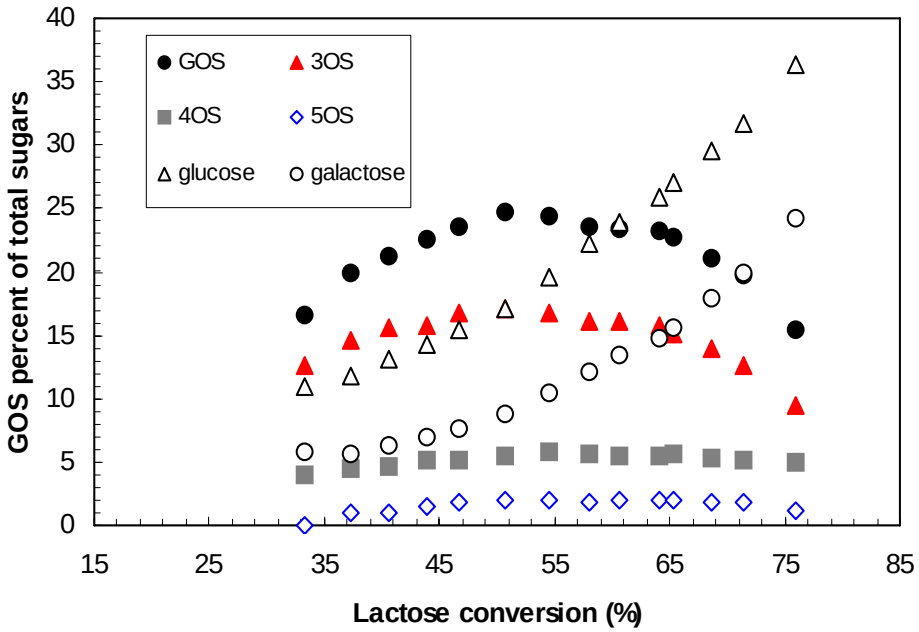
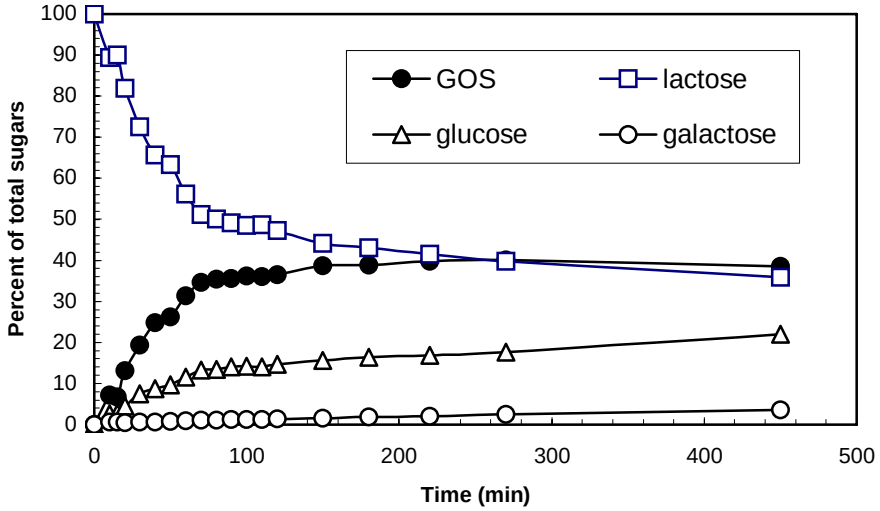


Figure 5.3: Reaccion kinetics for lactose hydrolysis and GOS formation a) as function of time, b) as function of lactose conversion catalyzed by immobilized enzyme ß-Galactosidase from *Aspergillus oryzae* in a recycle plug-flow reactor at 40 ^{0}C with initial lactose concentration of 400g/l.



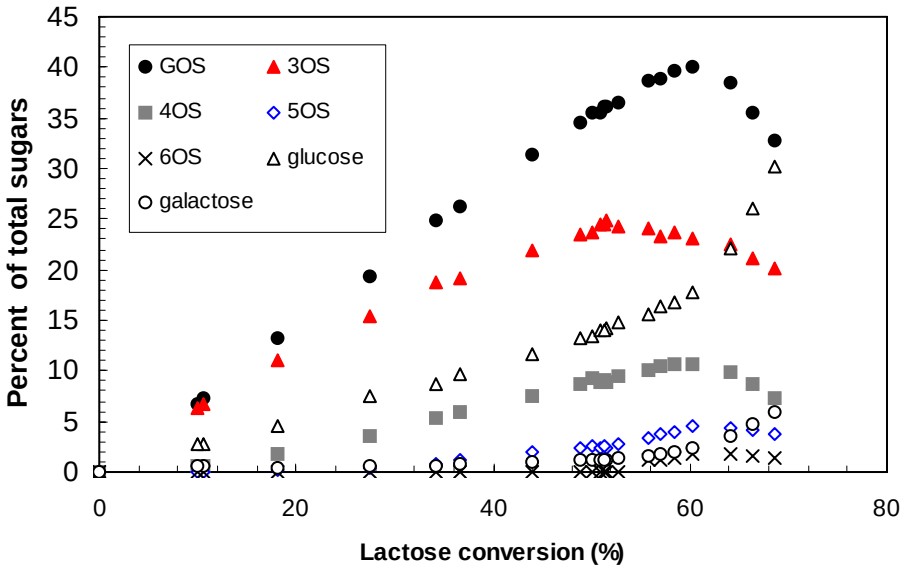


Figure 5.4: Kinetics of GOS formation and lactose hydrolysis a) as function of time, b) as function of lactose conversion catalyzed by immobilized enzyme ß-Galactosidase from *Bacillus circulans* in a recycle plug-flow reactor at 45 ^{0}C with initial lactose concentration of 400g/l.

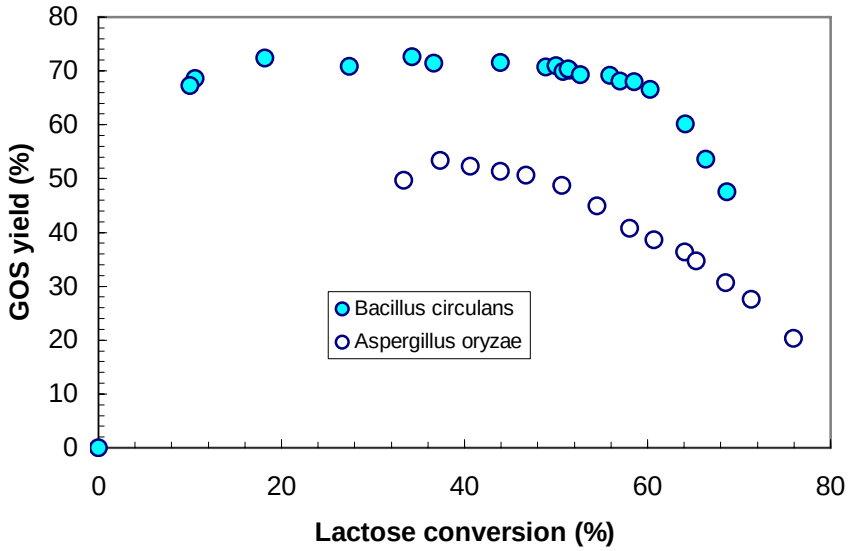


Figure 5.5: GOS yield during lactose hydrolysis as function of lactose conversion catalyzed by immobilized enzyme ß-Galactosidase from *Bacillus circulans* and *Aspergillus oryzae* in a recycle plug-flow reactor with initial lactose concentration of 400g/l.

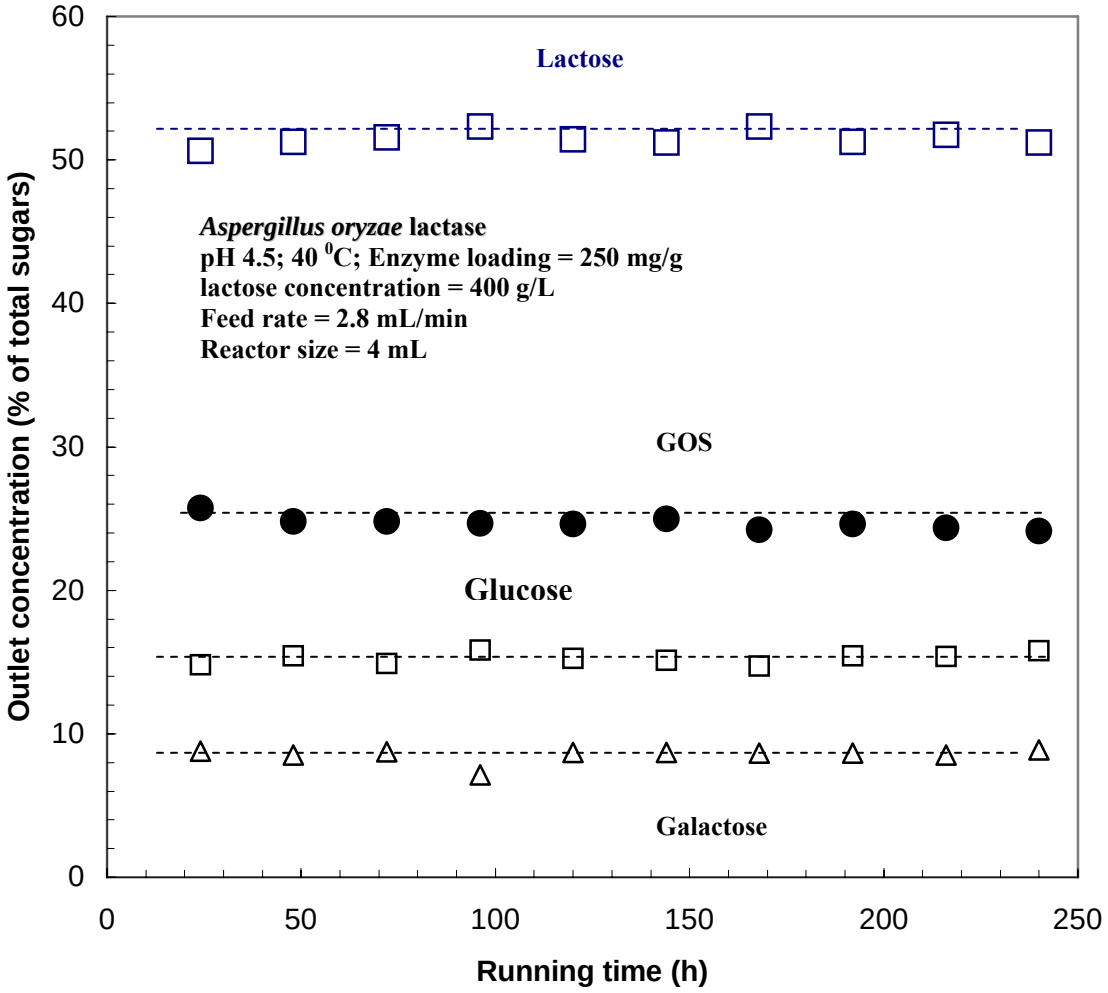


Figure 5.6: Continuous GOS production by immobilized ß-galactosidase from *Aspergillus oryzae* on cotton cloth in a single-path plug-flow reactor operated at 40 ^{0}C with a feed solution of 400 g/l of lactose.

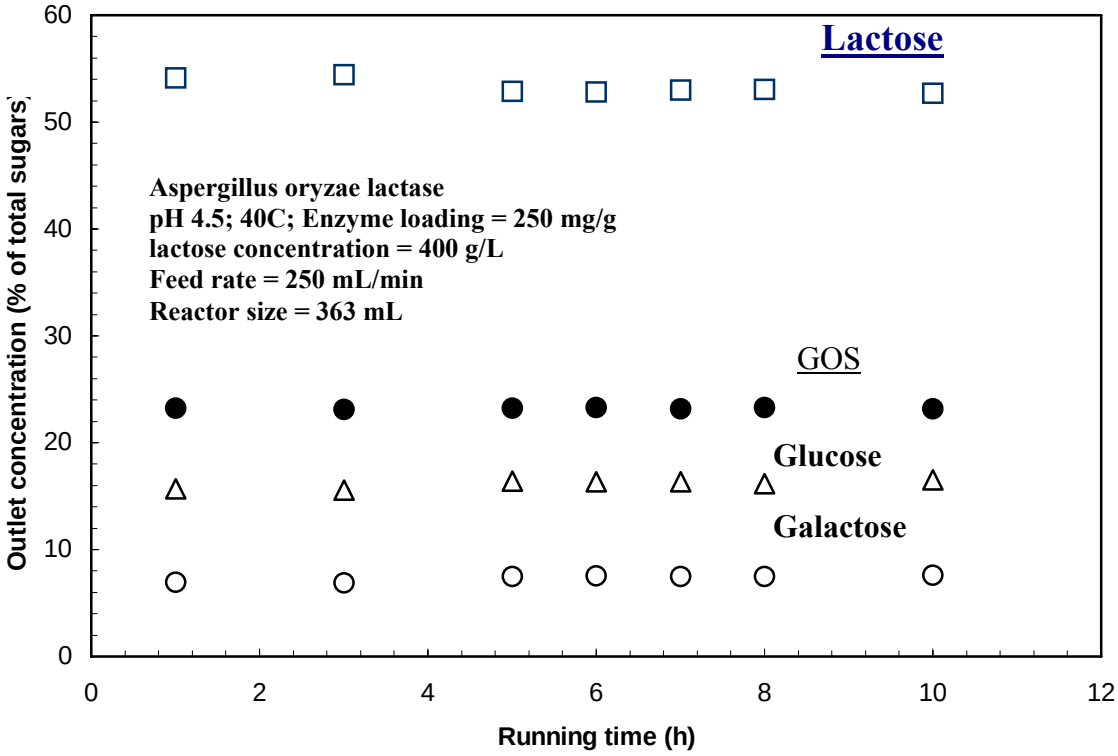


Figure 5.7: Continuous GOS production by immobilized ß-galactosidase from *Aspergillus oryzae* on cotton cloth in a single-path plug-flow reactor operated at 40 ^{0}C with a feed solution of 400 g/l of lactose.

