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UMI
PRODUCTION OF GALACTO-OLIGOSACCHARIDES FROM LACTOSE
BY ASPERGILLUS ORYZAE β-GALACTOSIDASE
IMMOBILIZED ON COTTON CLOTH

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Nedim Albayrak, M.S.

*****

The Ohio State University
2001

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ABSTRACT

Knitted cotton terry cloth as a novel and inexpensive fibrous matrix was evaluated for the development of fibrous bed biocatalyst reactor. *Aspergillus oryzae* β-galactosidase was immobilized on cotton cloth with two different methods. The first method used p-toluenesulfonyl chloride (tosyl) to activate cotton cloth and the enzyme was then coupled to the tosylated sites. Approximately 50 mg enzyme per gram of cloth was coupled with an immobilization yield of 55%. The second method involving polyethylenimine (PEI) relied on PEI-enzyme aggregate formation and coating of the aggregates on the fibrils of cotton cloth. Immobilized enzyme activity under optimized condition reached to 90-95% of free enzyme at a loading of 250 mg/g. Thermal stability of the immobilized enzymes were greatly increased, with tosyl immobilized enzyme showing 25-fold increase as compared with the free enzyme.

The production of galacto-oligosaccharides (GOS) from lactose was studied by cotton cloth immobilized enzyme by both methods. The total amounts and types of GOS produced were mainly affected by the initial lactose concentration in the reaction media. A maximum GOS production of 27 % (w/w) was achieved at 50 % lactose conversion with 500 g/L of initial lactose concentration. Trisaccharides were the major types of GOS
formed, accounting for more than 70% of the total GOS produced in the reaction. Monosaccharides produced during lactose hydrolysis severely delayed GOS formation and decreased 10-15% in GOS content.

A stable long-term continuous operation was demonstrated with the cotton cloth immobilized enzyme reactor. Overall, the high porosity (>95%) and low-pressure drop of cotton cloth allowed the reactor to be operated without any difficulties. Even with high enzyme loadings, cotton cloth immobilized enzymes showed no reduction in GOS formation and did not impose any significant diffusion limitation. GOS productivities in the reactor were 106 g/L/h with tosyl activated cotton cloth immobilized enzyme and 5200 g/L/h with PEI immobilized enzyme, which is 100-fold higher than those reported in the literature. The cotton-cloth enzyme reactor thus can be used for economical production of GOS from lactose. GOS product can be used as a prebiotic food ingredient because of its many health benefits.
Dedicated to the women of my life

my wife Gulnur, my daughter Elif and my mother Hava Albayrak
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CHAPTER 1

INTRODUCTION

Galacto-oligosaccharides (GOS) are prebiotic functional food ingredients that have been shown to beneficially affect the host well being by selectively stimulating the growth and/or activity of health-promoting bacterial species in the colon (Gibson and Roberfroid, 1995, Roberfroid, 1997). The foods containing GOS and other nondigestible oligosaccharides are already available for the consumer in Japan and France, where ‘the foods for specified health use’ (FOSHU) health claims are approved. In 1995, approximately 15,000 tons of GOS were produced worldwide (Crittenden and Playne, 1996). Americans are showing increasing interest in foods with beneficial health effects, and it has been estimated that foods containing functional additives will grow 7% annually to reach $322 million by 2009 in the US (Reisch, 2001). GOS are one of the most favorable candidates for this market. Besides their prebiotic effects, these oligosaccharides have low cariogenicity, low caloric values, and low sweetness (Sako et
al., 1999). Recently, GOS are included in the definition of dietary fiber by American Association of Cereal Chemists (Clemens, 2001).

GOS possesses several advantages over other nondigestible oligosaccharides. GOS are naturally found in human milk (Stahl et al., 1994, Yamashita and Kobata, 1974) and stable to food processing conditions, such as acidic conditions and high temperatures, as compared with fructo-oligosaccharides, which are obtained from sucrose (Sako et al., 1999). Moreover, GOS are conveniently obtained by enzymatic transgalactosylation from lactose, which is an abundant byproduct from cheese manufacturing. Currently, applications of lactose in food products are limited by its intolerance by some people, low solubility, and tendency to crystallize (Bourne et al., 1983, Shukla, 1975). Most of the whey lactose is utilized as animal feed. Considering the 3% annual increase in cheese production (Foda and Lopez-Leiva, 2000), the already problematic lactose is expected to be a major concern for the dairy industry. Therefore, conversion of whey lactose into a prebiotic food ingredient is of industrial significance (Playne and Crittenden, 1996). Although the commercial potential for the production and applications of GOS in food product lines is high (Crittenden and Playne, 1996, Shin et al., 1998), an economical and efficient production process still needs to be developed.

GOS are commercially produced from lactose in an enzymatic transgalactosylation reaction catalyzed by microbial β-galactosidases. Enzymes from different sources have different characteristics in not only pH and temperature optima, but also concentration and structures of GOS produced. β-Galactosidase from A. oryzae
was used in this study. This enzyme is commonly used in the food industry due to its acidic pH optima (pH 3.5-4.5) and relatively high temperature stability. Since the substrate for an industrial GOS production process would be whey permeate containing a high lactose concentration, the low pH used by *A. oryzae* is desirable. The enzyme is commercially available at a relatively inexpensive price compared to β-galactosidases from alternative sources. Of the oligosaccharides formed by *A. oryzae* β-galactosidase, ~70% are trisaccharides (as 6’ galactosyl lactose), which is also present in mother’s milk. Also, the enzyme gives high yield of GOS at low lactose conversions (~50%), and high GOS productivities over most bacterial and yeast enzymes (Shukla, 1975, Foda and Lopez-Leiva, 2000, Berger et al., 1995a).

There is no question that efficient and economical enzymatic conversion of lactose into GOS requires the use of immobilized enzyme, which allows the reuse of enzyme and continuous operation. GOS as an intermediate product of the enzyme is suitable for continuous operation, and commercial production of GOS will certainly benefited from a continuous production process (Mozaffar et al., 1986a). Nevertheless, continuous and stable GOS production from immobilized enzymes has not been addressed very well. Varieties of supports have been used for the immobilization of β-galactosidases by using various techniques. Many of the enzyme carriers are some types of microparticles, such as ion exchange resins and cellulose and glass beads. These particulate carriers are quite expensive (Anspach et al., 1994) and often cause high-pressure drops, reactor clogging and aggregation due to structural instability (Ichijo et al., 1985, Peng et al., 1987). It has been observed that the immobilized enzymes in these
supports often result in 20-30% reduction in GOS yield due to diffusion limitations (Prenosil et al., 1987b, Rugh, 1982, Sheu et al., 1998). Therefore, it can be concluded that these types of supports for enzyme immobilization are not appropriate for use in commercial production of GOS.

Some desirable reactor performances such as limited pressure drop, negligible mass-transfer resistance and good flow rates were noted with fibrous form of matrices. The matrices considered in biocatalyst applications include polyethylene terephthalate fibers (Elcin and Sacak, 1996), dimethylated superfine fibers (Ichijo et al., 1985, 1990a, b), silk fabrics (Demura et al., 1992, Furuhata et al., 1996, 1997) and cotton fabric (Sharma and Yamazaki, 1984, Maeshima et al., 1997). The main problem with these matrices, however, was that the level of enzyme immobilization was somewhat unsatisfactory.

The primary goal of this research was to develop a fibrous bed biocatalyst reactor using inexpensive fibrous support such as cotton cloth. As an inexpensive and widely available fibrous matrix, cotton cloth provides large specific surface area with high porosity (>95%), which allows immobilization of a large amount of enzyme. The reactor packed with cotton cloth in a spiral form has a low pressure drop, good flow rates, and little diffusion limitation. The enzyme immobilized in the fibrous matrix, therefore, should not impose any reduction in the GOS production as compared with soluble enzyme. Thus, the two specific objectives of this research were to develop enzyme immobilization systems for cotton cloth and to evaluate the performance of cotton cloth
immobilized enzyme reactor in GOS production from whey lactose. This research was also expected to lead to the development of a novel fibrous bed biocatalyst reactor with general applicability.

Native cotton fiber, which is composed of pure cellulose, lacks active groups to convey immobilization of enzymes. Previously, cotton cloth was activated with titanium chloride and used for the immobilization of A. oryzae β-galactosidase. Although 85% coupling yield was obtained, the enzyme gradually leached from the matrix during continuous operation (Bednarcik, 1998). Therefore, a stronger bond formation between the enzyme and cotton cloth was desirable for stable enzyme immobilization. In general, covalent bond formation yields stronger and more stable immobilization.

In this work, two novel techniques for enzyme immobilization on cotton cloth were developed. The first method involved the use of p-toluenesulfonyl (tosyl) chloride for the activation of cotton cloth to yield a strong covalent bond formation between the enzyme and cotton fiber. Details of this immobilization method are given in Chapter 3. The second method involved the use of polyethylenimine (PEI) to form PEI-enzyme electrostatic complex, which was followed by glutaraldehyde crosslinking. Detailed description of this immobilization method is discussed in Chapter 5. In both techniques, inexpensive and non-toxic chemicals were used at low levels. Thus, the cost of immobilization was low. Also, both methods require no special apparatus for enzyme immobilization and can be easily scaled up for commercial applications.
GOS formation kinetics and continuous reactor performances using immobilized β-galactosidase on cotton cloth were evaluated in packed bed reactors. Thermal stability of the enzyme immobilized on cotton cloth was also investigated. The effects of lactose concentration, temperature and pH on GOS formation kinetics were also studied, and the results are discussed in Chapters 4 and 5. Chapter 6 gives conclusions and recommendations for further research and development of the immobilized enzyme technology for GOS production.
2.1. Non-digestible Oligosaccharides (NDOs)

Carbohydrates are highly divergent in molecular size, monosaccharide unit structure, and linkage between units. Dietary carbohydrates can be classified by their degree of polymerization (DP; number of monosaccharide units combined) into monosaccharides, oligosaccharides (DP: 2 ~ 10), and polysaccharides (DP > 10) (Cummings et al., 1997). At the same time, based on the physiological properties of dietary carbohydrates, they can be classified as digestible or non-digestible.

The main characteristic of non-digestible oligosaccharides (NDOs) is that they resist hydrolysis by salivary and intestinal digestive enzymes because of the configuration of their glycosidic bonds (Roberfroid, 1997). NDOs are commonly classified according to their main monosaccharide components: FOS composed of fructose and glucose, or GOS composed of galactose and glucose. NDOs in a specific
group have a few variations in their chain length (trisaccharides, tetrasaccharides, etc.) and linkage type (e.g., β-1-4, α-1-6, and β-1-6 linkages), depending on the sources and production methods used.

There are more than 20 different types of NDOs available on the market worldwide. Table 2.1 lists major groups of NDOs and their production amounts. These oligosaccharides are either extracted from natural sources (e.g. rafinose and soybean oligosaccharides), or obtained by partial enzymatic hydrolysis of polysaccharides (e.g. xylo-oligosaccharides and isomalto-oligosaccharides), or produced by enzymatic transglycosylation (e.g. galacto-oligosaccharides and fructo-oligosaccharides) (Hidaka et al., 1991). The most abundantly supplied and utilized group of NDOs as food ingredients are GOS and FOS which are generally produced by enzymatic transglycosylation because of adequate supply of the raw materials and the high efficiency of the reaction (Sako et al., 1999).