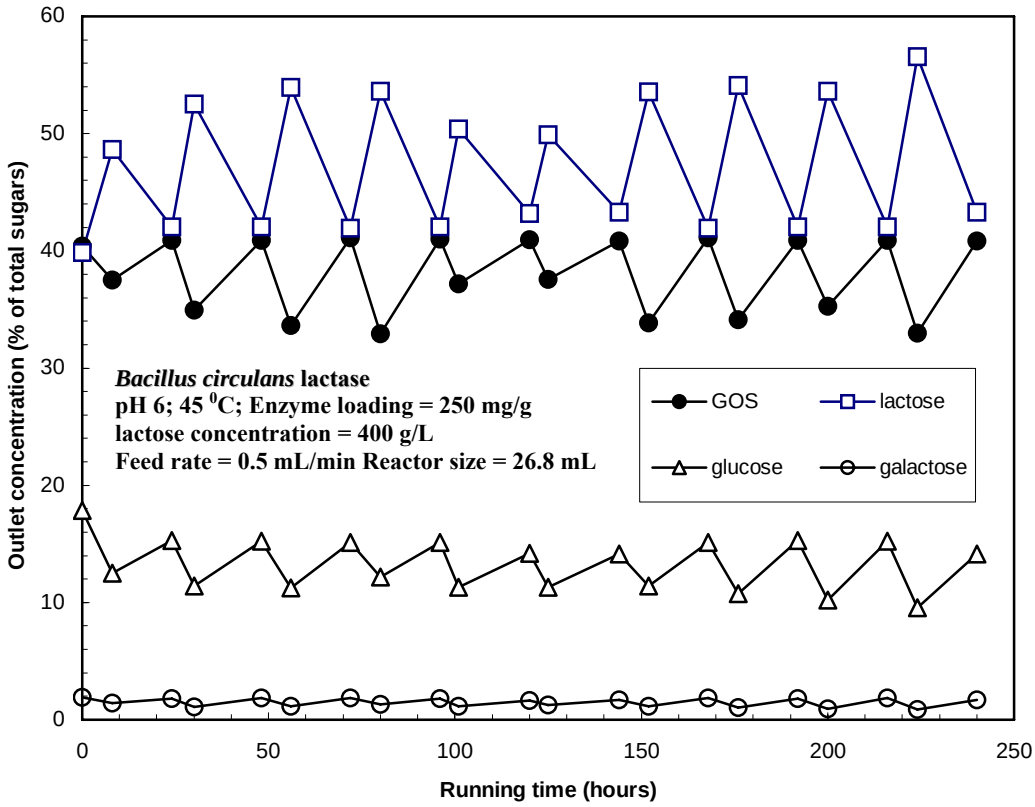


Figure 5.8: Continuous GOS production by immobilized ß-galactosidase from *Bacillus circulans* on cotton cloth in a single-path plug-flow reactor operated at 45 ^{0}C with a feed solution of 400 g/l of lactose

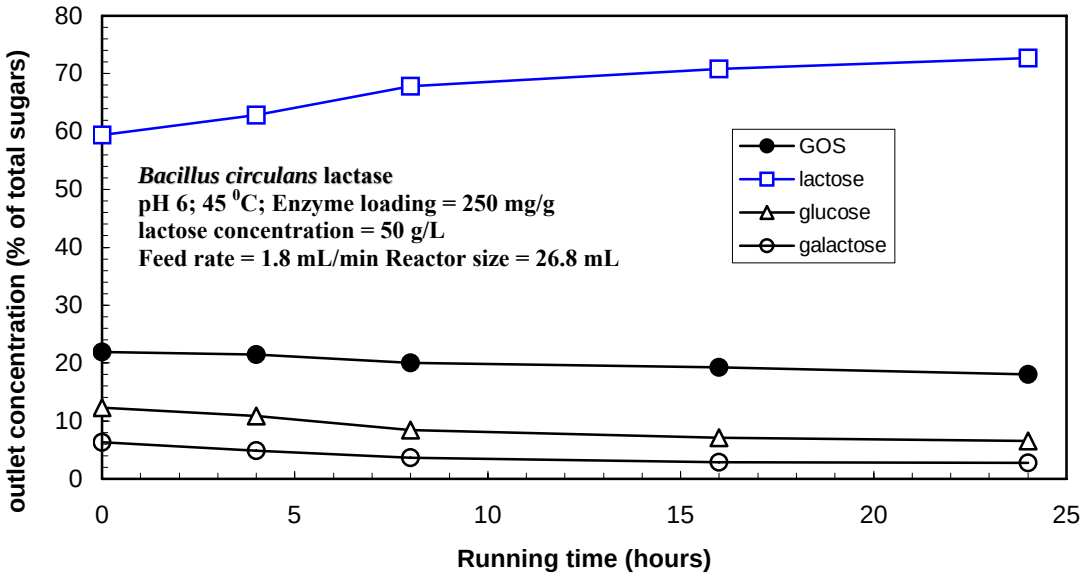
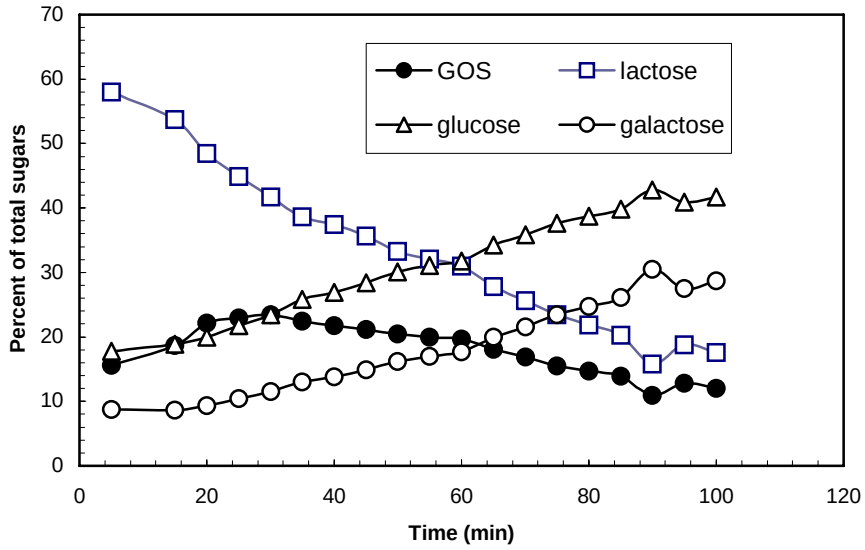


Figure 5.9: Continuous GOS production by immobilized ß-galactosidase from *Bacillus circulans* on cotton cloth in a single-path plug-flow reactor operated at 45 ^{0}C with a feed solution of 50 g/l of lactose.



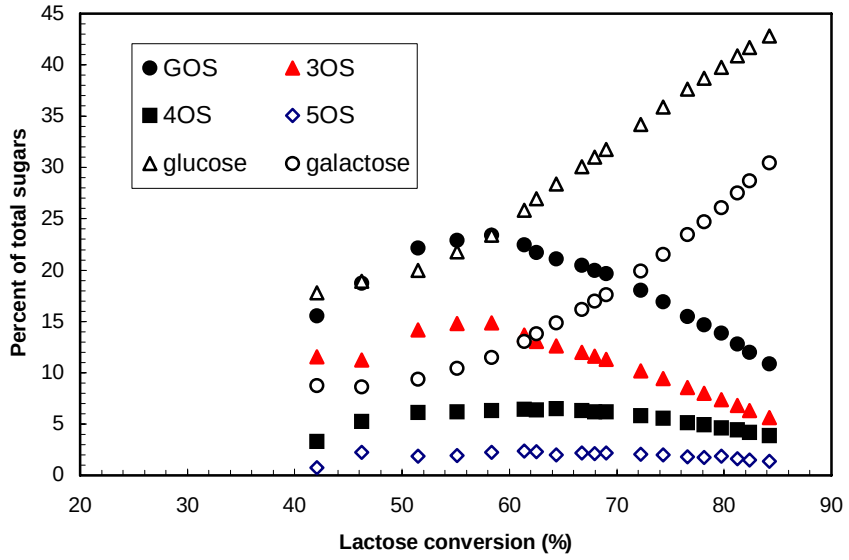


Figure 5.10: Reaction kinetics of lactose hydrolysis and GOS formation a) as function of time, b) as function of lactose conversion and c) GOS yield catalyzed by immobilized enzyme ß-Galactosidase from *Aspergillus oryzae* in a second recycle plug-flow reactor at 40 ^{0}C The feed was a GOS solution mixture of 500g/L containing: 14% GOS, 67% lactose, 13.5% glucose and 5.5% galactose (continued).

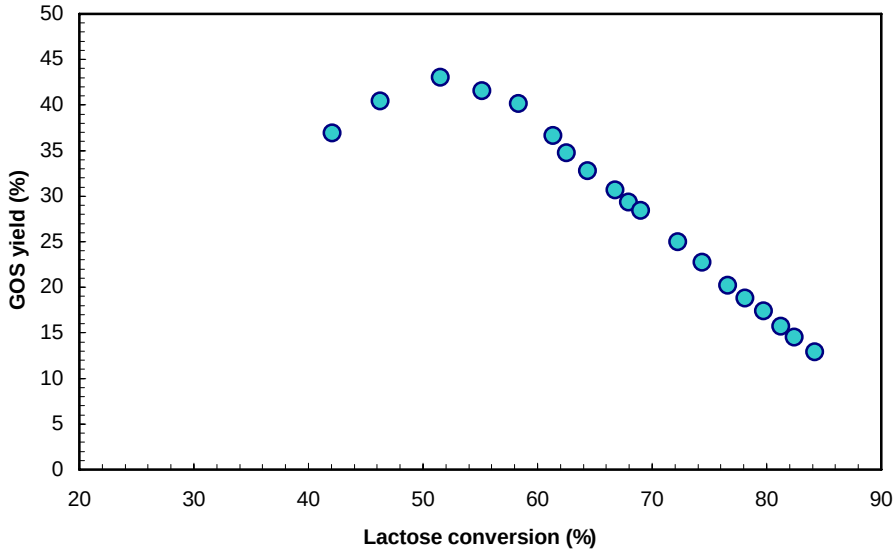
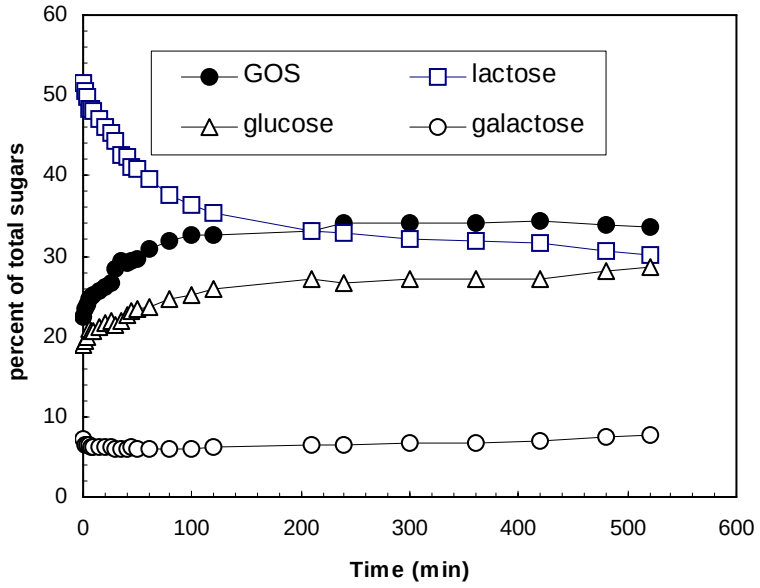


Figure 5.10: Continued



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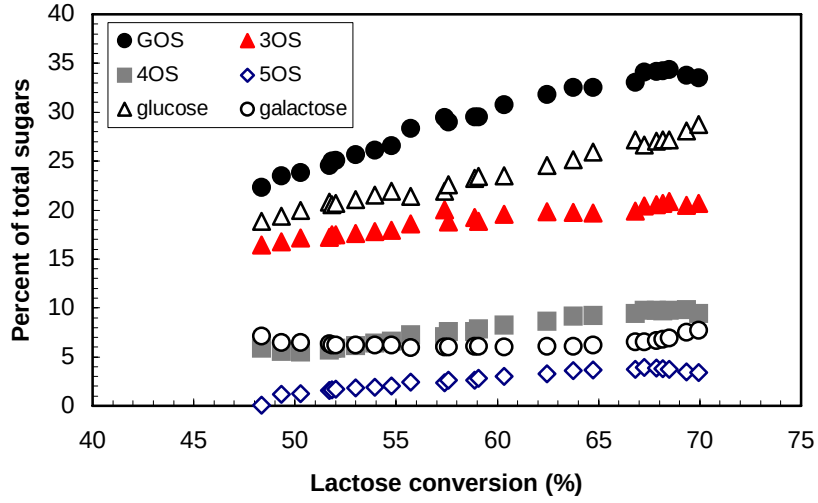

Figure 5.11: Reaction kinetics of lactose hydrolysis and GOS formation a) as function of time, b) as function of lactose conversion and c) GOS yield catalyzed by immobilized enzyme ß-galactosidase from *Bacillus circulans* in a second recycle plug-flow reactor at 45 ^{0}C. The feed was GOS solution mixture obtained from a first plug-flow reactor with the enzyme ß-Galactosidase from *Aspergillus oryzae.*

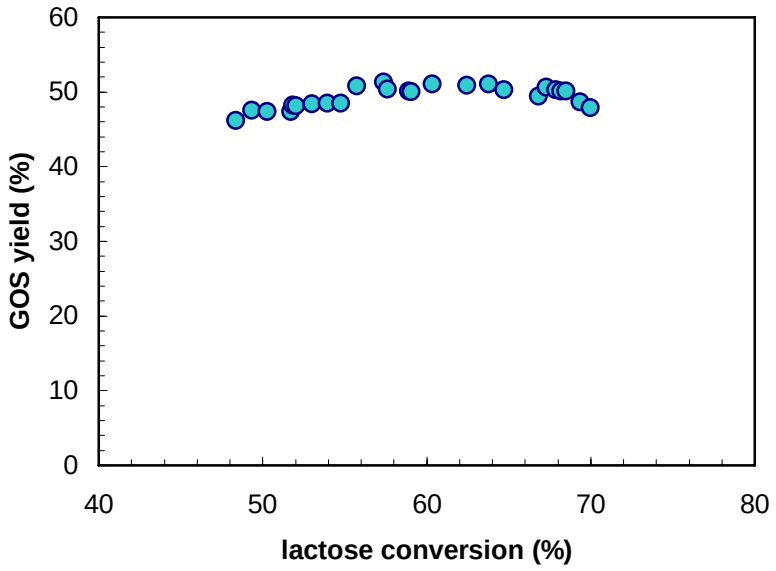

Figure 5.11: Continued.

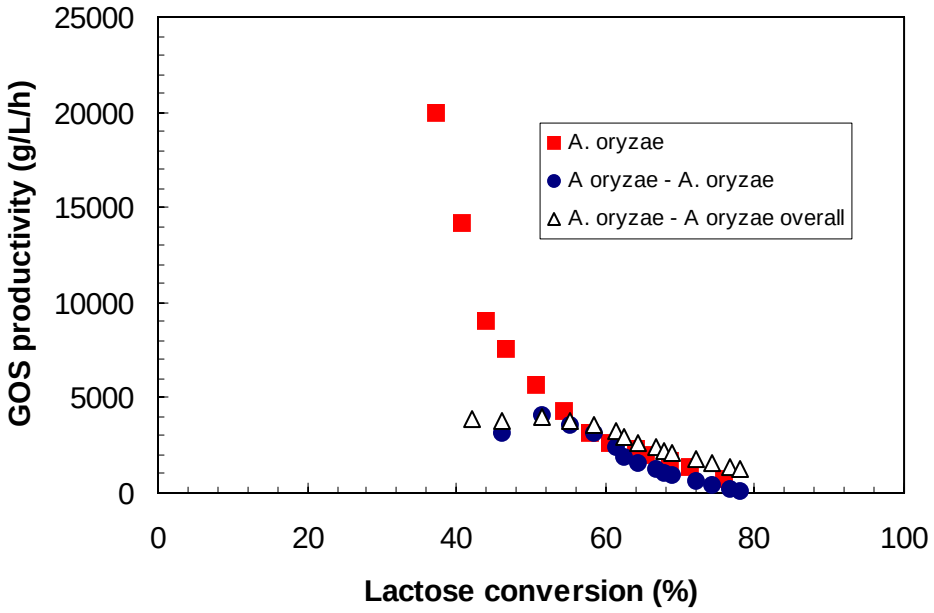

Figure 5.12: GOS productivities during lactose hydrolysis catalyzed by immobilized ß-galactosidase from *Apergillus oryzae* in a single and dual reactor.

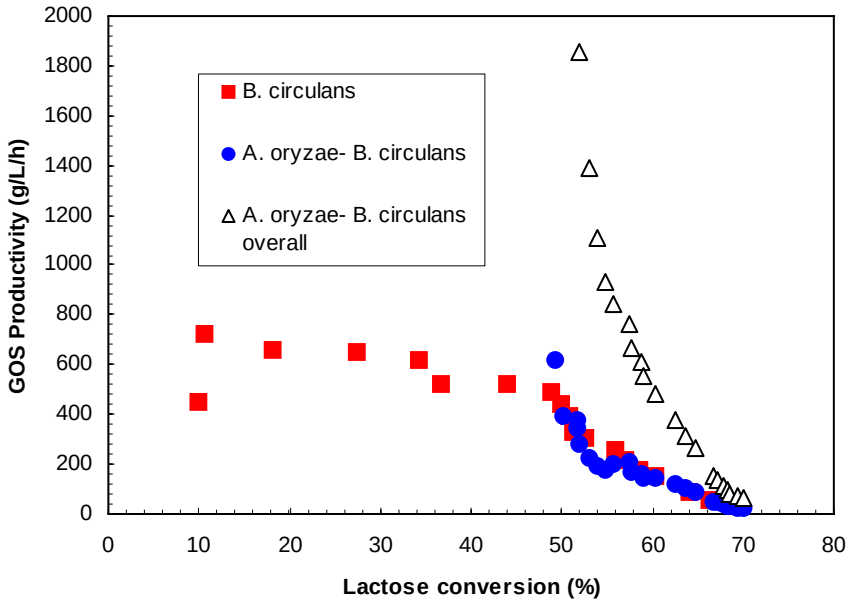

Figure 5.13: GOS productivities during lactose hydrolysis catalyzed by immobilized *Bacillus circulans* lactase in a single reactor and *Apergillus oryzae* and *Bacillus circulans* lactase in a dual reactor.

CHAPTER 6

SEPARATION OF GALACTO-OLIGOSACCHARIDES BY CHROMATOGRAPHIC TECHNIQUE

Abstract

A gel type cation exchange resin (Dowex 50W) was used for the sepration of GOS from a multicomponent sugar mixture. The degree of crosslinking of the resin had a larger effect on adsorption capacity and selectivity of sugars than cation type and temperature. The adsorption isotherms showed that increased crosslinking resulted in more selective sorption of sugars at the cost of sorption capacity. The resin with 4% crosslinking in Na^+ or K^+ ionic form was the best from the commercial resins available for GOS separation, presenting a balance between sugar adsorption and selectivity between sugars and achieving 95% separation of monosaccahrides from the GOS mixture. Furthermore, the order of sugar sorption under all different conditions studied was always the same as follows: galactose> glucose> lactose > GOS. Eddy diffusion and mass transfer resistance limited the most GOS separation.

6.1 Introduction

Oligosaccharides (OS), specifically galacto-oligosaccharides (GOS), are relatively new functional food ingredients that have great potential to improve the quality of many foods. In addition to providing useful modifications to food flavor and physicochemical characteristics, many of these sugars have properties that are beneficial to the health of consumers (Sako et al., 1999). GOS are large sugar molecules containing one unit of glucose and 2 to 5 units of galactose, joined together by glycosidic bonds (Oku, 1996). These sugars are produced from lactose in an enzymatic transgalactosylation reaction catalyzed by microbial β-galactosidases (Grosova, 2008). The first observed function of β-galactosidase was the hydrolysis of the disaccharide lactose into two monosaccharides: glucose and galactose. Later, it was discovered that the enzyme could also make glycosidic bonds between two saccharides in a reaction called transgalactosylation. This allows the enzyme to synthesize oligosaccharides, specifically GOS, from lactose (Zarate et al, 1990). However, because of the nature of the reaction, GOS are not produced purely. On the contrary, as reported in the previous chapter, the maximum amount of GOS produced using β-galactosidase from *Aspergillus oryzae* and *Bacillus circulans* were 25% and 40% GOS at 50%, and 60% lactose conversion, respectively, when a concentrated lactose solution (40% w/v) was used as the substrate. Also a certain amount of monosaccharides, products of the hydrolysis reaction, were present in the reaction mixture. Purification of GOS is important because by removing monosaccharides and lactose from GOS, there is a decrease in sweetness and calorie value. This relatively low sweetness is useful as bulking agent with reduced sweetness and can enhance other food flavours. Compared with mono- and disaccharides, the higher molecular weight of

galacto-oligosaccharides provides increased viscosity, leading to improved body and mouth feel (Crittedent et al., 1996). Also, there is an increase in enzymatic activity and/or yield resulting from the removal of byproducts (monosaccharides) in the reactor for GOS production (Boon et al., 2000).

The separation of carbohydrates plays an important role in food production and in cosmetic and pharmaceutical industries. Also, a high percentage of the cost in food production is related to separation processes (Ahlgren et al., 1997). The goal of this work was to separate GOS from other sugars, such as lactose, glucose and galactose, in the reaction mixture. Many of the existing separation methods such as crystallization (Hartel et al., 1993) and membrane separation (Iwasaki et al., 2000; Schweitzer, 1997) present different drawbacks. An alternative approach is to use a chromatographic technique for sugar purification. Liquid chromatography offers high selectivity, efficiency and loading capacity of the stationary phase and speed of process (Schulte et al., 2000). For analytical applications, many modes of chromatographic carbohydrate separations have been developed, including ligand-exchange (Stefansson et al., 1996), normal phase/hydrophilic interaction (Churms, 1996a), anion exchange (Lee, 1996), reversed phase (Rassi, 1996), and size exclusion chromatography (Churms, 1996b). In addition, carbohydrate chromatography is applied on an industrial scale in the starch industry to separate glucose/fructose, to remove glucose from malto-OS mixtures, to produce high-purity maltose from starch hydrolysate (Okada et al., 1992), and to produce high-purity palatinose-OS (isomaltulose-OS) (Crittenden and Playne 1996). However, for non-analytical separation of GOS, no information could be found for GOS separation by the chromatographic technique.

The goal in this work was to study a series of chromatographic variables to optimize the GOS separation. These parameters were studied in a fixed column to obtain a better understanding of the chromatographic relation between the sugars in solution and the stationary phase that could be applied later to model a simulated moving bed (SMB) system. A gel-type poly(styrene-co-divinylbenzene) (PS-DVB) strong acid cation exchange resin (Dowex 50W) was chosen as the solid phase since it has been used successfully in different carbohydrate separations (Lee, 2003; Angyal et al, 1999). The different factors in consideration are: a) degree of crosslinking of the stationary phase, defined as the percentage of the DVB (divinylbenzene) used during the synthesis of the resin (Figure 6.1), b) type of the counter-ion, defined as the ions attached to the resin with the ability to exchange with others of like charge or to form complexes of varying strength, c) temperature, and d) flow rate. Combining these parameters, the adsorption capacities and selectivities between sugars were calculated by obtaining the adsorption isotherms for samples of 25% GOS solution produced in an enzymatic reaction with *A. oryzae* lactase (see Chapter 3). Moreover, column efficiency was calculated for the separation of the different sugars.

6.2 Materials and Methods

6.2.1 Stationary phase and reagents

Gel type poly(styrene-co-divinylbenzene) (PS-DVB) strong acid cation exchange resins (Dowex 50W, Sigma-Aldrich) with a mesh size of 100-200 (particular diameter 75-140 μm), in H^+ form with an initial total exchange capacity of 1.1 meq/ml in 3 different degrees of crosslinking: 2%, 4% and 8%. Solutions of NaOH (1N), sodium

chloride (NaCl, 0.2 N), potassium chloride (KCl, 0.2 N) and calcium chloride ($CaCl_2$, 0.2N) were used to exchange cation forms. Dextran blue 2000 (with a weight average molecular weight of 2,000,000) from Sigma was used to calculate the internal porosity of the resin.

6.2.2 Effect of crosslinking of the stationary phase

The adsorption isotherms of GOS, lactose, glucose and galactose were studied using 25% GOS solution obtained from immobilized enzyme reactors with ß-galactosidase from *A. oryzae*. The adsorption isotherms were obtained by contacting the sugar solution, with a range of concentrations from 25 g/L to 400 g/L of total sugars, with the resins in a shaker incubator at room temperature. Resins with 3 different degrees of crosslinking (2%, 4% and 8%) were used in this study. From the adsorption isotherms, the adsorption coefficient for different sugars was obtained and the selectivity between different sugars was calculated.

6.2.3 Effect of counter-ion

Different ionic forms of the stationary phase (Dowex 50W) were used to obtain the adsorption isotherms. Originally, the resin was purchased in H^+ form. Before use, the resin was washed with deionized water. Resin was converted to different ionic form by contacting the resin for 30 minutes with 5 bed volumes of 0.2 N solution of the chloride salt of the particular cation being used (Na^+, K^+ or Ca^{2+}).

6.2.4 Effect of temperature

Two different temperatures (25 and 60 ^{o}C) were used to obtain the adsorption isotherms of GOS. The stationary phase used was a resin with 4% crosslinking in the Na form. Also, the adsorption isotherm of a solution of pure glucose in a wide range of concentrations (25-400 g/l) was obtained and compared with the isotherm of glucose from a multi-component mixture of 25% GOS to predict possible linearity of the sugars by using this specific resin (Dowex 50W).

6.2.5 Analytical Methods

6.2.5.1 Determination of isotherms

Samples of 25% GOS of total sugars for lactase from *A. oryzae* containing besides GOS: glucose, galactose and lactose were chosen for isotherm measurement with an initial total concentration of 400g/l. Also, isotherms for pure samples of glucose were obtained. Isotherms of sugar in liquid phase and resin were determined by contacting overnight water-washed, swollen resin samples (of about 5 g) with different sugar solutions (volumes of 5 and 10 mL) of initial known concentrations (from 25g/l to 400 g/l) in a shaker incubator at 2 different temperatures: 25 and 60 ^{0}C. Subsequently, the resin was separated from the sugar solution by vacuum filtration with a paper filter (Whatman, 5971). Then, the dry substance content of the resin was determined by drying overnight in an oven at 105 ^{0}C. The sugar concentrations in the liquid phase (C) before and after adsorption (Q) were determined with HPLC analysis. The sugar concentration in the solid phase was then calculated using the following equation:

$$Q = (C_{in} - C_{eq}) * V_{liq} / V_s \qquad (6.1)$$

where Q is the amount of sugar per unit volume of resin in equilibrium with a solution of concentration C_{eq} , C_{in} is the initial concentration in liquid phase, C_{eq} is the sugar concentration in the liquid phase after adsorption, V_{liq} is the volume of sugar solution and V_s is the volume of the resin calculated from the apparent density of the resin.

6.2.5.2 . Calculation of bed porosity

The bed porosity was determined with high molecular weight (2000 kg/mol) dextran T2000, also named dextran blue. It was assumed that the dextran molecules couldn't penetrate the resin interior due to size exclusion (Lameloise et al., 1994). The bed porosity was calculated from the retention time t_{ret} obtained from an injection volume of 0.5 mL of dextran blue with an initial concentration of 1 g/L. The flow rate (FR) was 0.9 mL/min and the bed volume (V) was 60 ml. The following equation was used to obtain the bed porosity:

$$\varepsilon = FR^* t_{ret}/ V \qquad (6.2)$$

6.2.5.3 Determination of apparent density of the resin

The apparent density was calculated for the 3 different degrees of cross-linking: 2%, 4% and 8% of the resin. A known amount of resins was put in a graduate cylinder with distilled water. The apparent density was calculated by dividing the weight of the resin (90 g) by the volume that occupied in the graduate cylinder. It is important to mention that the absorbent volume was measured after the resin was converted to the desired ionic form since the resin shrink after contacting a salt solution. The apparent density was used to calculate the amount of resin needed to pack the column.

6.2.5.4 Determination of selectivity

The selectivity of component (i) relative to component j was calculated as follows:

$$(Q_i/C_i)/(Q_j/C_j) \quad (6.3)$$

where Q and C are the sugar concentration of components i and j in liquid and solid phase, respectively.

6.2.5.5 HPLC analysis

The concentrations of sugars in sample solutions (glucose, galactose, lactose, and galactooligosaccharides) were determined by HPLC. An HPLC system consisting of a pump (LC-10AI Shimadzu), an automatic sampler injector (Shimadzu SIL10Ai), a carbohydrate analysis column (Phenomenex, Rezek RNM carbohydrate column, 7.8 mm-150 mm), a column oven (Shimadzu CTO-10A), a refractive index detector (Shimadzu RID-10Avp), and a Shimadzu CLASS-VP chromatography data system (version 7.2 integrator) was used. The eluent was pre-degassed distilled water (at 80 °C) at a flow rate of 0.4 mL/min. Distilled water was degassed by first boiling and then sonicating for 30 min. The column temperature was maintained at 80 °C, and the detector temperature was set at 45 °C. The concentrations (w/v) of these sugars (e.g., lactose, glucose, galactose, and oligosaccharides including tri-, tetra-, and pentasaccharides) presented as weight percentages of total sugars, were determined from peak heights and are reported in this paper.