Specific health claims are allowed in France and Japan where eight different NDOs have been licensed as food ingredients with ‘food for specified health use’ (FOSHU) status including GOS, fructo-oligosaccharides, lactosucrose, xylo-oligosaccharides, soybean oligosaccharides, rafinose, lactulose, and isomalto-oligosaccharides (Sako et al., 1999). It is also noted that 60% of FOSHU approved products contained NDOs (Berner and O’Dannell, 1998). In 1995, the estimated production of NDOs in the world was 85,000 tons, leading by GOS at 15,000 tons followed by FOS at 12,000 tons, respectively (Playne and Crittenden, 1996) (Table 2.1).
2.1.1. Health Benefits of NDOs

Several hundred different species of bacteria are thought to be present in the large intestine. Gram-negative rods belonging to the *Bacteroides* genus are the numerically predominant culturable bacteria in the colon. These microorganisms can constitute 30% of the total fecal flora (Gibson and Roberfroid, 1995). The other main groups consist of different (Gram positive) rods and cocci, such as bifidobacteria, clostridia, peptococci, streptococci, eubacteria, lactobacilli, peptostreptococci, ruminococci, enterococci, coliforms, methanogens, dissimilatory sulphate-reducing bacteria and acetogens. These bacteria are in competition for nutrients and all have their niches to survive.

The composition of the diet has a strong influence on intestinal physiology and the metabolism of the microflora and determines the nature and activity of gut microbiota (Gibson and Roberfroid, 1995). It is well documented that differences in the main food components, namely proteins, lipids, and carbohydrates, cause alterations in the composition of bacteria in the gastrointestinal tract. The carbohydrate composition of food is thought to be an important determinant of the composition of the intestinal flora (Sako et al., 1999). Thus, alteration in the composition of the colonic microbiota towards a healthier composition can be obtained with consumption of NDOs in general.

NDOs are known to be non-digestible and promote the growth of beneficial bacteria in the colon, and are thus recognized as prebiotics (Fuller and Gibson, 1997, Tanaka and Matsumoto, 1998). 'Prebiotics' are defined as non-digestible food ingredients
that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995).

In addition, ingestion of these oligosaccharides leads to maintenance of normal intestinal balance, improvement in constipation, hyperammonemia, endotoxemia and symptoms of non-compensatory liver cirrhosis, reduction of serum cholesterol level, indigenous synthesis of B-complex vitamins, enhanced absorption of dietary calcium and improvement of lactose utilization in lactose intolerance individuals (Teuri and Korpela, 1998). Other beneficial effects of NDOs include low sweetness, low or non-cariogenicity, low calorie rating and high water-holding capacity (Oku, 1996). All of the associated health benefits result from this shift of number and or metabolic activity of gut flora upon consumption of NDOs. Daily intake for GOS is 3 g (Terada et al., 1992), for fructo-oligosaccharides is 1 g (Tokunaga et al., 1990), and for xylo-oligosaccharides is 2 g (Okazaki et al., 1990). These results suggest that all of these oligosaccharides have the potential for being used for human ingestion, to enhance numbers of bifidobacteria (Tomomatsu, 1994, Oku, 1996). Thus, these oligosaccharides are of commercial interest and their production and applications (such as in prebiotic foods for specified health use) are therefore increasing rapidly.

2.2. Galacto-oligosaccharides (GOS)

GOS are natural constituents in human milk (Stahl et al., 1994) as well as in foods such as garlic and onions. Food grade GOS are commercially produced from lactose and
contain various oligosaccharides. Their chemical formula is \((\text{Galactose})_n\)-Glucose, with \(n\) ranging from 2 to 4. The galactose-galactose linkage is a \(\beta-(1\rightarrow3)\), \(\beta-(1\rightarrow4)\), or \(\beta-(1\rightarrow6)\) linkage, with the \(\beta-(1\rightarrow4)\) linkage being predominant (Oku, 1996); the galactose-glucose linkage is mainly \(\beta-(1\rightarrow4)\). Some disaccharides (e.g., allolactose and galactobiose) are also present in GOS. It was indicated that bacterial species fermenting 4\(\beta\)-GOS and 6\(\beta\)-GOS in gut overlap although some strain preferences were noted (Sako et al., 1999).

Kunz and Rudloff (1993) have reported in an article entitled "Biological Functions of Oligosaccharides in Human Milk" that the monomers of breast milk oligosaccharides are D-glucose, D-galactose, N-acetylglucosamine, L-fucose and sialic acid. With few exceptions, all of the breast milk oligosaccharides carry lactose at their reducing end. These oligosaccharides are referred to as bifidus factor. GOS are specifically known as "Bifidus growth factor" since they stimulate the growth of \(Bifidobacteria\) in the human body. GOS is currently produced from lactose in an enzymatic reaction catalyzed by microbial \(\beta\)-galactosidase and it has large commercial interest.

### 2.2.1. GOS Health Benefits

Due to their \(\beta\)-configuration, GOS are resistant to hydrolysis by human saliva and human gastric enzymes (Ohtsuka et al., 1990, Watanuki et al., 1996). In addition, the activity of lactase localized at the brush border membrane of the small intestine, which
has the potential to digest GOS, is usually weak or often deficient (Ito et al., 1990). The indigestibility of GOS in vivo has been shown by means of the hydrogen breath test in human studies (Tanaka et al., 1983, Ishikawa et al., 1995). Thus, GOS resists digestion and absorption in the small intestine and reaches the cecum and colon.

2.2.1.1. Bifidogenic Effect

2.2.1.1.1. In vitro

Effects of GOS on colon microbial ecology and host physiology have been most extensively analyzed in the last decade (Tanaka et al., 1983, Ishikawa et al., 1995, Ohtsuka et al., 1989). Table 2.2 displays utilization of GOS by various intestinal bacteria. It has been shown in many in vitro studies that GOS are selectively utilized by all strains the Bifidobacteria tested while many potential pathogens including Bacteroides spp. Clostridium butyricum, Clostridium difficile, Clostridium innocuum, Clostridium perfringens, Eubacterium aerofaciens, Eubacterium limosum, Enterococcus fecalis, Enterococcus faecium, Escherichia coli show no growth on GOS (Ohtsuka et al., 1989). Kim (1999) studied the growth rate of B. bifidum in MRS broth medium containing GOS and found that the growth rate increased up to 18%, 8%, and 7% with GOS compared to glucose, galactose, and lactose, respectively. Hopkins et al (1998) also observed that maximum specific growth rates and bacterial cell yields in many bifidobacteria species were higher on oligosaccharides compared to their monosaccharide units.
This selective and/or better utilization of GOS by bifidobacteria suggests that these microorganisms should be equipped with hydrolyzing enzymes that can handle a variety of bonds. In fact, it was indicated that many strains of *Bifidobacterium* produce glycolytic enzymes hydrolyzing different glycosidic bonds and a wide variety of monosaccharide units, whereas the enzyme activities from other enteric bacteria are less varied and have weaker activities than *Bifidobacterium* (Tochikura et al., 1986). The bifidogenic nature of GOS has been related to a linkage specificity of the *Bifidobacterium* β-galactosidase. Matsumoto et al (1989) isolated three different β-galactosidases from *B. breve*, and determined GOS hydrolyzing activities in comparison with lactose. One of the three enzymes isolated showed six times higher activity to GOS than to lactose.

### 2.2.1.1.2. *In vivo*: Effects of Ingestion on Intestinal Bifidogenic Activity

It is well known that indigestible oligosaccharides including GOS in human breast milk (Yamashita and Kobata, 1974) are growth-promoting factors responsible for the establishment of the Bifidus flora characteristic of breast-fed infants (Kawase et al., 1983, Tanaka and Matsumoto, 1998). Breast-fed infants have an intestinal flora with a significantly higher number of bifidobacteria (at least one log) than that of bottle-fed infants (Braun et al., 1981, Yukara et al., 1983, Yoshioka et al., 1991). With changing diet and aging, the number of bifidobacteria declines while Bacteroidesceae becomes dominant in a normal adult gut, followed by Eubacteria, Bifidobacteria and Peptococcaceae (Mitsuoka, 1982, 1990). In the elderly, decline of bifidobacteria continues with rises in coliforms, enterococci, and clostridiria (Hoover, 1993).
A large body of literature suggests that intake of GOS significantly increases the fecal count of *Bifidobacterium sp.* in a dose dependent manner (Tanaka et al., 1983, Ito et al., 1993). The increase in bifidobacteria is often accompanied by a decrease in gram negative *Bacteroidaceae* (Tanaka et al., 1983, Matsumoto et al., 1989, Mitsuoka, 1982, Narimiya et al., 1996) resulting in the change of the predominant genus in the fecal flora from *Bacteroidaceae* to *Bifidobacterium* (Bouhnik et al., 1997, Rowland and Tanaka, 1993, Yanahira et al., 1995, Tanaka et al., 1983, Hughes and Hoover, 1991). Direct consequence of this major shift in predominant flora of colon is reflected with metabolic products as well. Bifidobacteria produce strong acids such as acetic acid and lactic acid generally at a ratio of 1.5 to 2.0 (Ventling and Mistry, 1993, Dubey and Mistry, 1996). These organic acids not only lower the pH but also have antimicrobial properties.

### 2.2.1.2. Physiological Effects of GOS

Acetic, propionic and butyric acids, called short chain fatty acids (SCFA) are the major products of colon metabolism. After consumption of GOS, there is a significant increase in SCFA and organic acids like succinic (Hoshi et al., 1994) and lactic acids (Kikuchi et al., 1996) accompanied by a decrease in pH as observed in human subjects (Ishikawa et al., 1995, Dokkum et al., 1999) as well as human flora associated rat models (Chonan et al., 1995, Kikuchi et al., 1996, 1997, Chonan and Watanuki, 1996, Rowland and Tanaka, 1993). Approximately 95% of SCFA are rapidly absorbed by the human colonic epithelial cells and used as nutrients. They also function as the modulator of electrolyte transport, epithelial cell growth and intestinal motility (Yajima and Sakata, 1994).
1992). It was indicated that the organic acids could be the key substances for lowering the pH of the colonic content (Fukushima, 1996) since the rate of absorption of these organic acids is about one hundredth of SCFA (Umesaki et al., 1979).

2.2.1.2.1. Colon Toxicity and Colon Cancer Prevention

Biologically important functions of the colon include the absorption, secretion of certain electrolytes and water, as well as storage and excretion of waste materials. The human colon is composed of three parts, proximal, distal and rectal. The right or proximal colon is characterized by high substrate availability, lower pH (around 5.5-6.0) and a more rapid transit than the distal region. Bifidobacteria are most prevalent in the proximal region (Hoover, 1993). The left, or distal, area of the colon has a lower concentration of available substrate, the pH is approximately 6.5-7.0 and bacteria grow more slowly. The proximal region tends to be a more saccharolytic environment than the distal gut, the latter having higher bacterial proteolysis (Gibson and Roberfroid, 1995).

Although cancer of ‘proximal colon’ represents a minor type with uniform incidence rates throughout the world, the cancer in the ‘distal colon’ and ‘rectum’ are of higher incidence rates, especially in the Western world or the part of the world with a westernized dietary habits (Weisburger, 1996). Among other factors, secondary bile acids, and heterocyclic amine products are commonly blamed for this kind of cancer.
Diet controls the composition of microflora that generates these bile acids and more secondary bile acids were detected when a carbohydrate source was missing from the colon.