6.2.5.6 Determination of column efficiency

The column (XK16, maximum length 30.0 cm, internal diameter 16 mm, GE healthcare) was used to evaluate the column efficiency. The column was filled with resin using the slurry packing method. The column effluent was monitored by light absorption at 190 nm by using liquid chromatogram system Akta explorer 100 and the software Unicon 5.3 (Figure 6.2). The chromatographic efficiency of the resin Dowex50W (4% DVB content in K^+ and Na^+ as the cationic forms) to separated GOS from lactose glucose and galactose was evaluated by injecting a pulse of 0.5 ml of 25% and 40% GOS solutions with a total sugar concentration of 400g/l through a bed column of 60 mL at 5 different elution flow rates: 1.8, 1.2, 0.9, 0.6 and 0.3ml/min. The efficiency of the column was calculated by using the plate theory that relates the band broadening to a solute migration. This theory uses the concept of "theoretical plates", in which the column is considered to consist of a series of thin sections or "plates", each of which permits a solute to equilibrate between the mobile and the stationary phases. (Ladisch et al., 2001). The theoretical number of plates can be calculated under the following equation:

$$HETP = L / N \qquad (6.4)$$

where L is the length of the column, N is the number of plates and HETP represents the Height Equivalent to a Theoretical Plate. The number of "theoretical plates" that a column possesses for a solute can be calculated from the chromatogram by using the following equation (Grushka et al, 1975):

$$N = 16 (t_R / Wi)^2 \qquad (6.5)$$

where t_R is the retention time of the peak and Wi is the width of the peak from its base. From the number of plates the height of the plate (H) was calculated and related to the rate theory through the Van Deemeter equation to determine the optimum mobile phase flow rate (Figure 6.3) The Van Deemter equation gives H as a function of the mobile phase velocity (u):

$$H = A + B/u + C*u \quad (6.6)$$

where A, B and C represent the Eddy diffusion, longitudinal diffusion and resistance to mass transfer factors respectively which contribute to band broadening.

Finally, the resolution between two sugars A and B can be calculated from the retention times and peak widths in the chromatograms according to the following equation:

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B} \quad (6.7).$$

6.3 Results and Discussion

6.3.1 Effect of temperature

Temperature has critical effect on sugar adsorption on cation exchange resins; however, only few studies are available (Goto et al., 1993) and they were mainly reported in industrial patents (McCulloch, 1994), involving high temperatures (60 C or higher). Theoretically, using high temperatures in chromatographic separations results in less broadening of the elution curves due to an increased diffusion rate inside the resin particles. However, raising temperature indefinitely is not desirable because sugars may

changing their configuration and lose their chemical identity at high temperatures. Moreover, it could break complex formation between the resin and the sugars (Saska, 1992).

In this study 2 different temperatures were studied: 25 and 60 ^{0}C. These temperatures were not chosen randomly; 25 ^{0}C is the room temperature at which no energy is necessary to heat the system, and 60 ^{0}C is the temperature that is used in many industrial processes. Figure 6.4 shows the isotherms of GOS, lactose, glucose and galactose from a 25% GOS solution for a resin with 4% crosslinking in Na^+ form at 25 and 60 ^{0}C. As can be seen, the isotherms for the different sugars at both temperatures were linear or only slightly curve at high concentrations. For this reason, the isotherms could be fitted with an equation that is used to describe a concentration dependent distribution coefficient (Muralidharan et al. 1997):

$$Q = aC^2 + bC$$

where 'a' is an equilibrium parameter correcting for the concentration dependence of the distribution coefficient and 'b' is the slope of the isotherm at infinite dilution. Also, different isotherm equations such as Langmuir could fit the relation between the sugar and the resin at equilibrium. However, it has been reported that any chromatographic experiment can be carried out under linear conditions as long as $aC<<b$. Consequently, since this is the case, the isotherms were fitted with a linear isotherm curve where 'a' was neglected (Giochon et a., 2006). Nevertheless, it is possible that concentration dependence between different sugars could be found if higher initial sugar concentration were used. The fact of obtaining similar sugar adsorption at different temperatures is not

in agreement with Addachi et al., 1999, who reported that the sorption of glucose for fructose/glucose separation increases with increasing temperature. A possible reason for this difference is that he used a different type of support with smaller particle size.

Based on the adsorption isotherms from the different sugars, galactose and glucose were the compounds most adsorbed, followed by lactose, and GOS being the least adsorbed. Since there were no significant differences between the adsorption coefficients of the isotherms of each sugar at different temperatures, the selectivities (Table 6.1) for glucose/GOS, lactose/GOS and glucose/lactose and galactose/GOS were also similar. The selectivity of component i relative to component j was calculated as: $(q_i/c_i)/(q_j/c_j)$. The higher selectivity for glucose/GOS can be attributed to the higher difference in size between glucose and GOS. Moreover, in the case of a multicomponent mixture, such as 25% GOS samples containing GOS, lactose, glucose and galactose, competition between various sugars to access the sorption sites could change the adsorption coefficient of these sugars and consequently the adsorption isotherms. The isotherm of pure glucose in a range of concentration from 25g/l to 350g/l at 25 ^{0}C was obtained (Figure 6.5) and compared with the glucose from the GOS solution at the same temperature. As can be observed, the adsorption coefficient of the pure glucose and the coefficient obtained from the glucose present in the GOS mixture were similar. So, it can be concluded that there is no significant competition in this case between different components. Furthermore, based on the linear isotherm, it can be stated that the separation of GOS will be independent of the sugar concentration and the amount present in the mixture at the concentration range studied. However, as can be seen from the shape of the isotherm of pure glucose at high concentration, nonlinear isotherm would be

obtained since the isotherm was not totally lineal at the highest concentration used in this study. Since no significant difference for sugar separation was found between temperatures, 25 ^{0}C was used for the rest of the experiments.

6.3.2 Effect of cross-linking of the stationary phase

The degree of crosslinking of the PS-DVB resin can be varied by the amount of DVB (divinylbenzene) used during the synthesis of the resin. Crosslinking decreases the elasticity of the resin and thereby the swelling and the equilibrium water content (Tiihonen et al., 2001). Saccharides can be sorbed into the resin, either by distribution of the saccharides between the liquid inside the resin and the liquid outside the resin or by complexation (Adachi et al., 1999). To be able to distribute, the saccharide molecules have to be smaller than the size of the interstices of the resin. The size of the hydrated monosaccharides is in the same order of magnitude as the size of the pore diameter of a resin. The separation of oligosaccharides (GOS) may be improved by choosing the degree of crosslinking such that disaccharides and monosaccharides are able to be sorbed by the resin while GOS are excluded. Higher water content increases the sorption of the saccharides by distribution. The amount of cations per volume unit water increases with increasing the DVB content. Therefore, complexation driven sorption might improve relative to the sorption by distribution with increasing the DVB content. The DVB content not only influences the equilibrium sorption properties of the resin, but also the sorption kinetics and mechanical properties such as elasticity and attrition resistance. In this work, 3 different crosslinkings of the PS-DVB cation exchange resin were used to study their influence on the sorption and separation of sugars. Figures 6.6-6.8 show the

isotherms of GOS, lactose and glucose and galactose in a 25% GOS solution for Na+ loaded resins with different degrees of crosslinking: 2%, 4% and 8%. Sugar adsorption was greatly affected by the degree of crosslinking of the resin. The sorption of sugars was lower at higher crosslinked resin. This result can be explained by the decreased elasticity and swelling of the resin with increased crosslinking. This results in lower water content of the resin and decreases sorption of saccharides by distribution. On the contrary, at low crosslinking, the resin contains more water and the amount of cations is lower. Consequently, more adsorption can occur and the sugars have more opportunities to reach the inside pore of the resin. All figures show that at the same degree of crosslinking, the order of sorption of sugars was always the same and as follows: galactose>glucose>lactose>GOS. Both GOS and lactose were sorbed less than monosaccharides due to their larger size and hence the restricted accessibility to the resin interstices. Table 6.2 lists the selectivities of different sugars at different crosslinkings. In general, the selctivitiy increased with increasing the crosslinking. This result is in agreement with previous studies for the separation of malto-oligosaccahrides, which also showed that the selectivity improved by using a resin with a higher crosslinking between 2% and 6% (Adachi et al., 1989). The gain of selectivity can be attributed to the fact that higher crosslinking reduced available water in the resin and thus decreased the sorption by distribution but increased the amount of cations per unit volume, which increased complex formation for monosaccharides and less for lactose and GOS. Therefore, the selectivity of glucose and glactose/GOS increased the most from crosslinking 2% to 8%, followed by the selectivity of glucose/lactose. The lower selectivity for glucose/lactose is due to the fact that lactose is smaller than GOS and more lactose can be adsorbed by

complexion. The resin with 4% crosslinking was the best for GOS separation because it presented a balance between sugar adsorption capacity and selectivity. However, the optimal crosslinking could be between 4% and 8%.

6.3.3 Effect of counter ion

It is well known that the counter ion attached to a PS (poly-styrene) based cation exchanger affects the chromatographic behavior in sugar separation. The separation of sugar mixtures can be significantly improved by the correct choice of the counter ion. When sugars in the mobile phase come in contact with the counter ions in the stationary phase, they form coordination complexes of varying stability. The stability of the complex formed with the counter ion increases in relation to the availability for coordination (Goulding, 1975). It has been shown through nuclear magnetic resonance and electrophoresis studies that certain orientation of hydroxyl groups (the groups that form complex with the counter ions of the resin) in the sugars imparts stability to the complexes. Therefore, in a column chromatograph, the retention of sugars should increase with the number of pairs of favorably oriented hydroxyl groups present. Although the materials used for the separation are ion exchangers, ions are on purpose not exchanged in the process. In fact, water molecules from the hydrated cations are exchanged for (hydrated) sugar molecules to form different coordination complex (Stefansson et al., 1996). In this work, 3 different counter ions (Na^+, K^+ and Ca^{++}) were chosen to investigate their influence on the adsorption and selectivity of sugars. The choice of the counter ions was based on its use in previous separation process (Antosova et al., 1999; Yang et al., 1995). It has been reported that cations such as Ag^+ and Pb^{2+}

gave better results for sugar separation. However, these heavy metals cations cannot be used in food because of their toxicity (Pirisino, 1984). Figures 6.7, 6.9 and 6.10 present the isotherm data of galactose, glucose, lactose and GOS for the resin with 4% crosslinking in the Na^+, K^+, and Ca^{2+} forms from a solution of 25% GOS at 25 ^{0}C. As can be seen, linearity was not affected by the choice of counter ion. These figures show that the sugar adsorption capacity of the resin in K^+ and Na^+ forms were higher than that of the resin in Ca^{++} form. Differences in capacity between different cationic forms of the resin can be explained as follows. As it was mentioned above, for complex formation between hydrated sugar molecules and hydrated cations, replacement of (some of) the water molecules is required. It is known that the interaction between sugar and cation is controlled by the modification of the hydration of the molecules (Morel-Desrosiers et al., 1999). We expect that the competition between water molecules and sugar molecules for the sorption sites is weaker in the case of weak water sorption. K^+ binds water molecules weaker than Na^+, and Na^+ binds water weaker than Ca^{2+}. Weakly bonded water is available for hydrogen bond formation with sugar molecules, which results in the order $K^+ > Na^+ > Ca^{2+}$ for sorption of sugars in the resin (Walton, 1985). This order is partially in agreement with the experimental results, since in our case it was not found any significant difference on sugar sorption between resins in Na^+ and K^+ forms. The observed order of sugar sorption for all ions was as follows: galactose> glucose> lactose > GOS. As expected, the monosaccharides (galactose, glucose) show higher isotherms than the other sugars (lactose and GOS), which may be explained by the larger size of the disaccharides and GOS and, hence, increased exclusion. The differences between the isotherms of the monosaccharides may be due to the steric orientation of the OH groups

of the sugar molecules. Glucose has only one equatorial-axial (eq-ax) oriented OH group on α-D-glucopyranose and none on ß-D-glucopyranose. Galactose has two eq-ax oriented OH groups in the α-pyranose form and one in the ß-pyranose form. Not surprisingly, galactose shows higher adsorption than glucose. Similar results were obtained when resins with 2% and 8% crosslinking were used. Table 6.3 shows how the selectivities between different sugars were independent of the cation used. In general, the effect of selectivity in resin loaded with different cations was minimum. However, since K^+ and Na^+ were the cations that offered higher sugar adsorption capacity, they were chosen as the optimum cation for separation.

6.3.4 Efficiency of the separation: column efficiency

The efficiency of the column to separate sugars can be calculated by knowing the number of plates. As shown in Table 6.4, the number of plates obtained for GOS, lactose and glucose from a solution of 25% GOS increased with decreasing the flow rate (from 1.8 to 0.3 mL/min). Also, comparing the different sugars at the same flow rate, glucose presented the highest number of plates, followed by lactose and GOS. This result is in agreement with the previous experiments since glucose was the most retained component and GOS the least. The higher the number of plates the more efficient is the column to separate any particular sugar. The same pattern in results were obtained in Table 6.5 for a solution of 40% GOS. The chromatograms from Figures 6.11-6.13 were used for the calculation of the different numbers of plates. Based on these results, it can be concluded that in this range of flow rates, the lowest flow rate (0.3ml/min) gave the best separation for all sugars. Van Deemter Plot, which equation is $H = A + B/u + C*u$, is used to obtain

the optimum mobile velocity to separate any compound, or at least, the factors affecting the band broadening. These factors will be the limiting variables of the separation process. Figure 6.14 shows the Van Deemter plot (plate hight vs mobile velocity) for the separation of different sugars from a solution of 25% GOS. The usual curve shape of the Van Deemter plot is hyperbolic. However, in this case, a straight line was obtained. The type of line obtained was y = a + c*x or H = a + c* u. Based on these results, it can be concluded that the main factors affecting GOS separation are, (a) eddy diffusion, which accounts for the time that 2 different particles from the same compound takes to traverse a packed column, and (c) mass transfer resistance, which accounts for the time that a mobile phase needs to equilibrate between the stationary phase and the mobile phase. The Eddy diffusion is independent of the mobile velocity. However, resistance to mass transfer depends on it. Consequently, as shown the graph the effect of mass transfer affected the most the separation of all sugars. Nevertheless, it had more influence in the separation of GOS than the other sugars. Similar results were obtained from a solution of 40% GOS (Figure 6.15). These results are in agreement with earlier published data by Saska, 1992 for the separation of sucrose from sugarcane molasses. Hyperbolic curve was not obtained because during this study flow velocities where relatively high, neglecting in this case the longitudinal diffusion (term b from the Van Deemter equation). However, using a lower flow rate to obtain the optimum velocity at which it can be predicted the best separation was not the aim of this research. The final goal of this study is to optimize our system to apply it to a SMB system. In this type of systems high flow rates are used for separation. For this reason, finding the parameters that were affecting our system was more important. Furthermore, to quantify the separation of the different sugars the

resolution between GOS/glucose, GOS/lactose and lactose/glucose was calculated using equation 6.7. Figures 6.11-6.13 show the chromatograms for the separation of GOS, lactose, glucose and galactose by using resin in Na^+ and K^+ form at different flow rates, from a solution of 25% and 40% GOS. Figure 6.16 shows that the separation of GOS from monosaccharides was 98% at the lowest flow rate (0.3 mL/min); proving that the removal of monosaccharides is possible by using this type of resin. However the separation of GOS from lactose was only 44%. Higher resolution was obtained between GOS/glucose followed by lactose/glucose and GOS/lactose. Furthermore, the resolution between all sugars increased by decreasing the flow rate. Figure 6.17 shows similar results when a solution of 40% GOS was used. Finally, Figure 6.18 compares the separation of GOS/lactose at different flow rates between a resin in Na^+ and K^+ form. As it can be seen, both resins provided similar separation at all flow rates studied, with it is in agreement with the results obtained previously for the isotherm calculation.