Unlike carbohydrate metabolism, the products from proteolysis are toxic (Fooks et al., 1999). These putrefactive metabolites include ammonia, phenol, p-cresol, indole, isobutyric acid and isovaleric acid (Sako et al., 1999). In addition, it was suggested that several bacterial enzymes, such as β-glucuronidase, β-glucosidase, and nitroreductase may play a role in colon carcinogenesis by converting pre-carcinogens to proximal carcinogens (Rowland, 1988). It has been demonstrated that ingestion of GOS in human subjects significantly decreased the fecal concentrations of those putrefactive metabolites and the activities of β-glucuronidase, β-glucosidase, and nitroreductase along with significant increase in the fecal count of Bifidobacterium and a decrease in Enterobacteria (Rowland and Tanaka, 1993) and Bacteroidaceae (Ito et al., 1993, Tamai et al., 1994).

Acidic environment itself creates another selectivity factor for bifidobacteria and lactobacilli, which are more resistant to acidic environment than harmful bacteria such as Bacteroidaceae and Clostridia (Oku, 1996). It was suggested that a decrease in pH due to acid production may have direct effects on the protonation of ammonia and amines to the positively charged counterparts which are non-diffusible to body, as well as indirect effects on the activities of those harmful enzymes (Gibson and Roberfroid, 1995) in addition to lower production rate of those enzymes (Oku, 1996). Reduced fecal activities
of these enzymes (Ito et al., 1993) and fecal physiological parameters such as pH, ammonia, p-cresol, and indole are considered risk factors not only for colon cancer development but also for systemic disorders. These alterations may be considered beneficial in reducing the risk of cancer development, and by decreasing the toxicity of the colon contents, which significantly contribute to cancer developments in the large intestine and other tissues (Rowland and Tanaka, 1993).

2.2.1.2.2. Improvement of Constipation

Constipation is a condition in which a person has uncomfortable or infrequent bowel movements. It was shown that SCFA production contributed to improvement of constipation by increasing osmotic pressure and stimulating peristalsis (Ishikawa et al., 1995). In a study using diabetic patients, a correlation was found between the improvement in constipation and the decrease in fecal Bacteroidaceae after ingestion of GOS (Narimiya et al., 1996). Deguchi et al (1997) demonstrated that with a daily ingestion of 5 g GOS, the defecation frequency increased, feces became softer, and bowel movement increased. It was concluded that 5.0 g or 10.0 g of GOS improved defecation of persons with a tendency for constipation. Hoshi et al (1994) observed a correlation between cecal enlargement and increase in succinic acid in the cecal content in rats fed galactosylsucrose and xylosylfructose.
2.2.1.2.3. Lactose Utilization

The presence of a high number of bifidobacteria in gut significantly reduced symptoms from lactose malabsorption (Jiang et al., 1996). It was indicated that β-galactosidase activities of Bifidobacteria are inducible by lactose (Jiang et al., 1996). Since β-galactosidases are inducible, and more β-galactosidase activity will be present in the gut upon GOS intake, thus has a higher probability to ease the lactose tolerance compared to other types of NDOs which do not induce β-galactosidase activity in the gut.

2.2.1.2.4. Resistance against Infectious Diseases

Diarrhea is a common symptom of gastroenteritis caused by infectious microorganisms. Some bacteria such as *Salmonella, Shigella, E. coli* and *Campylobacter* are common etiological agents in gastroenteritis and diarrheal diseases. These bacteria invade the lining of the intestine and damage the underlining cells. Upon diarrheal infections, microflora balance in the gut is severely disturbed due to proliferation of harmful bacteria, and it becomes harder for indigenous bacteria to colonize the intestinal mucosa (Homma, 1988).

It was demonstrated that many antibiotics cause alterations of the balance among bacteria in the gut, thus allowing certain disease causing bacteria to multiply. The most common problem-causing bacterium is *Clostridium difficile*, which produces toxins that can damage the protective lining of the bowel (Merck Manual, 1997, Pothoulakis, 2000). It was suggested that Bifidobacteria adhere easily to the intestinal epithelial cells better.
than most of the other microorganisms and protect against invading pathogens such as *Salmonella* and *E. coli* (Homma, 1988). The inhibition of growth of some human enteropathogens such as *Salmonella*, *Campylobacter* and *E. coli*, due to bifidobacterial antagonistic activity has been shown (Gibson and Wang, 1994, Oyarzabal and Conner, 1995).

Children are generally more sensitive to diarrheal infections. Hoover (1993) indicated that maintenance of high number of bifidobacteria prevented or minimized the enteric diarrheal infections in children. Moreover, administration of an indigestible oligosaccharide reduced the duration and number of recurrent episodes of diarrhea in children (Dohnalek et al., 1998). Thus, administration of GOS helps to restore the balance of microbiota and prevents the growth of harmful bacteria due to the selective stimulation of bifidobacteria.

### 2.2.1.2.5. Mineral Absorption

The absorption of calcium and magnesium takes place in both small and large intestines (Hardwick et al., 1991), and was stimulated by ingestion of NDOs. Chonan and Watanuki (1995) showed that the apparent absorption of calcium in rats was stimulated by feeding GOS. This effect was accompanied by a reduction in cecal pH and increases in cecal and cecal digesta weights. In the presence of NDOs in the cecum, cecal bacteria produce a large amount of SCFA and other organic acids such as lactic acid and succinic acid, then the solubility of calcium is improved. Yanahira et al (1997) found that there
were significant correlations between calcium and magnesium absorption ratios and total SCFA concentrations in the cecum. They also found that acetic acid concentration had the most stimulatory effect on the absorption of calcium.

Therefore, the stimulation of calcium absorption seems to be caused by the increased stability and solubility of calcium in the SCFA-rich acidic conditions (Chonan and Watanuki, 1995, 1996, Chonan et al., 1995) with a possible mechanism of chelating of minerals with carboxylic acids. In addition, GOS also recovered the absorption of magnesium and thus suppressed the calcification of the heart and the kidney under the conditions of high phosphorus and calcium dietary concentrations (Chonan et al., 1996). They speculated that the use of magnesium increased by feeding 4’GOS to a limited extent prevented the lower magnesium status and the severity of calcification of the kidney and heart caused by excess dietary phosphorous and calcium.

2.2.1.2.6. Summary of Health Benefits

Because human gut microflora can play a major role in host health, there is currently some interest in the manipulation of the composition of the flora towards a potentially beneficial community (Ziemer and Gibson, 1998). Prebiotics are nondigestible food ingredients that beneficially affect the host health by selectively stimulating the growth and/or activity of one or a limited number of bacterial species that already reside in the colon (Gibson and Roberfroid, 1995, Roberfroid, 1997). NDOs in general, including GOS, are prebiotics which can significantly modulate the colonic
microflora by increasing the number of specific bacteria and thus changing the composition of colon flora (Ito et al., 1993). Selective fermentation of GOS by bifidobacteria leads to an increase in the activity and/or the number of these bacteria and to the production of acetic acid and lactic acid as fermentation end products, resulting in a lower pH in the colon and providing a means to suppress the growth of harmful bacteria such as *Escherichia coli*, *Clostridium perfringens* and *Clostridium difficile*, and their metabolic by-products and toxins they produce (Sako et al., 1999). Furthermore, the modification leads to ease of constipation, reduction in the activities of harmful enzymes, better absorption of minerals (Yanahira et al., 1997), resistance to pathogenic colonization and invasion onto gut mucosa and prevention of diarrhea caused by these pathogens (Dohnalek et al., 1998).

Although the majority of the literature reviewed supports the view stated above, there have been several studies with conflicting results that failed to show the beneficial effects of NDOs, especially in young healthy subjects. For example, recently, Alles et al (1999) concluded that GOS, although completely fermented in the human colon, did not beneficially change the composition of the intestinal microflora, the amount of protein fermentation products in feces, or the profile of bile acids in fecal water. Also, Heuvel et al (1998) found that GOS and FOS have no effect on the absorption of minerals in healthy man subjects.
2.2.2. GOS Production

GOS are produced from lactose by the transgalactosylation activity of β-galactosidases. The linkage between the galactose units, the efficiency of transgalactosylation and the components in the final products depend on the enzymes and the conditions used in the reaction. A highly concentrated solution of lactose is used as a substrate solution for GOS production. The main products are trisaccharides, namely 4'- or 6'-galactosyllactose, and longer oligosaccharides consisting of 4 or more monosaccharide units. Although tri- to hexa-saccharides with 2-5 galactose units are the main products of the reaction, transgalactosylated disaccharides (TDs) consisting of galactose and glucose with different β-glycoside bonds from lactose and two galactose units are also produced depending on the type of enzyme used (Table 2.3). TDs are considered NDOs, since they have similar physiological characteristics to longer GOS (Matsumoto et al., 1990, Ishikawa et al., 1995).

2.2.3. Physicochemical Characteristics of GOS

In standardized large scale productions using the β-galactosidase derived from B. circulans, more than 55% of the lactose is converted to GOS (Ishikawa et al., 1995). Food ingredients are rarely needed to be pure substances. Purification of GOS product increases the price greatly. It is rather preferable to have a mixture since, as a functional food ingredient with a maximum 10 g suggested daily intake, GOS products from enzymatic conversion suffice the need. In fact, commercially available GOS are mixtures of several molecular species of oligosaccharides (55%), lactose (20%), glucose (20%),
and a small amount of galactose (Sako et al., 1999). Oligomate 55 is a GOS product on the Japanese market contains at least 55% 4β-GOS including TD in the solid materials. The relative sweetness of the product is about 35% that of sucrose. Oligomate 55 solutions are slightly more viscous than high fructose corn syrups (HFCS) and have similar osmotic pressure to sucrose solutions of the same concentrations (Sako et al., 1999). GOS are stable under the most food processing conditions. They remain unchanged after treatment at 160 °C for 10 min at neutral pH and after treatment at 120 °C for 10 min at pH 3. The stability of GOS is better than that of FOS that is sensitive to heat and acidic conditions (Voragen, 1998).

2.2.4. Market and Applications of GOS

Four companies, three located in Japan and one in the Netherlands, currently produce GOS. In 1995, approximately 15,000 tons of GOS were produced (Crittenden and Playne, 1996). In Japan, OS are already available through addition to over 450 foods. In the United States and North America, oligosaccharides also should have many marketable areas since Americans are demanding “natural” foods with beneficial health effects. In the last decade, consumers’ demand for healthy foods led to a dramatic increase in the development of NDO containing foods in the world since NDOs are recognized as important food ingredients to maintain and improve our health. Besides their prebiotic effects, low cariogenicity, low caloric values, and low sweetness were the most important characteristics of these oligosaccharides (Sako et al., 1999).
At the same time, NDOs generally have preferable physicochemical characteristics applicable to various processed foods. In particular, the stability of GOS in acidic and high-temperature conditions enables GOS to be applied without decomposition in a wider variety of foods. GOS are now used as sweeteners by themselves, and in fermented milk products, breads, jams, confectionery, beverages, etc. For example, bread is a suitable candidate for GOS inclusion. During fermentation with yeast and the baking of bread, GOS are not broken down, and render the bread excellent in taste and texture. Fermented dairy products are another good example of GOS inclusion.

Fermented milk products containing probiotics with added GOS are commercially available in Japan as well as in Europe. Baby foods and specialized foods for elderly and hospitalized people are promising fields of application of GOS in the next millennium, since these people are more susceptible to changes in their intestinal environment. A further possibility in microflora management procedures is the use of synbiotics, where probiotics and prebiotics can be used in combination. The live microbial additions may be used in conjunction with a specific substrate for growth, for example GOS with a *Bifidobacterium* strain. The result should be improved survival of the probiotic, which has a readily available (and specific) substrate for its fermentation, as well as the individual advantages that each should offer. The approach could have particular application in babies and the elderly who suffer most from the imbalance of microbiota in the gut.

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2.3. β-Galactosidase

β-Galactosidase or lactase (EC 3.2.1.23) is one of the first of the class of oligosaccharide-hydrolyzing enzymes isolated and purified from various organisms, including plants, fungi, yeasts, bacteria, and animal organs. β-Galactosidase from different sources varies considerably in many properties although the specificity of the enzyme remains essentially the same (Richmond et al., 1981). The enzyme lactase has long been used to hydrolyze lactose in the manufacture of some dairy products (Gekas and Lopez-Leiva, 1985, Axelsson and Zacchi, 1990, Bakken et al., 1990). The enzyme catalyzes the hydrolysis of β-galactosidic linkages such as those found in lactose (Prenosil et al., 1987a, b, Zarate and Lopez-Leiva, 1990, Yang, and Tang, 1988).

2.3.1. Reaction Mechanism

The biological and natural substrate for β-galactosidase is lactose. Lactose, however, is not the only substrate, and not always the best. In general, the mechanism of enzymic lactose hydrolysis is of transgalactosyllic nature. As shown in Figure 2.1, the active site of lactase has been proposed to be an imidazole and a sulphhydryl group. The sulphhydryl group acts as a general acid to protonate the galactosidic oxygen atom and the imidazole group acts as a nucleophile that attacks the nucleophilic center at the first carbon of the galactose molecule. The reaction corresponds to a S_N2-like replacement mechanism (Richmond et al., 1981). Huber et al (1976) postulated that lactose is first taken from the galactose side and the glucose molecule vacates the active site of the enzyme leaving galactosyl group on the enzyme. The enzyme transfers the galactose...
moiety of a β-galactoside to an acceptor containing a hydroxyl group. When this acceptor is water, galactose is formed and released from the active site. In the presence of other sugars such as lactose acting as an acceptor for the galactose, oligosaccharides or new sugars are formed (Prenosil et al., 1987a,b).

Up to twenty different GOS have been found, primarily trisaccharides and disaccharides, but tetrasaccharides, pentasaccharides and even larger ones have also been reported (Mahoney, 1998, Zarate and Lopez-Leiva, 1990). The reaction kinetics has been well characterized for lactase from various sources. The enzymatic hydrolysis of lactose follows the Michaelis-Menten kinetics with competitive inhibition by galactose (Yang and Okos, 1989). A reversible reaction kinetic model and a more complex model involving the mutarotation of galactose, which results in a stronger competitive inhibitor, α-galactose, have also been proposed (Flaschel et al., 1982). It was reported that anomers of galactose have separate rate of inhibition reactions (Bakken et al., 1991). That is, they have different degree of inhibition of the enzyme reaction. Other models that also describe the transgalactosylation reactions also exist (Bakken et al., 1992). An extended Michaelis-Menten model with trisaccharide intermediates has also been used to model the reactions (Prenosil et al., 1987b). A general reaction scheme is shown in Table 2.3.
2.3.2. Factors Affecting GOS Production

2.3.2.1. Enzyme Source

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: pH optima, temperature optima, Km for lactose and type and distribution of GOS (Table 2.5). Concentration and structures of GOS produced are dependent on the source of the enzyme. Regardless of the enzyme source, the maximum GOS content (or process yield) 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. Some OS, especially disaccharides, are formed from intramolecular transfer reactions. For example, Shin et al (1998) reported that of total 55% GOS formed with the enzyme from Bullera singularis were 18% disaccharides and 37% trisaccharides.

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°C (Berger et al., 1995a, b) and 85°C (Onishi and Tanaka, 1998, Onishi et al., 1995), respectively. Concerning the potential for industrial use, β-galactosidases from most sources are not commercially available or approved for use in food. Although thermostable enzymes are desirable, their advantages in sugar conversions are of limited value due to browning of the product at high temperatures.
Glycoside bonds between two galactose units are mainly β, 1-4 bonds (4'-GOS) when β-galactosidases derived from Bacillus circulans (Mozaffar et al., 1984) or Cryptococcus laurentii (Ozawa et al., 1989) are used, and β, 1-6 bonds (6'-GOS) when enzymes derived from A. oryzae or Streptococcus thermophilus (Matsumoto, 1990) are used. 6’GOS (6'-galactosyllactose), one of the oligosaccharides found in human milk, constitutes about 70% of total GOS formed by β-galactosidase from A. oryzae (Deya, 1990), which makes the enzyme more advantageous.

In general, some bacterial (Shin and Yang, 1998, Berger et al., 1995a) and yeast enzymes (Shukla, 1975, Foda and Lopez-Leiva, 2000) maximize GOS at higher conversion of lactose (70-80 %), thus achieving higher processing yields of GOS (above 50%). However, due to the increase in the inhibitory monosaccharides as lactose hydrolysis increases, the reaction of conversion of lactose to any product is greatly reduced. This means that with those enzymes, higher GOS yields are achieved at the expense of lower productivities of GOS, which is between 2.2 and 6.6 g GOS/ L/ h (Shin et al., 1998).

When it comes to economical analysis of the GOS production, however, the productivity is far more important than the yield because lactose is an inexpensive by-product. In addition, considering a very low suggested daily intake of GOS (max 10 g), pure or highly concentrated GOS products may not be so desirable as a food ingredient.
In terms of productivities of GOS, the enzyme from *A. oryzae* is far more superior (5 to 10 times greater) to some of the bacterial or yeast enzymes (Iwasaki et al., 1996, Shin et al., 1998). Rather low yield of max GOS as 25-35% (Matsumoto et al., 1989) with β-galactosidase from *A. oryzae* is compensated with very high GOS productivity. In addition, as can be seen from Table 2.5, GOS composition is mostly trisaccharides and higher unit sugars, which is desirable since they last longer in the large intestine. *A. oryzae* has acidic pH optima (pH 3.5-4.5) and relatively high temperature optima and stability, both of which are desired in industry since a typical substrate for an industrial production process would be concentrated whey. The operational stability of immobilized enzyme from *A. oryzae* was found to be 5- to 14-fold higher compared to that of *K. fragilis* (Kminkova et al., 1988). In addition, this enzyme is already used in food products, commercially available, and relatively inexpensive as compared to β-galactosidase from alternative sources.

2.3.2.2. Lactose Concentration

Initial lactose concentration is by far the most significant factor that affects GOS formation. Iwasaki et al (1996) indicated that β-galactosyl group has a higher probability of attaching to lactose than water as an acceptor at increasing lactose concentrations. In the complicated enzymic reaction of lactose, it was generally observed that the hydrolysis and transferase reactions occur simultaneously. What dominates the product profile of the reaction largely depends on the lactose concentration. At low lactose concentrations hydrolysis reaction products dominate, while at high lactose concentrations GOS
formation dominates. Iwasaki et al (1996) modeled the GOS formation kinetics and found that the enzymic reaction process is strongly influenced by the viscosity of the lactose solution. They found that maximum GOS conversion indicated as molar ratio of GOS to lactose reacted was 0.31 and 0.32 at 380 and 571 g/L lactose concentrations with the calculated viscosities being 2.12 mPa and 6.6 mPa, respectively. Thus, increase in GOS production becomes limited by the viscosity of lactose concentration beyond approximately 400 g/L.

Compared to lactose concentration and lactose conversion, other process parameters, such as enzyme concentration, pH and temperature, have minimal effects on GOS production (Monsan and Paul, 1995, Iwasaki et al., 1996), although they affect the reaction rates. For example, Iwasaki et al (1996) found that increasing temperature from 30 to 60 °C resulted in slightly increase in the conversion of lactose but decrease rapidly over 60°C due to the inactivation of enzyme. With increasing temperatures, the molar ratio of oligosaccharides produced to lactose converted decreased at low lactose concentrations but it was almost constant at high lactose concentrations. They found that the effects of pH and temperature on GOS formation were relatively small at high lactose concentrations.

2.3.3. Chemical Characteristics of β-Galactosidase from Aspergillus oryzae

β-Galactosidase, an extracellular enzyme from A. oryzae, has acidic pH optima of 4.0-5.0, and thermophilic temperature optima of 45-65 °C (Park et al., 1979, Gargova et
al., 1995, Ozbas and Kutsal, 1990, Kminkova et al., 1988). Like other microorganisms (Mozaffar et al., 1984), *A. oryzae* also produces multiple β-galactosidases with different characteristics (Ogushi et al., 1980), which may explain the different results reported in the literature. The enzyme also bears quite high stability against wide range of pH, where catalytic activity was unaffected between pH 4.0 and 8.0 at room temperature for overnight incubation (Park et al., 1979, Watanabe et al., 1979, Ogushi et al., 1980, Gargova et al., 1995).

The enzyme contains a relatively high portion of acidic amino acids with 10.8 % aspartate and 9.2% glutamate. Isoelectric point of the enzyme is 4.6 (Ogushi et al., 1980). It was indicated that there were 12 amino groups exposed on the surface of the enzyme (Naoi et al., 1984). The molecular weight is approx. 100-110 kDa (Liao and Horwath, 1990, Sugiura et al., 1979). Presence of carbohydrates on the enzyme was reported (Ogushi et al., 1980, Nakao et al., 1987), constituting 10-15% of its molecular weight. Nakao et al (1987) identified the carbohydrate chains as short and long chain oligosaccharides, which consisted of mannose, galactose, glucose, and glucosamine. It was also indicated that 1.3-3% of the total carbohydrate chains contained phosphate in mono- and diester linkages. Yang et al (1994a) demonstrated that the enzyme was quite sensitive to aggregation in response to ionic strength of the buffer, in which low ionic strength led to salting out, while high ionic strength led to salting in.
2.3.4. Immobilization of β-Galactosidase

β-Galactosidases from different organisms have been immobilized on a variety of supports using various techniques. Although 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 because until recently GOS were considered undesirable by-product during hydrolysis of lactose (Prenosil et al., 1987a).

It is important to note that what is favored for the hydrolysis of lactose is quite different from that for GOS production. For example, Portaccio et al (1998) immobilized β-galactosidase from A. oryzae on chitosan beads and a nylon membrane (Immunodyne) to study the effect of the nature of the support on catalytic activity and inhibition by galactose. They found a higher inhibitor constant for the β-galactosidase/chitosan system was than that for the β-galactosidase/Immunodyne (nylon). It was suggested that the former system is more appropriate to perform lactose hydrolysis.