6.4 Conclusions

Dowex 50W, a strong acid cation exchange resin was a suitable adsorbent to separate GOS from a sugar mixture. It was found that the degree of crosslinking of the resin was the factor that most influenced the adsorption of sugars. Increasing the crosslinking resulted in less sorption of sugars. However, the selectivity between sugars also increased at higher crosslinking. The resin with 4% crosslinking was the best from the commercial resins available for GOS separation since presented a balance between sugar adsorption and selectivity between sugars. However, a resin with 6% crosslinking could present a better separation of GOS. Other factors such temperature or counter ion

showed minimal effect on both sorption capacity and selectivity of the different sugars. However, K^+ and Na^+ ionic forms were chosen as the optimum since they provided higher adsorption capacity than resin in Ca^{++} form. Furthermore, the order of sugar sorption under all different conditions was always the same and as follows: galactose> glucose> lactose > GOS. The lower adsorption of GOS and lactose were explained by the size exclusion mechanism. The order of monosaccharides was in line with the number of equatorial-axial oriented sugar OH groups for complex formation with the cation. The isotherms obtained under the different conditions were linear over a wide range of sugar. Finally, the factors that most limited the GOS separation were Eddy diffusion and resistance to mass transfer.

6.5 References

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Temperature	25 ^{0}C	60 ^{0}C
glucose/lactose	1.25	1.17
lactose/GOS	1.19	1.16
glucose/GOS	1.48	1.32
galactose/GOS	1.51	1.48

Table 6.1: Selectivity of different sugars in a sample of 25% GOS by using a resin Dowex50 with 4% crosslinking in Na^+ form at 25 ^{0}C and 60 ^{0}C.

resin type	X2	X4	X8
glucose/lactose	1.1	1.25	1.38
lactose/GOS	1.05	1.19	1.23
glucose/GOS	1.16	1.48	1.71
galactose/GOS	1.19	1.61	1.95

Table 6.2: Selectivity of different sugars in a sample of 25% GOS by using a resin Dowex50 with 2%, 4% and 6% crosslinking in Na^+ form at 25 ^{0}C.

Cation form	Na^+	K^+	Ca^{++}
glucose/lactose	1.25	1.21	1.18
lactose/GOS	1.19	1.16	1.14
glucose/GOS	1.48	1.48	1.35

Table 6.3: Selectivity of different sugars in a sample of 25% GOS by using a resin Dowex50 with 4% crosslinking in Na^+, K^+ and Ca^{++} form at 25 ^{0}C.

Flow rate Q (mil/min)	GOS	lactose	glucose
1.8	73	94	177
1.2	115	126	221
0.9	125	206	261
0.6	149	227	272
0.3	169	380	492

Table 6.4: Number of plates (N) for GOS, lactose and glucose from a sample of 25%GOS by using a resin with 4% crosslinking in Na^{+} at different flow rates.

Flow rate Q (mil/min)	GOS	lactose	glucose
1.8	76	113	165
1.2	91	148	211
0.9	108	183	256
0.6	115	202	287
0.3	159	384	336

Table 6.5: Number of plates (N) for GOS, lactose and glucose from a sample of 40%GOS by using a resin with 4% crosslinking in Na^{+} at different flow rates.

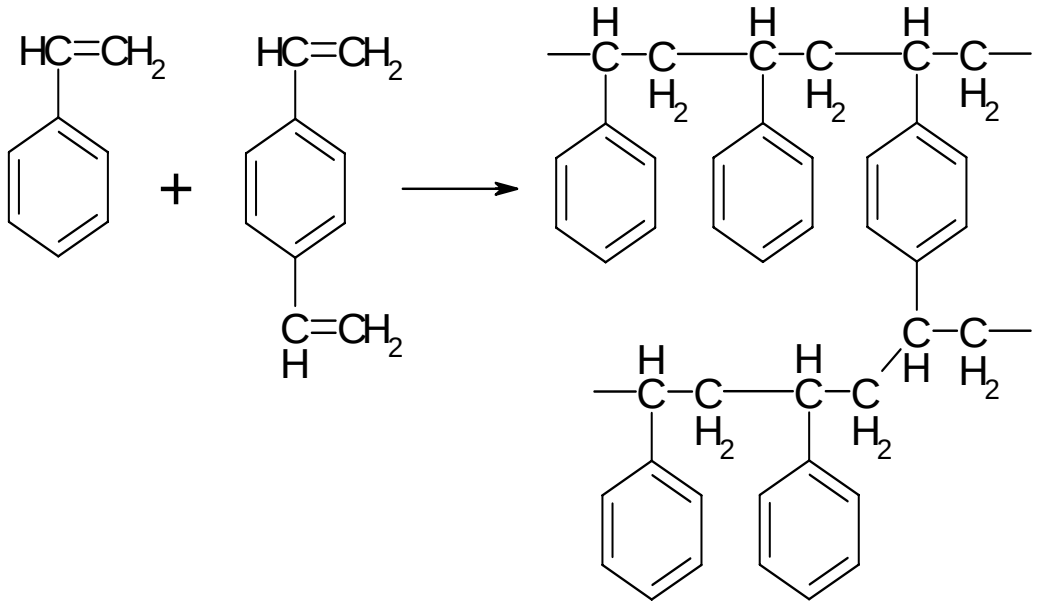


Figure 6.1: Reaction of styrene with divinylbenzene to produce styrene-divinylbenzene

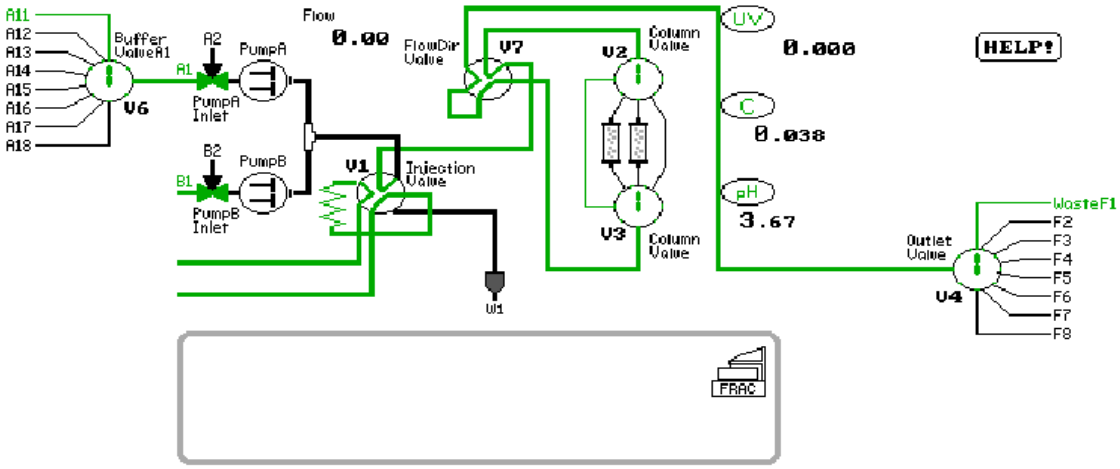


Figure 6.2: Liquid chromatographic system AKTA explorer 100

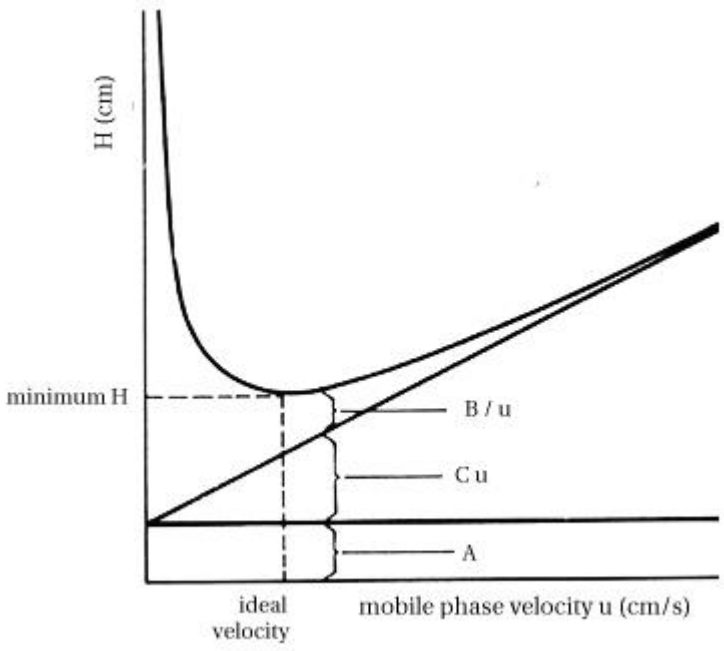


Figure 6.3: Typical Van Deemter Plot

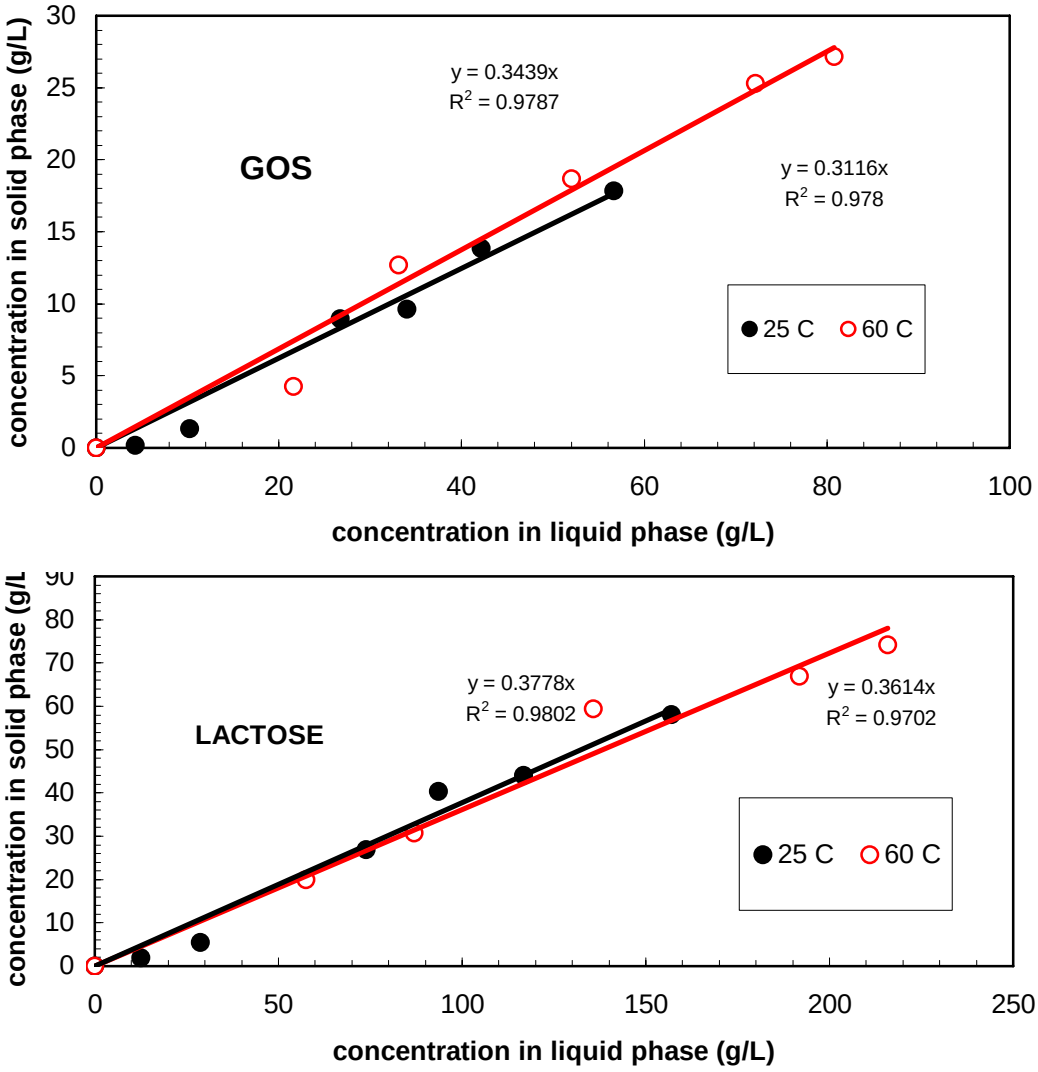
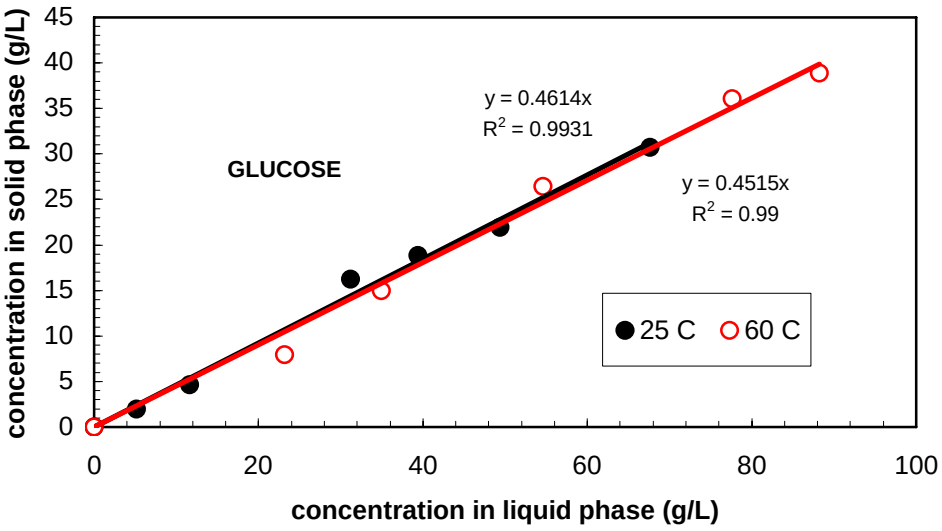


Figure 6.4: Isotherms from a sample of 25% GOS using 4% cross-linking PS-DVB resin in Na^+ form at 25 ^{0}C and 60 ^{0}C for: a) GOS, b) lactose, c) glucose and d) galactose (continued).