Only a few authors have looked at β-galactosidase immobilization specifically for creating GOS as opposed to lactose hydrolysis. The supports as well as techniques and their effects on enzymes are reviewed below. Among techniques and supports used, particulate carriers, such as resins and beads, and glutaraldehyde fixation have been commonly used. Mozaffar et al (1986a, b) used Duolite ES-762 (phenol formaldehyde resin) and Merckogel (controlled-pore silica gel) for β-Galactosidase-II from Bacillus circulans. β-Galactosidase from A. oryzae has been immobilized to an ion-exchange resin

These methods are in general simple and easy to carry out. However, their industrial applications would be quite difficult (Ichijo et al., 1985). First, many of the carriers used for immobilization of β-galactosidase are developed for chromatographic purposes (Peng et al., 1987), which are highly engineered and reactive groups were introduced previously. Also, commonly used porous macroparticles such as cross-linked dextran or agarose beads have quite high surface areas, which allows space for enzyme immobilization and have higher coupling yields (Peng et al., 1987). However, these resins and beads are highly expensive considering industrial scale support needs (Howlett et al., 1991). 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 (Ichijo et al., 1985, 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). It is indicated that self-compression of immobilized enzyme causing a clogging problem is hard to avoid in bead forms (Peng et al., 1987). Therefore, flow rate has to be slowed
down to reduce the pressure and the rate of the enzymatic reaction becomes limited by diffusion. Thus, advantages of high load of enzyme could not be fully realized due to operational instability. For these reasons, these particulates or bead-immobilized systems are difficult to scale up for industrial size reactors (Ichijo et al., 1985).

2.4. Enzyme Immobilization

Immobilization of enzyme can be defined as restriction of enzyme's mobility in a fixed place (Shuler and Kargi, 1992). Since enzymes only catalyze the reaction, they can be reused as long as they are not denatured. The presence of the free enzyme contaminates the final product. Otherwise, the recovery of free enzyme from reaction mixture for reuse is not feasible due to its difficulty and price (Bernath and Venkatasubramanian, 1986). Enzyme immobilization allows a continuous process, easier downstream processing of the products, and a greater control over the reaction. In general, immobilized enzymes are more stable than free enzyme against denaturation conditions, such as working at high temperature or using organic solvents (Nilsson and Mosbach, 1984a). In general, the enzyme is the most expensive components of a given enzymatic process. Thus, using the enzyme only once affords a relatively expensive process and product. It was found that enzyme immobilization is economically feasible despite a very high cost for the enzyme attachment. Among reactors, the plug-flow tubular reactor gave the lowest cost ($0.48 \text{ Kr/kg lactose}$) evaluated for the hydrolysis of lactose (Axelsson and Zacchi, 1990, Daniels, 1987).
2.4.1. Methods for Enzyme Immobilization

Immobilization of enzymes can be prepared in almost an unlimited range of compositions and morphologies. Various classifications based on carrier or support and binding technique have been made. The method for enzyme immobilization can be classified into two major categories as bound and entrapped. Adsorption (ionic, hydrophobic, etc.) and covalent binding of enzymes (between support and enzyme) on water-insoluble carriers are the major types of the bound category. Cross-linking involving a covalent bond formation between enzymes and/or support with a bifunctional reagent can be considered as a sub-category within covalent bindings. Matrix and membrane entrapment are main groups of entrapment of enzymes, which is physical enclosure rather than binding (Swaisgood and Horton, 1989).

Physical adsorption of an enzyme onto a solid is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid. A major advantage of adsorption as a general method of insolubilizing enzymes is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap, easily carried out, and tends to be less disruptive to the enzymic protein than chemical means of attachment, the binding being mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. In this respect, the adsorption method bears the greatest similarity to the situation found in biological membranes in vivo and has been used to model such systems.
The most intensely studied of the insolubilization techniques is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be insolubilized, the choice is limited by the fact that the binding reaction must be performed under conditions that do not cause loss of enzymic activity, and the active site of the enzyme must be unaffected by the reagents used. Since the enzyme is covalently linked to the support matrix, very little desorption is likely using this method.

2.4.2. Factors Affecting Enzyme Immobilization

Immobilization of enzymes has been considered as an art since many researchers tailored the method they used for success. The ideal enzyme immobilization method would (1) employ mild chemical conditions; (2) allow for large quantities of enzyme to be immobilized; (3) provide a large surface area for enzyme/substrate contact within a small total volume; (4) minimize barriers to mass transport of substrate and product, and (5) provide a chemically and mechanically robust system (Swaisgood and Horton, 1989). Although there is no priori requirements per se, the combination of these characteristics are considered important for the quality of performance of the enzyme in desired applications.

The efficacy of immobilization is generally expressed by three factors: protein binding, activity retention, and immobilization yield (Confort et al., 1989). Protein binding relates to the amount of protein bound out of the protein exposed to per amount,
volume or surface area of the support. Activity retention is the percent of the bound protein present in the active form. The yield of immobilization is the product of the protein binding and the activity retention, and is equivalent to the ratio of enzyme activity present to enzyme activity exposed.

Several variables need to be optimized for successful immobilization or coupling of an enzyme. The concentration of enzyme in the immobilization solution may affect the protein binding, activity retention, and the yield of immobilization. In general, as the enzyme concentration increases, the protein binding also increases but the activity of the bound enzyme usually does not follow that increment. Activity retention sometimes levels off or sometimes decreases due to the blockage of substrate diffusion. The pH and ionic strength of the enzyme solution also affects the efficiency of immobilization. Coupling pH is dictated by the necessary conditions as it relates to the charge distribution for the immobilization to take place and by the optimal pH range for enzyme stability (Confort et al., 1988). In some cases, presence of a substrate of the enzyme or a competitive inhibitor may have a protective function during immobilization, which presumably contributes towards retention of tertiary structure of the enzyme.

2.4.3. Fibrous Materials for Enzyme Immobilization

Fibrous matrices have been of interest in biocatalyst applications. Among fibrous matrices, polyethylene terephthalate fibers (Elcin and Sacak, 1996), dimethylated superfine fibers (Ichijo et al., 1985, 1990a, b), cotton fabric (Sharma and Yamazaki,
1984, Maeshima et al., 1997), nylon fiber (Seng et al., 1980), nonwoven nylon fabric (Shemer et al., 1979), silk fibers (Grasset et al., 1979), silk fabrics (Demura et al., 1992, Furuhata et al., 1996, 1997), porous glass fiber (Toldra et al., 1986), etc., have been studied for immobilization of various enzymes. Good flow rates, limited pressure drop and negligible mass-transfer resistance were commonly observed in studies. These desirable characteristics are attributed to relatively open structures and high mechanical strength of the fibrous matrices (Sharma and Yamazaki, 1984, Ichijo et al., 1990b).

However, some problems associated with these supports were also reported. The most commonly indicated difficulties appear to be related to low enzyme activity or low immobilization efficiency. Regardless of the type of fibrous matrices, the immobilization technique used generally makes a significant difference. Glutaraldehyde fixations were usually applied for these materials regardless of suitability of this nonspecific cross-linking agent. Toldra et al (1986) obtained 1.5 mg protein/m² surface area for α-glucoamylase with porous glass fiber by immobilizing using silane glutaraldehyde coupling. Invertase and alkaline phosphatase were immobilized through ionic bonding on silk fabrics grafted with polyethylenimine, with 55 and 10 mg enzyme protein per g support, respectively, where, however, only 3 and 5% of the expected activities were obtained (Furuhata et al., 1996, 1997). Therefore, it is desirable that better methods of immobilization need to be developed for porous fibrous matrices.
2.4.4. Choice of Enzyme Reactor

Although many reactor designs and their potential advantages have been reported, the fixed-bed reactor is generally the engineers' choice for commercial and industrial applications because of its efficiency, ease of operation and general simplicity (Shin et al., 1998). Among many forms of fixed-bed reactors, the most common is a packed-bed of particular material to which enzyme is immobilized. In a packed bed, substrate can flow downward or upward or it could be recycled continuously, which is advantageous when the linear velocity of the substrate solution affects the reaction rate. The recycling method allows the substrate solution to be passed through the column at the desired velocity. For industrial applications, upward flow is generally preferred. This is because downward flow causes the compression of the beds of enzyme columns.

Packed beds provide simple and ease of operation but problems such as diffusion resistance arise due to granular shape supports used (Peng et al., 1987, Ichijo et al., 1985). In these systems, it is also necessary to consider the effect of the column dimensions on the reaction rate. The productivity of the reactor increases by reducing the width of the channel because linear velocity increases with the reduction of the width and that contributes to the decrease in mass transfer resistance (Ichijo et al., 1985).

2.4.5. Characteristics of Cotton Fiber

Cellulose is the most abundant compound in nature, regarded as "natural, environmentally friendly and a renewable resource", which explains that it has distinct
advantages over synthetics. Cotton cellulose differs from wood cellulose primarily by having a higher degree of polymerization and a higher degree of crystallinity. Crystallinity indicates that the fiber molecules are closely packed and parallel to one another. The cellulose chains within the cotton fibers tend to be held in place by hydrogen bonding. These hydrogen bonds occur between the hydroxyl groups of adjacent molecules and are more prevalent between the parallel, closely packed molecules in the crystalline areas of the fiber.

Cellulose is a macromolecule made up of anhydroglucose units united by 1-4, oxygen bridges. The anhydroglucose units are linked together as β-cellobiose; therefore, anhydro-β-cellobiose is the repeating unit of the polymer chain. The degree of polymerization, the number of repeating cellobiose units, of cotton fiber is 9000 - 15,000 (Sturgeon, 1988). Only one primary and two secondary hydroxyl groups in each repeating cellobiose unit of cellulose are chemically reactive groups that can undergo substitution reactions in procedures designed to modify the cellulose fibers. The hydroxyl groups also serve as principal sorption sites for water molecules and make the support hydrophilic. The strength of cotton generally increases with increased moisture, which is explained in terms of intermolecular hydrogen bonding between cellulose chains and their degree of crystallinity. Average crystallinity measured by x-ray diffraction for cellulosic fibers of cotton is about 73% (Sarko, 1987).

The cotton fiber when observed in its entirety is a flat twisted ribbon that has 50-100 convolutions per inch. Throughout the fiber structure, there are variously sized pores
or capillary spaces between the variously sized fibrils. Consequently, the cotton fiber can be viewed as a physical microscopic sponge with a complex porous structure. This internal structure makes cotton fibers accessible to liquids and vapors. The capillary action of the fibrils in the fiber pulls in the liquids by capillary action where it is held in pores between the fibrils. This accounts for cotton's wickability and its unique absorbing capacity (Sturgeon, 1988).

2.4.5.1. Cotton Cloth as Fibrous Matrix

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 due to compaction and clogging by fine units (Sharma and Yamazaki, 1984). Margel and Sturchak (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 and Yamazaki, 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 (microbiological) 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. Hydrophilicity of cotton fabric is advantageous in that it not only prevents unintended adsorption of protein during covalent immobilization (Elsner et al., 1999), but also provides an environment in which the enzyme is more stable against denaturation, which is generally associated with hydrophobic support.

Km increase along with diffusion limitations is inevitable in many immobilization procedures, especially more with gel entrapment than cross-linking techniques. It was reported that enzyme immobilization on cloth would be particularly suited for transformation of large-size substrates since the enzyme is exposed on the surface of the support (Sharma and Yamazaki, 1984). Sharma and Yamazaki (1984) showed that cotton cloth adsorbed as much protein as agarose did. Although this was hydrophobic adsorption, ionic binding of enzymes into cotton cloth was shown to be as effective as hydrophobic binding (Howlett et al., 1991). These findings indicate that the surface area of cotton cloth is as good as agarose.

2.4.5.2. Cellulose Activation

The hydroxyl groups of cellulose are its only functional groups, yet they are not reactive to bind another molecule covalently. Moreover, not many of those hydroxyl groups are readily available for further reactions. Therefore, cellulosic materials are
generally pretreated to expose more hydroxyl groups or to disrupt crystallinity in the cellulose structure. The most common treatment is an inorganic alkali application, which is referred to as mercerization. A concentrated alkali solution, such as sodium hydroxide or carbonate, is employed to induce swelling in the cellulose fiber and increase the availability of hydroxyl groups for the activation reaction. It has been observed by many researchers that the yield of the substitution reactions of cellulose increased dramatically when cellulose was pretreated with sodium hydroxide solution (Confort et al., 1989).

2.4.5.2.1. Cellulose Activating Reagents

The critical issues in the design of an immobilized enzyme system are the support material and the immobilization chemistry. Hydroxyl groups in general can be activated with cyanogen bromide, tosyl chloride, cyanuric chloride or oxidation reagents such as chromium trioxide, sodium periodate and dimethylsulfoxide-carbodiimide before the enzymes can be immobilized. The inert hydroxyl groups are thus converted into more active cyanate ester, tosylate, reactive acyl-like chlorines, and carbonyl groups, respectively, which readily react with nucleophilic groups of proteins such as amino and sulphhydryl (-SH) groups (Nilsson and Mosbach, 1981).

It is obvious that every method has advantages and disadvantages, and there is no single method working for every type of support or enzyme. For instance, cyanogen bromide (CNBr) method has been commonly used for the activation of polysaccharides containing hydroxyl groups. The reaction is rapid and requires titration at a narrow range
of pH with intensive stirring for uniform activation. The cellulose activated with CNBr forms cyclic imidocarbonate due to the vicinity of hydroxyl groups as opposed to cyanate esters with agarose. Although cyanate ester is very reactive, cyclic imidocarbonate is not so reactive. Thus, cellulose, in comparison with agarose, requires a more alkaline enzyme solution for coupling (Melius and Wang, 1974). The optimum coupling pH is near 9.0 - 10.5 range, which is not suited for many enzymes including β-galactosidase. Even then, due to reversible characteristic of the linkage with cellulose, significant leakages of enzyme from the support have been observed (Confort et al., 1989, Peng et al., 1986). Thus, coupling may have a lower efficiency and takes a longer time at lower pH values. Confort et al (1988) compared tresyl chloride, oxirane and CNBr for activation of agarose, and found that CNBr produced the least stable bond causing enzyme leakage. Furthermore, CNBr is toxic so it not only requires special care during activation but also creates concerns for possible presence of cyanate residues in the production of food ingredients.

Periodate (NaIO₄) oxidation of cellulose results in dialdehyde cellulose due to vicinal hydroxyl groups of the parent polymers. The support containing aldehyde groups can be used for immobilization of proteins. Upon coupling of amino ligands, e.g. proteins, Schiff base products are formed between amino group of the ligand and the aldehyde group of the support (Margel and Sturchak, 1999). The Schiff base products are unstable in aqueous solution since they are in equilibrium (reversible) with the interacting reagents. Instability of the Schiff base bonds in the aqueous solution results in leakage of
the bound protein. But they may be stabilized by reduction of the Schiff base bonds with an appropriate reducing agent, e.g. NaBH$_4$ or NaCNBH$_3$ (Margel and Sturchak, 1999).

2.4.5.2.1.1. Activation of Cellulose with Organic Sulfonyl Chlorides

Among sulfonyl chlorides, tosyl chloride (4-toluenesulfonyl chloride) and tresyl chlorides (2,2,2-trifluoroethanesulfonyl chloride) are commonly used for activation of hydroxyl group containing compounds such as dextran (Eisner et al., 1999), agarose (Confort et al., 1988, Nilsson and Mosbach, 1984b), cellulose (Confort et al., 1989), and synthetic polymers (Nilsson and Mosbach, 1981). These can be used to convert hydroxyl groups into sulfonates that are good leaving groups. On reaction with nucleophiles, a stable linkage is formed between the nucleophile and the initial hydroxyl group carrying carbon (Sturgeon, 1988). The most reactive nucleophiles to displace with sulfonate esters are thiol groups and then primary amino groups. Imidazole and tryrosyl groups also act as attacking nucleophiles for the esters. In addition to proteins any compound possessing these groups can be introduced to the support for subsequent indirect coupling reactions.

The activation reaction with sulfonyl chlorides is carried out in organic solvents containing a desired concentration of activating reagent. Aprotic organic solvents, such as acetone or dioxane, that do not contain hydroxyl groups on the structure are usually employed. Since water can act as a nucleophile, activation has to be carried out in anhydrous conditions. Thus, solvents as well as support materials need to be dried before the reaction. During the reaction, HCl is released into the medium, which may cause
hydrolysis of the support, especially under anhydrous conditions. Thus, in order to neutralize the liberated HCl the reaction medium often contains an organic base, such as pyridine, triethylamine, etc. (Margel and Sturchak, 1999).

Sulfonated or sulfonate activated supports are very stable under aqueous acidic conditions (Sturgeon, 1988). At high pHs, sulfonates become less stable since the hydroxyl groups of water can act as nucleophiles at alkaline pHs. Several types of sulfonyl chlorides are available, although most commonly used ones are tosyl chloride (p-toluenesulfonyl chloride) and tresyl chlorides (2,2,2-trifluoroethanesulfonyl chloride). Depending on the side chain or R-group, they vary a lot not only in their reactivity, solubility and reaction conditions, but also in their prices. Reactivity refers to the rate and extent of formation of sulfonates with hydroxyl groups and ease of replacement by nucleophiles in terms of behaving as a leaving agent. Tosyl chloride has much lower reactivity than tresyl chloride, which is about 100 times more reactive than tosyl chloride to form sulfonates. Tosyl chloride is one of the least expensive reagents. It is several thousand times cheaper than tresyl chloride. Tosyl activation level can be followed by UV spectroscopy, which is not possible with tresyl chloride (Nilsson and Mosbach, 1981, Sturgeon, 1988)

Activation with sulfonyl chlorides proceeds in a concentration-dependent manner in which the higher the concentration used, the more activation levels are achieved, which in turn provides a readily predictable and reproducible extents of activation. This linear activation is difficult to achieve with the cyanogen bromide method. Sulfonyl
chlorides form very stable linkage between the enzyme and the cellulose support in that the amino nucleophile replaces the hydroxyl group of cellulose. Thus a carbon-nitrogen amide (peptide) linkage is formed (Confort et al., 1989, 1988). The stability of the bond formed prevents any significant leakage of bound protein. The structure of the cellulose backbone does not change during the sulfonyl chloride method of immobilization. Thereby, its basic chemical and physical properties, i.e. solubility, mechanical properties, non-biodegradability, etc, remain almost unchanged.

2.4.6. Polyethyleneimine

Polyethyleneimine [PEI; (C\textsubscript{2}H\textsubscript{5}N)\textsubscript{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 and Venkatasubramanian, 1986). The two forms of PEI known are the linear crystalline type and the more important amorphous branched structure with a distribution of primary, secondary, and tertiary amino groups in the ratio 1:2:1 while branching at every 3 to 3.5 nitrogen atoms (Zemek et al., 1982). 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. Thus, PEI has been used as a protein precipitant (Gestrelius, 1980, Dissing and Mattiasson, 1998) and as a nucleic acid precipitant (Atkinson and Jack, 1973, Oehler and Clark, 1996, Dissing and Mattiasson, 1996).
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 (Sundaram, 1977) coupled to support. In order to covalently couple PEI to support, glutaraldehyde (Isgrove et al., 2001) and carbodiimide (Tang et al., 1998) were employed. Further activation was carried out with plasma (Zhu et al., 2000, Chuang et al., 1997), glutaraldehyde (Isgrove et al., 2001) and thiophosgene (Zemek et al., 1982). When adsorbed, PEI is generally crosslinked with a bi- or multi-functional crosslinking agent, usually glutaraldehyde (Isgrove et al., 2001, Bardeletti, 1997, Sundaram, 1977) or epichlorohydrin (Zemek et al., 1982), to prevent the removal of PEI from the support.

Recently PEI has been used in interesting enzyme immobilization procedures. In order to build effective enzyme electrode, the ‘layer-by-layer’ method has received a lot of attention (Caruso et al., 2000, Chuang et al., 1997, Onda et al., 1996, 1999, Mateo et al., 2000). This procedure involves using PEI for the formation of films, or covering a metal electrode in a stepwise fashion to introduce several enzymes such as glucose oxidase and peroxidase (Chuang et al., 1997, Onda et al., 1999). An excellent and very fast reaction response was obtained with the enzyme electrodes made with PEI-immobilized enzymes.

PEI has also been an essential ingredient of many enzyme immobilization procedures where PEI serves as a complexing agent with another negatively charged polymer to form nanomicron size particles. These negatively charged polymers include alginate (Jiang et al., 2000, Cao et al., 1996), polyacrylic acid (Dissing and Mattiasson,
1996, Yang et al., 1994b, Pommersheim et al., 1994) and poly(carbamoyl)sulfonate (Patel et al., 2000). The polyion complexes formed are used for enzyme immobilization. Of course, these particles are activated for further functionalization with desired groups for final enzyme immobilizations. In these applications, almost no function for electrostatic attraction of enzyme is intended. PEI was also used to introduce or enrich amine groups of support, including silk fabric (Furuhata et al., 1996) and Nylon (Isgrove et al., 2001).

Since PEI forms ionic complexes with macromolecules containing acidic domains (nucleic acids and some proteins), the interaction behavior is affected by salt concentration, pH, and the concentration of precipitable components (Jendrisak, 1987). In general, stabilization of enzyme was reported in these entrapped enzymes coated with PEI (Lee et al., 1993). In order to enhance effective complex formation with PEI, a polyaspartic acid tails were fused to the glucoamylase enzyme gene (Suominen et al., 1993) and β-galactosidase (Parker et al., 1990, Zhao et al., 1990).

The Food and 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. Glutaraldehyde although now known to be somewhat toxic, provided appropriate precautions are taken, is approved for use in food manufacturing operations (Isgrove et al., 2001).
<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Structure&lt;sup&gt;(a)&lt;/sup&gt;</th>
<th>Type of Bond</th>
<th>Est. Production 1995 (ton)</th>
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<td>Lactulose</td>
<td>Ga-Fr</td>
<td>β  -1,4</td>
<td>20 000</td>
</tr>
<tr>
<td>Lactosucrose</td>
<td>Ga-Gu-Fr</td>
<td>β  -1,4</td>
<td>1600</td>
</tr>
<tr>
<td>Rafinose</td>
<td>Ga-Gu-Fr</td>
<td>α  -1,6</td>
<td>200</td>
</tr>
<tr>
<td>Galacto-oligosaccharides</td>
<td>(Ga)&lt;sub&gt;n&lt;/sub&gt;-Gu</td>
<td>β  -1,4, 1-6</td>
<td>15 000</td>
</tr>
<tr>
<td>Fructo-oligosaccharides</td>
<td>(Fr)&lt;sub&gt;n&lt;/sub&gt;-Gu</td>
<td>β  -1,2</td>
<td>12 000</td>
</tr>
<tr>
<td>Soybean oligosaccharides</td>
<td>(Ga)&lt;sub&gt;n&lt;/sub&gt;-Gu-Fr</td>
<td>α  -1,6</td>
<td>2000</td>
</tr>
<tr>
<td>Isomalto-oligosaccharides</td>
<td>(Gu)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>α  -1,6</td>
<td>11 000</td>
</tr>
<tr>
<td>Xylo-oligosaccharides</td>
<td>(Xy)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>β  -1,4</td>
<td>300</td>
</tr>
<tr>
<td>Palatinose-oligosaccharides</td>
<td>(Gu-Fr)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>α  -1,6</td>
<td>5000</td>
</tr>
<tr>
<td>Glycosylsucrose</td>
<td>(Gu)&lt;sub&gt;n&lt;/sub&gt;-Fr</td>
<td>α  -1,4</td>
<td>4000</td>
</tr>
<tr>
<td>Malto-oligosaccharides</td>
<td>(Gu)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>α  -1,4</td>
<td>10 000</td>
</tr>
<tr>
<td>Cyclodextrins</td>
<td>(Gu)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>α  -1,4 cyclic</td>
<td>4000</td>
</tr>
<tr>
<td>Gentio-oligosaccharides</td>
<td>(Gu)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>β  -1,6</td>
<td>400</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>85 300</strong></td>
</tr>
</tbody>
</table>

<sup>(a)</sup> Ga: galactose; Gu: glucose; Fr: fructose; Xy: xylose.