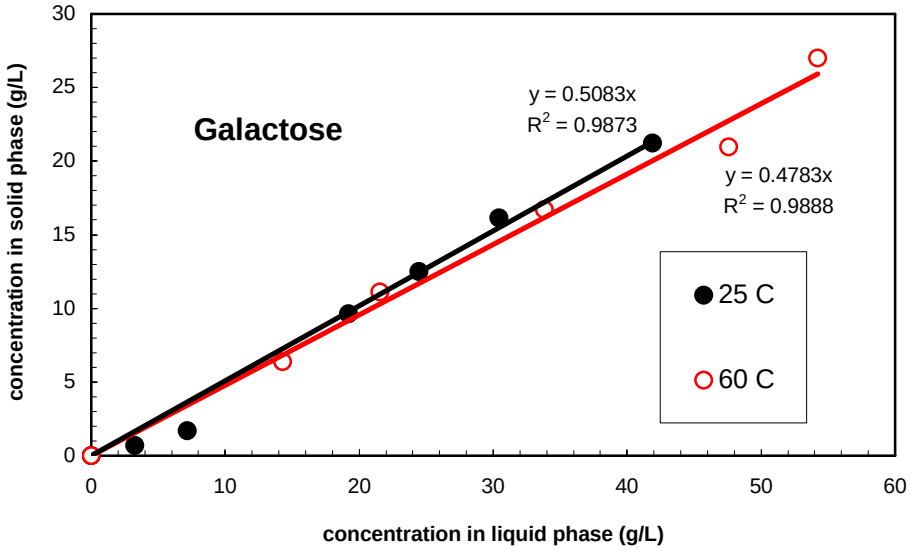


Figure 6.4: (Continued).

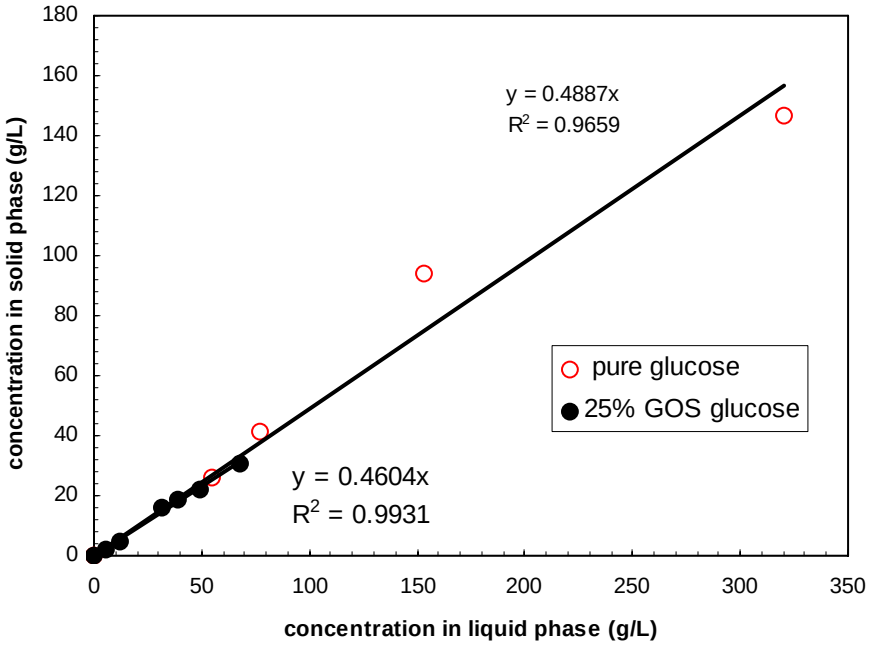


Figure 6.5: Isotherms of pure glucose and from a sample of 25% GOS for 4% cross-linking on PS-DVB resin in Na^+ form at 25 ^{0}C.

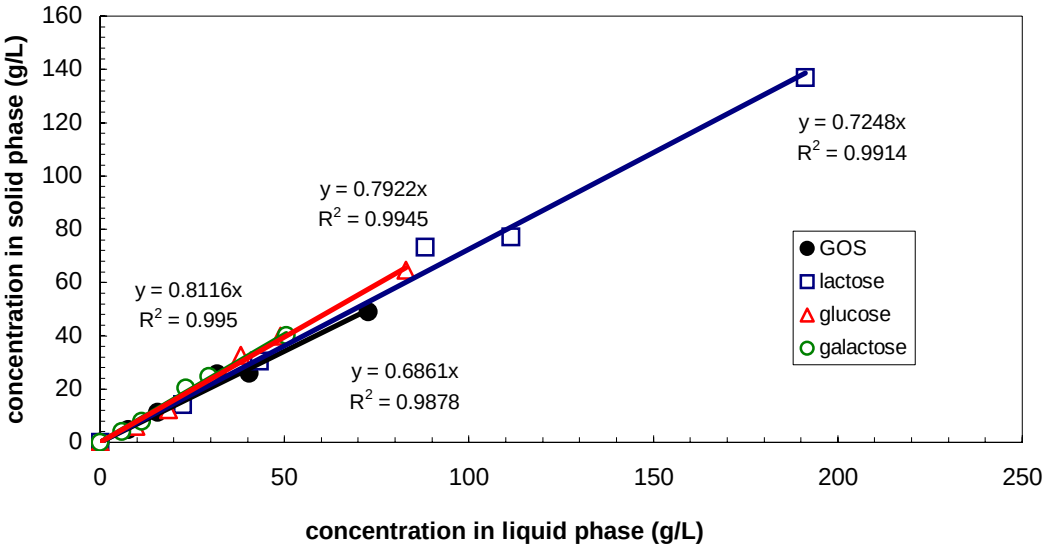


Figure 6.6: Isotherms of GOS, lactose and glucose and galactose from a sample of 25% GOS for 2% cross-linking on PS-DVB resin in Na^+ form at 25 ^{0}C.

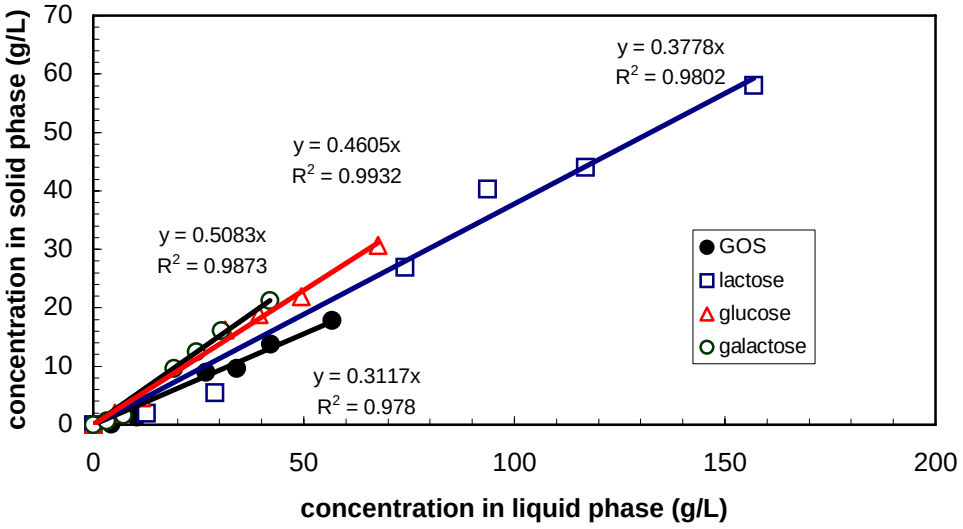


Figure 6.7: Isotherms of GOS, lactose and glucose and galactose from a sample of 25% GOS for 4% cross-linking on PS-DVB resin in Na^+ form at 25 C.

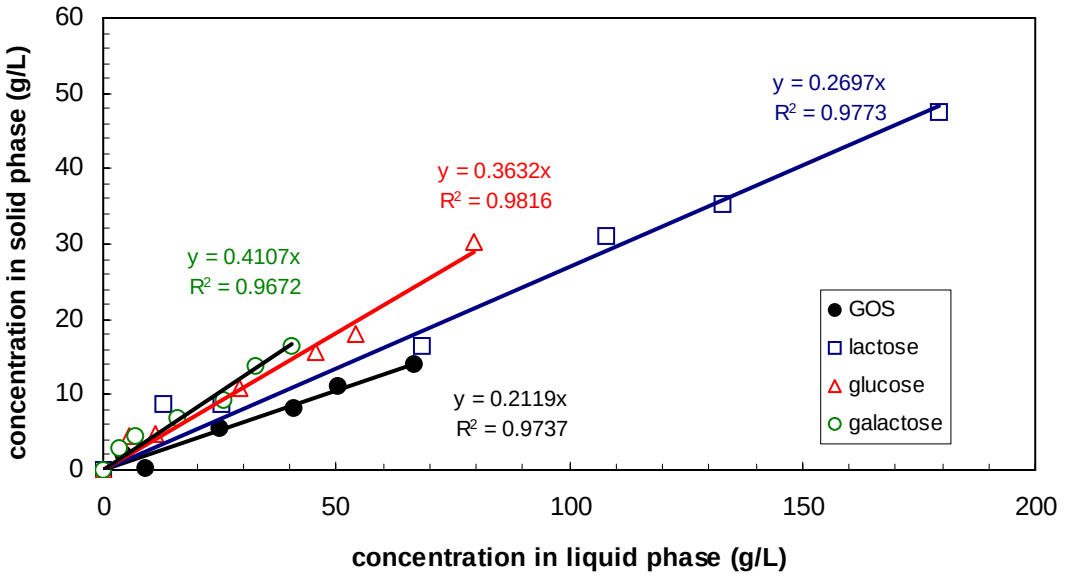


Figure 6.8: Isotherm of GOS, lactose and glucose and galactose from a sample of 25% GOS for 8% cross-linking on PS-DVB resin in Na^+ form at 25 ^{0}C.

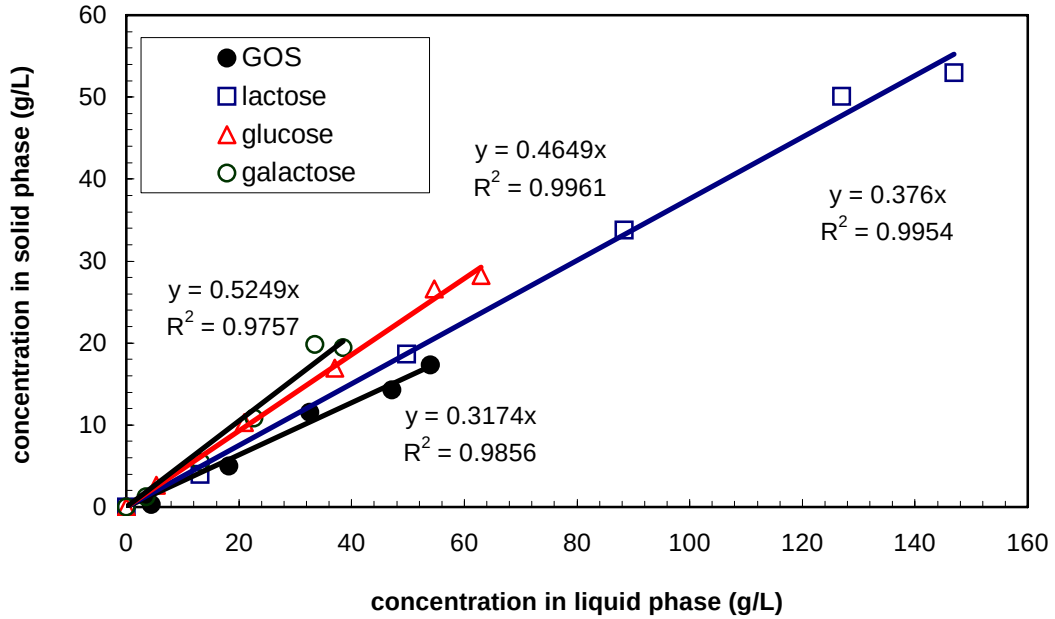


Figure 6.9: Isotherm of GOS, lactose and glucose from a sample of 25% GOS for 4% cross-linking on PS-DVB resin in K^+ form at 25 ^{0}C.

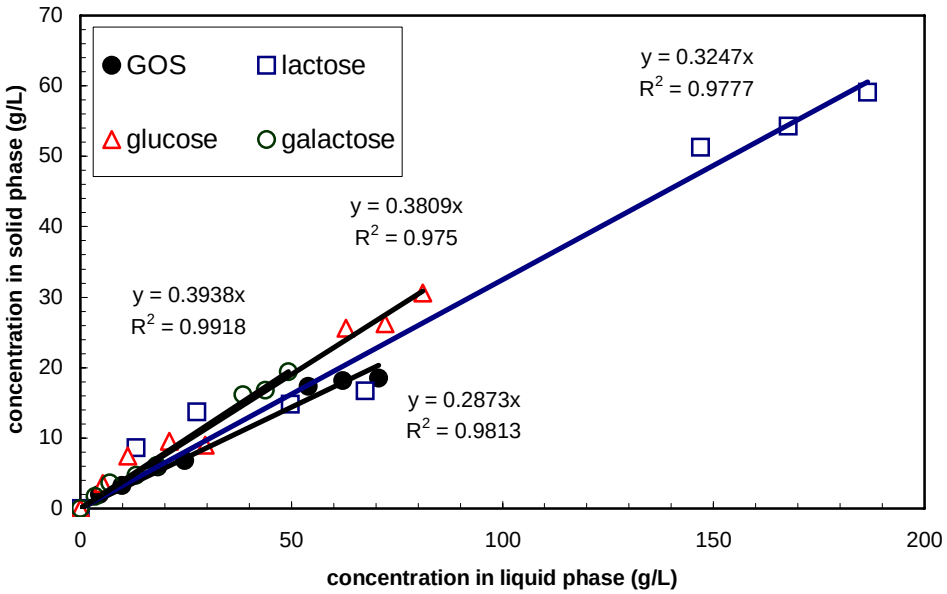


Figure 6.10: Isotherm of GOS, lactose and glucose and galactose from a sample of 25% GOS for 4% cross-linking on PS-DVB resin in Ca^{++} form at 25 ^{0}C.