Table 2.1. List of non-digestible oligosaccharides with their chemical characteristics and estimated production in 1995 (from Sako et al., 1999).
<table>
<thead>
<tr>
<th>Intestinal Bacteria</th>
<th>Strains</th>
<th>Glucose</th>
<th>Lactose</th>
<th>Lactulose</th>
<th>Rafinose</th>
<th>4'-GOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium adolescentis</td>
<td>7</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>6</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Bifidobacterium breve</td>
<td>3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Bifidobacterium infantis</td>
<td>2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Bifidobacterium longum</td>
<td>8</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>2</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>2</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactobacillus gasseri</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactobacillus salivarius</td>
<td>2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bacteroides distasonis</td>
<td>5</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>2</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Bacteroides ovatus</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bacteroides vulgatus</td>
<td>8</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mitsuokella multiacidus</td>
<td>3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rikenella microfusus</td>
<td>1</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Megamonas hypermegas</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Clostridium butyricum</td>
<td>1</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>1</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium innocuum</td>
<td>1</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium ramosum</td>
<td>2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Eubacterium aerofaciens</td>
<td>6</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eubacterium limosum</td>
<td>2</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peptostreptococcus prevotii</td>
<td>1</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Peptostreptococcus productus</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Propionibacterium acnes</td>
<td>1</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fusobacterium varium</td>
<td>1</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Veillonella alcaescens ss. dispar</td>
<td>1</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Megaphaera elsdenii</td>
<td>1</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus fecalis ss. Fecalis</td>
<td>3</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>2</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>6</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Indication for the growth of bacteria: +++; same as that on glucose, +: less than that on glucose, +: slight growth, -: no growth (from Ohtsuka et al., 1989).

Table 2.2. Utilization of 4'-galactosyl lactose (4'GOS) by various intestinal bacteria.
<table>
<thead>
<tr>
<th>Reactions</th>
<th>Symbols</th>
</tr>
</thead>
<tbody>
<tr>
<td>L + E ↔ (E-L) → (E-Gal) + Glu</td>
<td>L: lactose</td>
</tr>
<tr>
<td>(E-Gal) + H₂O ↔ E + Gal</td>
<td>E: β-galactosidase</td>
</tr>
<tr>
<td>(E-Gal) + L ↔ (E-3OS) ↔ E + 3OS</td>
<td>Gal: Galactose</td>
</tr>
<tr>
<td>(E-Gal) + 3OS ↔ (E-4OS) ↔ E + 4OS</td>
<td>Glu: Glucose</td>
</tr>
<tr>
<td>(E-Gal) + 4OS ↔ (E-5OS) ↔ E +5OS</td>
<td>3OS: trisaccharide</td>
</tr>
<tr>
<td>Gal + (E-Gal) ↔ (E-GB) ↔ E + GB</td>
<td>4OS: tetrasaccharide</td>
</tr>
<tr>
<td>Glu + (E-Gal) ↔ (E-AL) ↔ E + AL</td>
<td>5OS: pentasaccharide</td>
</tr>
<tr>
<td>(E-X): Enzyme-X complex</td>
<td>GB: galactobiose</td>
</tr>
<tr>
<td></td>
<td>AL: allolactose</td>
</tr>
</tbody>
</table>

Table 2. 3. Outline of GOS formation reactions of β-galactosidase.
**Reaction Conditions**

<table>
<thead>
<tr>
<th>Enzyme Sources</th>
<th>Initial Lactose Conc. (g/L)</th>
<th>T (°C)</th>
<th>pH</th>
<th>Max. GOS (w %)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>200</td>
<td>45</td>
<td>4.5</td>
<td>18.9</td>
<td>Kim et al., 1990</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>400</td>
<td>40</td>
<td>4.5</td>
<td>32</td>
<td>Iwasaki et al., 1996</td>
</tr>
<tr>
<td>Bacillus circulans</td>
<td>456</td>
<td>40</td>
<td>6</td>
<td>40</td>
<td>Mozaffar et al., 1986a</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>200</td>
<td>10</td>
<td>7</td>
<td>18</td>
<td>Rahim and Lee, 1991</td>
</tr>
<tr>
<td>Bullera singularis</td>
<td>100</td>
<td>45</td>
<td>4.5</td>
<td>55(*)</td>
<td>Shin et al., 1998</td>
</tr>
<tr>
<td>Caldocellum saccharolyticum</td>
<td>700</td>
<td>80</td>
<td>6.3</td>
<td>42</td>
<td>Stevenson et al., 1996</td>
</tr>
<tr>
<td>Cryptococcus laurentii</td>
<td>100</td>
<td></td>
<td>4.7</td>
<td>47</td>
<td>Ohtsuka et al., 1988</td>
</tr>
<tr>
<td>Kluyveromyces fragilis</td>
<td>350</td>
<td>35</td>
<td>6.2</td>
<td>45</td>
<td>Shukla, 1975</td>
</tr>
<tr>
<td>Kluyveromyces lactis</td>
<td>200</td>
<td>45</td>
<td>7</td>
<td>31</td>
<td>Foda and Lopez-Leiva, 2000</td>
</tr>
<tr>
<td>Penicillium simplicissimum</td>
<td>600</td>
<td>50</td>
<td>6.5</td>
<td>30.5</td>
<td>Cruz et al., 1999</td>
</tr>
<tr>
<td>Pyrococcus furiosus</td>
<td>450</td>
<td>75</td>
<td>5</td>
<td>29</td>
<td>Boon et al., 1999</td>
</tr>
<tr>
<td>Rhodotorula minuta</td>
<td>360</td>
<td>60</td>
<td>6</td>
<td>64</td>
<td>Onishi and Yokozeaki, 1996</td>
</tr>
<tr>
<td>Saccharomyces fragilis</td>
<td>350</td>
<td>35</td>
<td>6.2</td>
<td>45</td>
<td>Roberts and Pettinati, 1957</td>
</tr>
<tr>
<td>Saccharopolyspora rectivirgula</td>
<td>600</td>
<td>70</td>
<td>7</td>
<td>41</td>
<td>Nakao et al., 1994</td>
</tr>
<tr>
<td>Sirobasidium magnun</td>
<td>360</td>
<td>60</td>
<td>6</td>
<td>67</td>
<td>Onishi et al., 1996</td>
</tr>
<tr>
<td>Sporobolomyces singularis</td>
<td>100</td>
<td></td>
<td>5</td>
<td>50</td>
<td>Gorin et al., 1964</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>100</td>
<td>37</td>
<td>7</td>
<td>25</td>
<td>Greenberg and Mahoney, 1983</td>
</tr>
<tr>
<td>Sterigmatomyces elviae(^{*})</td>
<td>300</td>
<td>80</td>
<td>4.5</td>
<td>25</td>
<td>Onishi et al., 1995</td>
</tr>
<tr>
<td>Sterigmatomyces elviae(^{**})</td>
<td>360</td>
<td>60</td>
<td>4.5</td>
<td>64</td>
<td>Onishi et al., 1995</td>
</tr>
<tr>
<td>Sterigmatomyces elviae(^{***})</td>
<td>360</td>
<td>60</td>
<td>4.5</td>
<td>60</td>
<td>Onishi and Tanaka, 1998</td>
</tr>
<tr>
<td>Thermus aquaticus</td>
<td>160</td>
<td>70</td>
<td>4.6</td>
<td>35</td>
<td>Berger et al., 1995a</td>
</tr>
<tr>
<td>Trichoderma harzianum</td>
<td>150</td>
<td>30</td>
<td>7</td>
<td>32</td>
<td>Prakash et al., 1987</td>
</tr>
</tbody>
</table>

\(^{(*)}\) Includes disaccharides  
\(^{(*\prime)}\) Cells were used to produce GOS  
\(^{(*\prime\prime)}\) Cells were grown in lactose solution

**Table 2.4.** GOS formation conditions of β-galactosidases produced by various microorganisms (from Yang and Bednarcik, 2001).
<table>
<thead>
<tr>
<th>Source</th>
<th>pH opt.</th>
<th>Temp. Opt. (°C)</th>
<th>Km for lactose (mM)</th>
<th>Metal ion required</th>
<th>Mol. Weight</th>
<th>Oligosaccharides production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kluyveromyces fragilis</td>
<td>6.5</td>
<td>35-40</td>
<td>14</td>
<td>+</td>
<td>201,000</td>
<td>Di &gt; Tri</td>
</tr>
<tr>
<td>Kluyveromyces lactis</td>
<td>7.0</td>
<td>35-40</td>
<td>15</td>
<td>+</td>
<td>135,000</td>
<td>Di &gt; Tri</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>7.2</td>
<td>40</td>
<td>2</td>
<td>+</td>
<td>540,000</td>
<td>Di &gt;&gt; Tri</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>3.0-4.0</td>
<td>55-60</td>
<td>85</td>
<td>-</td>
<td>124,000</td>
<td>Di &gt; Tri</td>
</tr>
<tr>
<td>Lactobacillus bulgaricus</td>
<td>7.0</td>
<td>45-50</td>
<td>5</td>
<td>+</td>
<td>195,000</td>
<td>Di , Tri</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>6.5</td>
<td>55</td>
<td>6</td>
<td>+</td>
<td>540,000</td>
<td>Di , Tri</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>4.5-5.1</td>
<td>55</td>
<td>37</td>
<td>-</td>
<td>110,000</td>
<td>Di &lt;&lt; Tri -</td>
</tr>
<tr>
<td>Bacillus circulans II</td>
<td>6.0</td>
<td>60</td>
<td>50</td>
<td>-</td>
<td>160,000</td>
<td>Di &lt;&lt; Tri -</td>
</tr>
</tbody>
</table>

Di, disaccharide; Tri, Trisaccharides; Tri ~, Trisacharides and higher oligosaccharides.