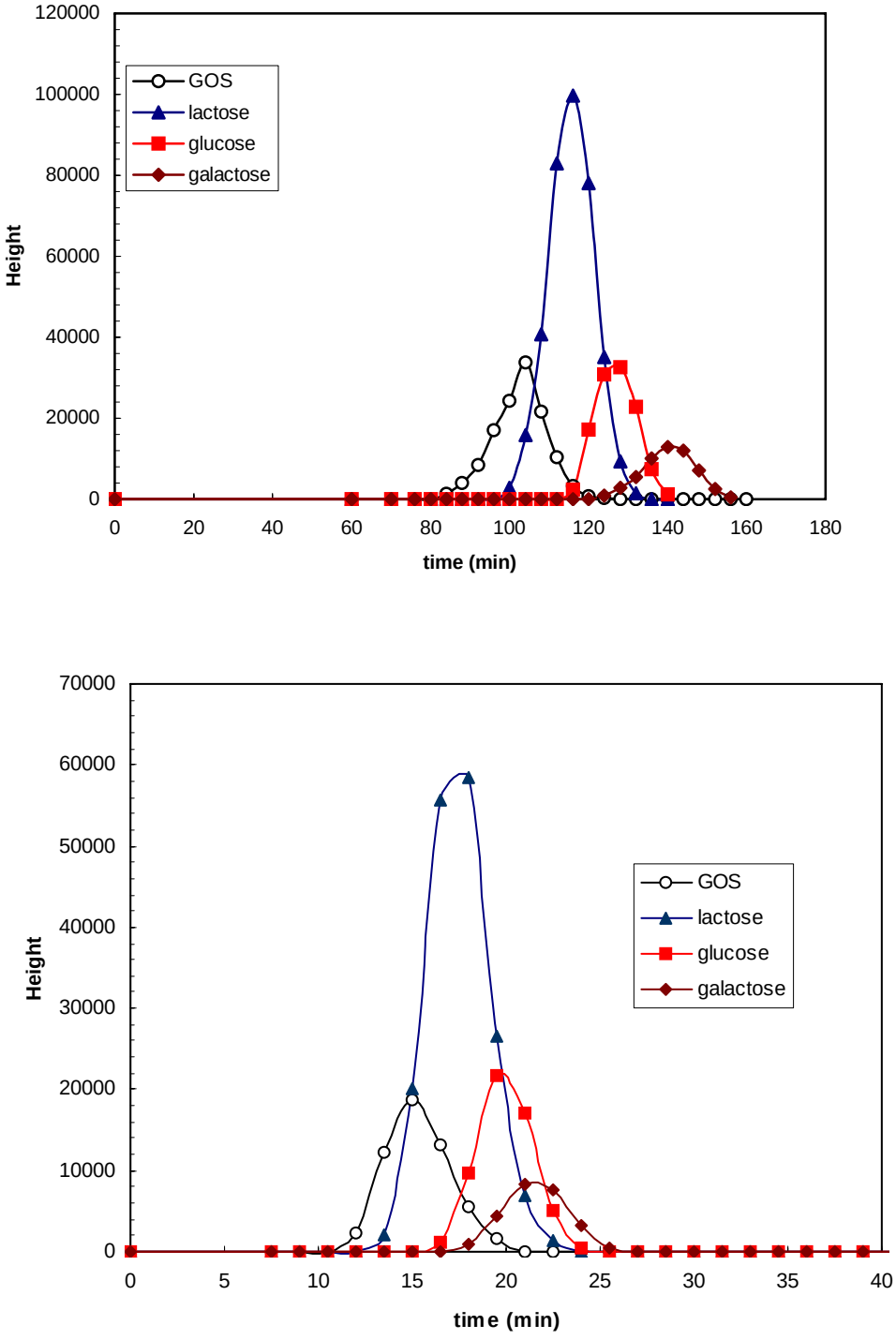
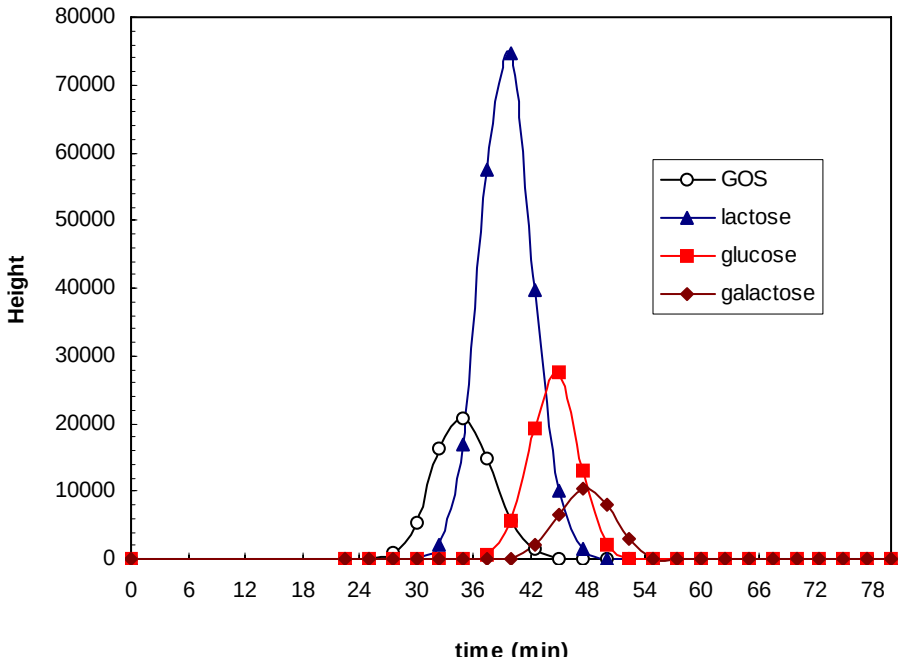


Figure 6.11: Chromatogram of GOS, lactose, glucose and galactose separation from a sample of 25%GOS a) at flow rate of 0.3 ml/min and b) 1.8 ml/min by using resin with 4% crosslinking in Na^+ form.



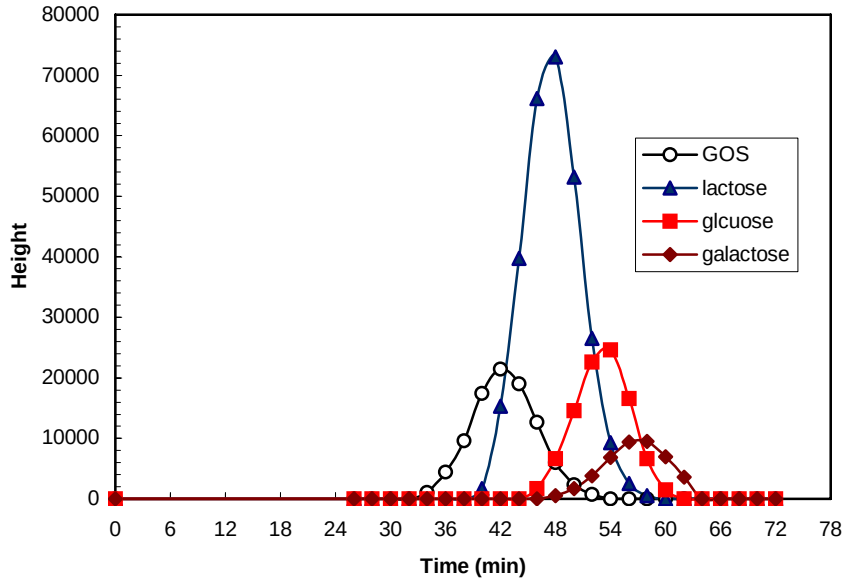


Figure 6.12: Chromatogram of GOS, lactose, glucose and galactose separation from a sample of 25%GOS at flow rate of 0.9 ml/min by using resin with 4% crosslinking in a) Na^+ form and b) K^+ form

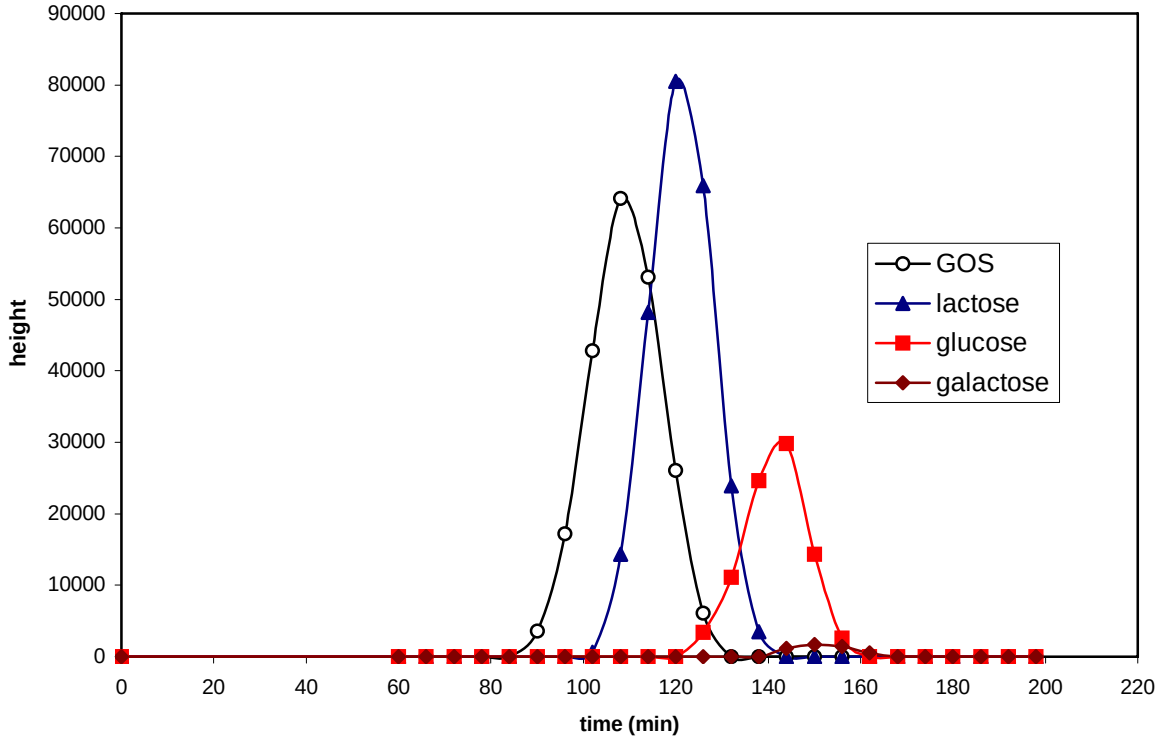


Figure 6.13: Chromatogram of GOS, lactose, glucose and galactose separation from a sample of 40% GOS by using a resin with 4% crosslinking in Na^+ form.

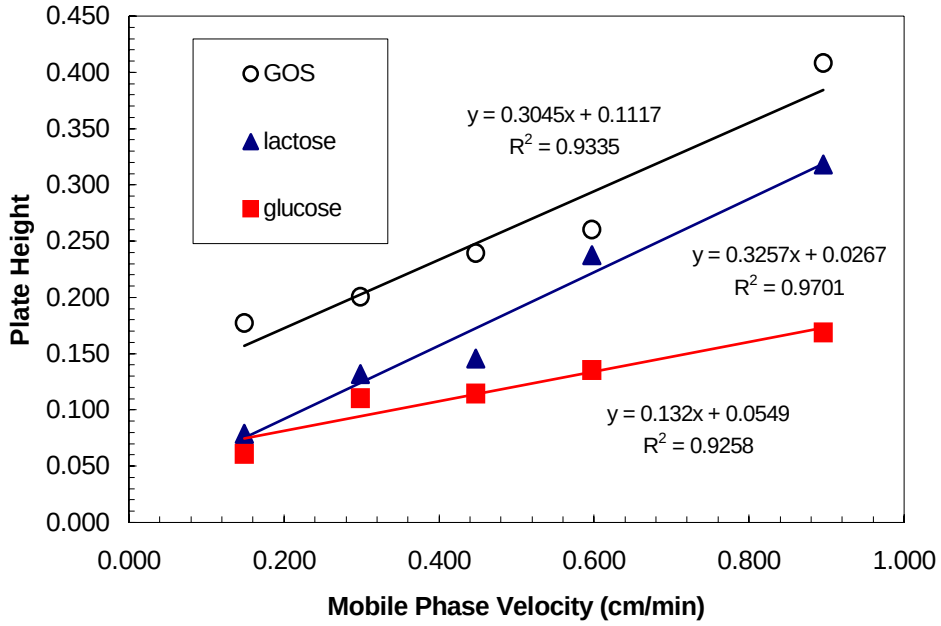


Figure 6.14: Van Deemter plot for GOS, lactose and glucose from a 25% GOS sample by using a resin with 4% crosslinking in Na^+ form at different average linear velocities

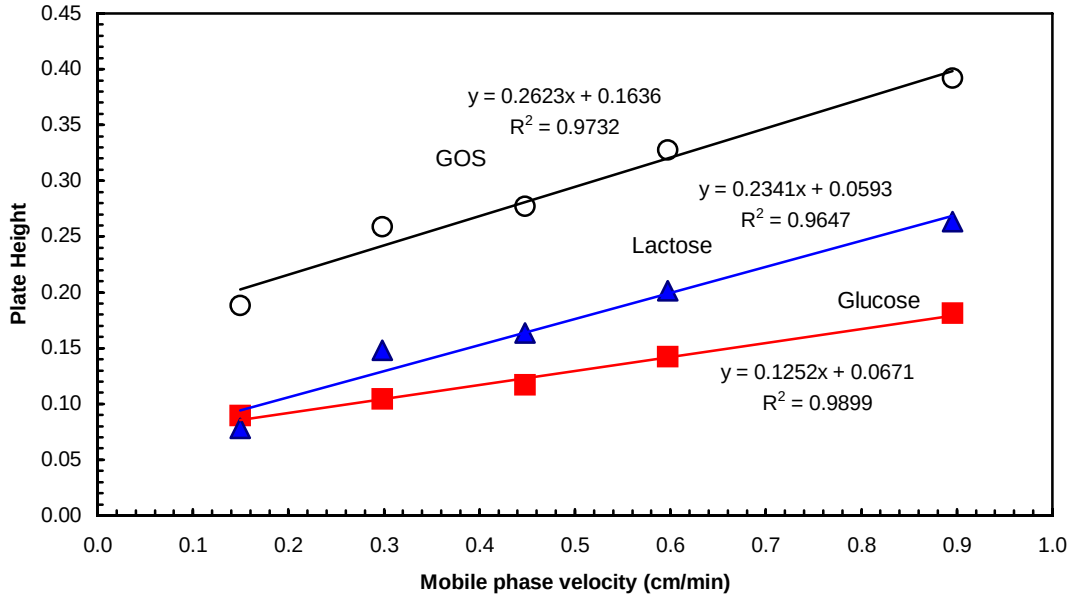


Figure 6.15: Van Deemter plot for GOS, lactose and glucose from 40% GOS sample by using a resin with 4% crosslinking in Na^+ form at different average linear velocities

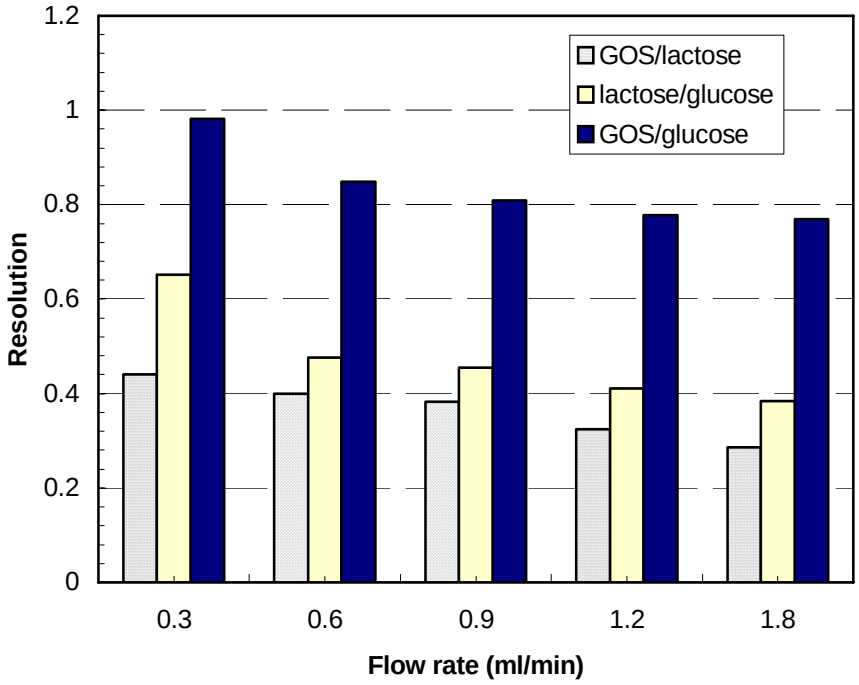


Figure 6.16: Resolution of GOS, lactose and glucose from a sample of 25% GOS by using a resin with 4% crosslinking in Na^+ form at different flow rates.

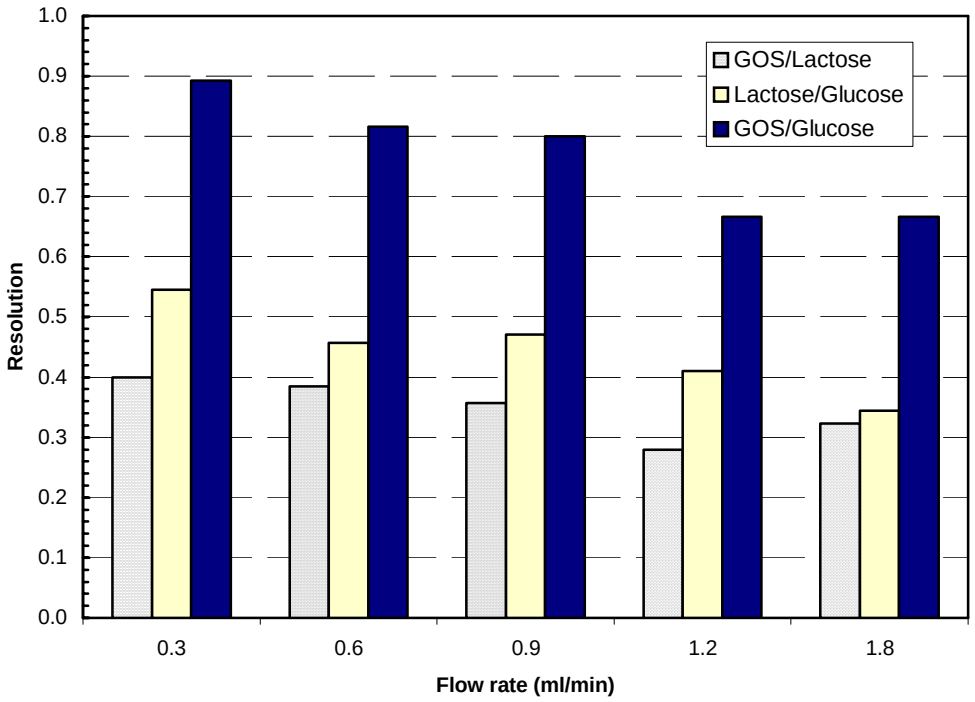


Figure 6.17: Resolution of GOS, lactose and glucose from a sample of 40% GOS by using a resin with 4% crosslinking in Na^+ form at different flow rates.

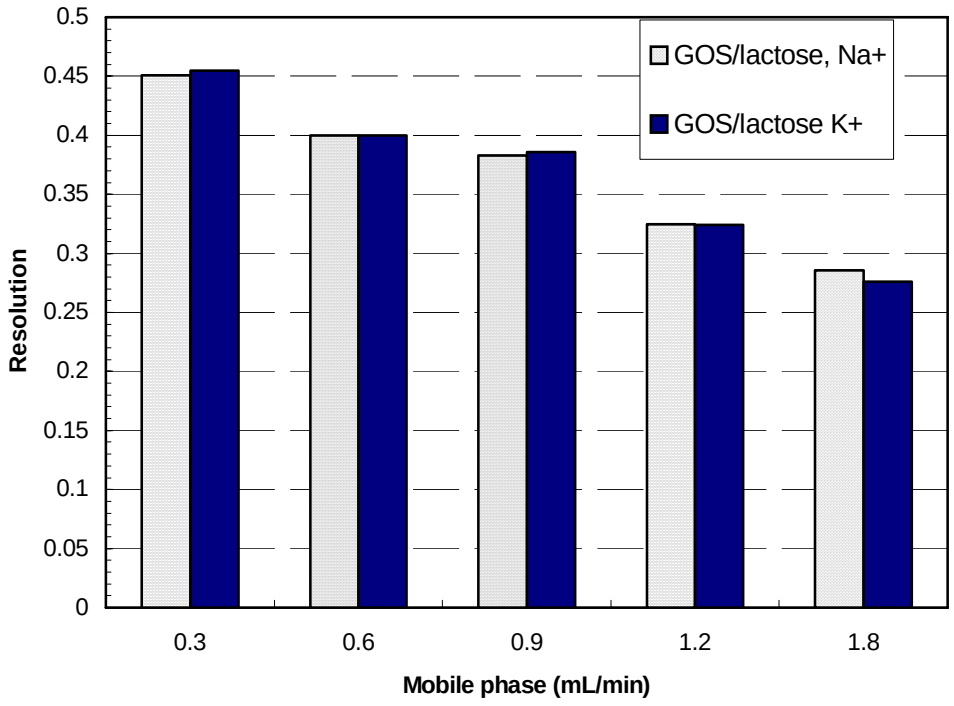


Figure 6.18: Resolution of GOS and lactose from a sample of 25% GOS by using a resin with 4% crosslinking in Na^+ and K^+ form at different flow rates.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The immobilization of ß-galactosidase on cotton cloth for the purpose of galacto-oligosaccharides (GOS) production and posterior chromatographic separation by using a commercial cation exchange resin (Dowex 50W) was addressed in this study. Lactases from *Aspergillus oryzae* (fungus), *Kluveromyces lactis* (yeast) *and Bacillus circulans* (bacterium) presented not only different optimal conditions for GOS production but also different amounts, types and sizes of GOS. The formation of GOS was mainly affected by lactose concentration while the solution pH affected the rate of sugar formation for all lactases. *B. circulans* lactase produced the highest and the most diverse types of GOS with 40% (w/w) at 60% lactose conversion from 400 g/L lactose solution, which appeared to be the optimal concentration. Then, *K. lactis* lactase produced 31% (w/w) GOS at 75% lactose conversion and *A. oryzae* produced 27% (w/w) GOS at 50% lactose conversion under the same concentration. The enzyme was unable to convert free galactose for GOS formation. Furthermore, the rate of the reaction was reduced when galactose was added in the lactose solution

When, *B. circulans* lactase reacted with whey permeate, instead of lactose, as the substrate higher hydrolytic activity was observed. Lactases were immobilized on cotton cloth by adsoption, using polyethyleneimine (PEI) and crosslinked with glutaraldehyde (GA). The optimal conditions for immobilizing different lactases were also different. ß-galactosidases from *A. oryzae and B. circulans* were immobilized at high enzyme loading of 250 mg/g with 0.22% and 0.04% (w/v) PEI, respectively, at pH 9 and 0.2% (w/v) GA at pH 7. The most important factor in the enzyme immobilization was the PEI-enzyme ratio with 1/20 and 1/125 for *A. oryzae and B. circulans* lactases, respectively. The contact time necessary between enzyme-cotton for the immobilization of *B. circulans* lactase was longer (20 h) compared with *A. oryzae* lactase (10 minutes). Furthermore, the activity yield obtained was 90% and 35% for *A. oryzae and B. circulans* lactase, respectively. Thermal stability of the immobilized enzymes on cotton cloth increased significantly as compared with free enzyme.

Since the maximum amounts of GOS from different lactases were obtained at different lactose conversions, GOS production by using two sequential plug flow reactors with immobilized ß-galactosidase from *A. oryzae* and *B. circulans* on cotton cloth was developed because it could lead to increased GOS yield and reactor productivity. Initially, a single reactor with immobilized lactase from *A. oryzae and B. circulans* were fed continuously with 400 g/l lactose during 10 days, obtaining GOS productivities of 4300 g/L·h and 180 g/L·h, respectively. Lactase from *A. oryzae* produced 25% (w/w) GOS with no apparent decrease in the production level over time. However, *B. circulans* lactase produced initially 40% (w/w) GOS but a clear steady state was never achieved

due to enzyme inactivation and a drop between 3% and 8% of the initial 40% GOS was observed during the 10 days of continuous run. However, enzyme deactivation was reversible since most of the enzyme activity was recovered after running a buffer solution through the reactor for several hours. It seemed that the inactivation of the enzyme was caused by the accumulation of GOS on cotton cloth, which blocked the enzyme active site from contacting with the substrate. Furthermore, when two consecutive reactors with immobilized *A. oryzae and B. circulans* were run, higher productivities were achieved as compared with a single reactor at the same lactose conversion.

Commercial cation exchange resins were suitable adsorbents to separate GOS from the reaction mixture, especially monosaccharides: glucose and galactose. The degree of crosslinking of the resin had a larger effect on adsorption capacity and selectivity of sugars than cation type and temperature. The optimal conditions for sugar separation were achieved with a resin with 4% crosslinking in Na^+ or K^+ form, separating at least 95% of monosaccharides from GOS in the sugar mixture.

7.2 Recommendations

The experimental data from this study indicated that the immobilization of *B. circulans* lactase was accomplished with 35% activity yield. Other proteins present in the commercial enzyme powder, besides the enzyme, were also immobilized, and consequently reduced the immobilization efficiency and yield. Improvement of the immobilization could be achieved by purifying the enzyme to separate it from the other proteins. The enzyme could be purified using a gel chromatography and gel electrophoresis as previously reported (Hsu, et al., 2005; Hernaiz et al., 2000).

Furthermore, based on the results obtained from the continuous production of GOS with immobilized lactase from *B. circulans*, a clear steady state was never achieved due to enzyme inactivation. This enzyme inactivation was due in part to the accumulation of GOS on the cotton cloth, which blocked the enzyme from contacting with the substrate. Using a different type of reactor such as continuous stirred tank reactor (CSTR) could help to avoid the accumulation of GOS on the cotton for longer time.

Using a different source of lactase from *Bifidobacterium* or *Lactobacillus* spp. is another promising way to produce GOS since it was suggested that ß-galactosidases from probiotic microorganisms might produce GOS structures that have special prebiotic effects, specifically targeting selected probiotic strains (Rastall et al., 2002). The maximum GOS obtained from *Bifidobacterium longum* lactase was 32.5% (w/w) GOS at 59.4% lactose conversion with an initial lactose concentration of 400 g/L (Hsu et al., 2007). Furthermore, Splechtna et al. (2007) produced 36% (w/w) GOS at 80% lactose conversion.

Pure lactose was mainly used in this study for GOS formation. However, slightly lower GOS percentage was obtained with concentrated whey permeate as compared with lactose solution as the substrate. Continuous GOS production from whey permeate feed is of industrial significance and should be further investigated. Whey permeate contains high salt and residual protein contents, and may constitute a challenge for immobilized enzyme reactors. Problems such as reactor clogging and decrease in enzyme activity should be studied in continuous GOS production from whey permeate.

The experimental data from this study showed that cation exchange resin Dowex 50W could separate monosaccharides from GOS in the reaction mixture with a small

injection of 0.5 mL in a column of 60 mL. However, factors such as loading capacity and particle size should be investigated to further optimize the GOS separation. Furthermore, conventional chromatography is not an efficient method for separation as compared to other chromatography modes such as simulated moving bed (SMB). Conventional adsorption chromatography is a discontinuous process with poor use of the adsorbent and high product dilution. SMB systems should be evaluated for the separation of GOS since the process is continuous and no total resolution has to be achieved at the column outlet since only parts of the fronts of the axial concentration profile are withdrawn in pure form from the system. Furthermore, the product dilution is lower and pure fractions are withdrawn (Schulte et al., 2000). SMB systems have already been used in the sugar industry, especially in the separation of glucose from fructose in the production of high fructose corn syrup obtaining purities of 97% (Yu et al., 2003).

This study showed that the separation of monosaccharides (glucose and galactose) is achievable. However, the separation of lactose due to the proximity in size to 3OS is more difficult to achieve. Another method, besides SMB, that can separate GOS from lactose is the oxidation of lactose to lactobionic acid using fungal cellobiose dehydrogenases (CDH). Maischberger et al. (2008) produced GOS from lactose containing 48% monosaccharides, 26.5% lactose and 25.5% GOS with *Lactobacillus reuteri* lactase. The final product after the oxidation process contained less than 0.3% lactose with a GOS yield of 60.3%.

7. 3 References

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