Table 2.5. GOS formation and enzymatic characteristics of β-galactosidases produced by various microorganisms (Dombo et al., 1997).
Figure 2.1. Proposed reaction mechanism of β-galactosidase. The first step is hydrolysis of lactose and second is formation of a 3-unit galacto-oligosaccharide (reproduced from Prenosil et al., 1987a, and Richmond et al., 1981).
CHAPTER 3

IMMOBILIZATION OF ASPERGILLUS ORYZAE β-GALACTOSIDASE ON TOSYLATED COTTON CLOTH

3.1. Introduction

With the demand for green and environmentally friendly technology on the rise, biocatalysis has gained increasing attention for synthesis of bio-based industrial products ranging from chemicals to neutraceuticals. The biocatalyst or enzyme used in bioprocessing often constitutes the major manufacturing cost and sometimes can be prohibitively expensive to use. Enzyme immobilization provides easy recovery and reuse of the enzyme and many other advantages, including ease in product separation and continuous operation (Filbert and Pitcher, 1976). In general, continuous immobilized enzyme reactors give higher productivities as compared with reactors using free enzyme, and can minimize downtime, enzyme costs, reactor size, and capital investment. However, large scale applications of immobilized enzymes are rare (Axelsson and
Zacchi, 1990), largely because the support materials used for enzyme immobilization are either too expensive or difficult for industrial use. Therefore, development of new techniques for enzyme immobilization on inexpensive and industrially applicable carriers are of economical significance.

For successful development and application of an immobilized biocatalyst, the enzyme support is generally considered as the most important component contributing to the performance of the reactor system. Fibrous matrices with high porosity, open structures and high mechanical strength have long been an interest in biocatalysis due to their advantages over particulate materials, which include high specific surface area, low pressure drop, and negligible mass-transfer resistance (Sharma and Yamazaki, 1984, Ichijo et al., 1990a). Various fibrous materials, including polyethylene terephthalate fibers (Elcin and Sacak, 1996), dimethylated superfine fibers (Ichijo et al., 1985, 1990a, b), cotton fabrics (Sharma and Yamazaki, 1984, Maeshima et al., 1997), nylon fiber (Seng et al., 1980), nonwoven nylon fabrics (Shemer et al., 1979), silk fibers (Grasset et al., 1979), silk fabrics (Demura et al., 1992, Furuhata et al., 1996, 1997), and porous glass fiber (Toldra et al., 1986) have been successfully used for enzyme immobilization. However, various problems associated with these supports were also reported. One common problem in using fibrous materials for enzyme immobilization is the lack of active sites on the polymer. Consequently, most previous studies used glutaraldehyde as a nonspecific cross-linking agent to fix the enzyme on the matrix, but the results were often unsatisfactory with low immobilization efficiency and low final enzyme activity (Furuhata et al., 1996, 1997, Toldra et al., 1986).
The main objective of this study was to explore the effectiveness of cotton cloth as a fibrous matrix for the development of a fibrous-bed biocatalyst reactor. Knitted cotton fabric such as terry cloth, is inexpensive and widely available, and has been successfully used in cell immobilization and fermentation studies (Huang and Yang, 1998, Talabardon et al., 2000, Yang and Lo, 1998, Yang et al., 1995). Recently, cotton cloth has also been used as a support matrix for enzyme immobilization. Several enzymes, including glucose oxidase (Kumar et al., 1997), urease (Kamath et al., 1988, Das and Kayastha, 1998) and invertase (Yamazaki et al., 1984), were immobilized on polyethylenimine-coated cotton cloth by ionic adsorption followed by crosslinking with glutaraldehyde. β-Galactosidase from E. coli was immobilized by adsorption on hydrophobic cotton cloth, which was made by introducing phenol groups to the supports (Sharma and Yamazaki, 1984). Although the activity yields were above 50%, total activity achieved per gram of support was low. Cotton fiber, having a cellulose backbone, can be activated with a variety of reagents such as cyanogen bromide, sulfonyl chlorides and periodate to form a covalent bond with enzyme (Groman and Wilchek, 1987, Scouten, 1987, Scouten et al., 1987). It has been shown that cyanogen bromide activation, though commonly employed to activate hydroxyl group bearing support so as to yield a covalent bond, forms a labile linkage leading to leakage of coupled catalyst especially with cellulose (Confort et al., 1988, 1989, Scouten, 1987). p-Toluenesulfonyl chloride (tosyl) is one of the least expensive reagents that can be used to activate hydroxyl groups of carriers under mild conditions (Nilsson and Mosbach, 1984b). While tresyl chloride is more reactive than tosyl, it is extremely expensive and too volatile to be conveniently used as tosyl (Nilsson and Mosbach, 1984b, Scouten et al., 1987). Unlike
cyanogen bromide, tosylated material is stable in dilute acid environment and the covalent bond formed upon displacement of tosyl should be strong and stable (Ballesteros et al., 1986). Therefore, it is our interest to develop and optimize tosylation of cotton cloth for enzyme immobilization.

In this work, methods for tosylation of cotton cloth and immobilization of *A. oryzae* β-galactosidase on the tosylated cotton cloth were developed. The optimal condition for tosyl activation of cotton cloth and the mechanism in the enzyme immobilization on tosylated cotton cloth were investigated. The effects of mercerization of cotton fiber with NaOH, addition of pyridine, and pH on the degree of tosylation and subsequent enzyme immobilization were also studied and are reported in this Chapter. Comparison of various fibrous supports with cotton cloth in terms of performance for activation and enzyme immobilization are also discussed. Finally, the *A. oryzae* β-galactosidase immobilized on the tosylated cotton cloth was characterized in its thermal stability and reaction kinetics. The immobilized enzyme can be used for continuous production of galacto-oligosaccharides from lactose (Albayrak and Yang, 2001).
3.2. Materials and Methods

3.2.1. Enzyme and Reagents

*Aspergillus oryzae* β-galactosidase obtained from Genencor International (Rochester, New York, USA) was used in this study. Each gram of the enzyme contained 100,000 fungal lactase units. One unit is defined as the amount of enzyme that liberates one μmol o-nitrophenol from o-nitrophenyl-β-galactopyranoside (ONPG) per min at pH 4.5 and 37 °C (Genencor). *p*-Toluenesulfonyl chloride (tosyl chloride, 98%) was obtained from Sigma (St. Louis, MO). Acetone (99.9%) and pyridine (100.0%) from Mallinckrodt (Paris, Kentucky) were dried over molecular sieve 4A (1/16” pellets, Advanced Specialty, S. Plainfield, NJ.) before use. Buffers used were acetate (pH 4.0 – 6.0), phosphate (pH 6.0 – 7.0) phosphate/carbonate (pH 8.0) and carbonate (pH 9.0 – 10.0) of corresponding sodium salts at 0.1 M concentration.

3.2.2. Tosylation of Cotton Cloth and Enzyme Immobilization

The immobilization procedure consisted of four main steps: mercerization of cotton with NaOH, pyridine pretreatment, tosyl activation of cotton, and enzyme coupling to cotton fibers. In order to determine the effect of each step for optimal activation of cotton cloth and immobilization of the enzyme, major variables in each step such as concentrations and reaction times were investigated. The ranges of variables tested and the conditions used are summarized in Table 3.1. For mercerization, cotton cloth was soaked in NaOH solution at 40 °C for several hours. Mercerization for about 2
to 4 h was sufficient and further incubation did not affect the results. The cloth was then rinsed thoroughly with distilled water to remove NaOH. The wet cotton was blotted between paper towels to remove as much water as possible. The blotted cloth was subsequently rinsed with acetone and dry acetone to further remove water. The acetone exchanged cotton cloth was then bathed in dry pyridine. For tosylation of the cloth, a dry acetone solution of tosyl was introduced into the cotton-pyridine mixture. When tosylation was completed, the cotton cloth was removed from the reaction mixture and washed with first acetone and then excess amounts of 5 mM HCl solution to remove tosyl and pyridine residuals from the cloth. Tosyl activated cloth was kept in 5 mM HCL solution and stored at 4 °C until used for enzyme immobilization. Before immobilization, the activated cotton cloth were removed from 5 mM HCL and rinsed with distilled water and 0.1 M acetic acid buffer (pH 4.5). Enzyme immobilization was carried out by immersing the cloth overnight in 0.1 M acetic acid buffer (pH 4.5) containing the enzyme. After immobilization, the cloth was rinsed with copious amounts of the same buffer and kept in it until enzyme activity was determined. All reactions during tosyl activation and enzyme immobilization were carried out in either 125- or 250-mL Erlenmeyer flasks at room temperature in a shaker-incubator at 250 rpm. Each flask was closed tightly with a rubber stopper to prevent evaporation of reactants and solvents. The effect of each variable tested was evaluated by monitoring the tosyl content of the cotton cloth and activity of the immobilized enzyme.
3.2.3. Characterization of Immobilized Enzyme

The immobilized enzyme on cotton cloth was characterized by evaluating the potential effects of immobilization on its thermal stability and reaction kinetics, especially in comparison with free enzyme. Thermal stability of the immobilized enzyme on cotton cloth was evaluated at 40, 50, and 60 °C. Cotton cloth (~1.25 g) containing immobilized enzyme was packed in a glass column reactor (9 mm internal diameter). The reactor was continuously fed with a lactose solution (200 g/L in 0.1 M acetic acid buffer, pH 4.5) at a constant flow rate (1 mL/min) and temperature for a period of up to three weeks. The concentration of glucose in the reactor effluent was monitored to determine the enzyme activity in the reactor. The thermal stability of free enzyme was also studied. The enzyme was dissolved in 0.1 M acetate buffer (pH 4.5) and incubated in a constant-temperature water bath. Samples were taken at appropriate time intervals and the residual enzyme activity was assayed.

GOS formation kinetics with the immobilized enzyme was also studied in a similar reactor operated at a recycle batch mode. The lactose solution (200 g/L in 0.1 M acetic acid buffer, pH 4.5) in the flask (total solution volume, 50 mL) was continuously recirculated through the immobilized enzyme reactor at a flow rate of 90 mL/min at 40 °C, and samples were taken at proper time intervals and assayed for their sugar contents using high performance liquid chromatography (HPLC).
3.2.4. Analytical Methods

3.2.4.1. Determination of Tosyl Content

Tosyl contents of activated cottons were determined after hydrolysis of tosyl with NaOH (Ballesteros et al., 1986, Nilsson et al., 1981). About 0.05 – 0.1 g tosylated cotton pieces were placed in 10 ml of 3N NaOH solution in test tubes for 24h. The UV spectrum of the solution containing the hydrolyzed tosyl from the cotton cloth was obtained with a spectrophotometer (Shimadzu UV-1601). The UV scans of p-toluenesulfonic acid and tosyl removed from the activated cotton by NaOH hydrolysis yielded almost identical spectrums. As can be seen in Figure 3.1, the major tosyl peak was identified at 261.5 nm. For quantification purpose, the peak height at 261.5 nm was measured and compared to a standard curve constructed with p-toluenesulfonic acid (Sigma) solutions with known concentrations (See Figure A 6 in Appendix A).

3.2.4.2. Determination of Protein Coupling Yield

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 the enzyme (protein) solution was measured with a spectrophotometer (Shimadzu UV-1601). The enzyme (protein) concentration was determined by comparing with the standard curve constructed using enzyme (protein) solutions with known concentrations as seen in Figure A 1 and A 2 in Appendix A. The amount of the enzyme (protein) coupled onto the tosylated cotton was determined from the initial protein amount present in the enzyme coupling solution subtracting the final total protein amounts present in the remaining
coupling solution and the washing solution (Alcantara et al., 1990). 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 of the enzyme present in the coupling solution.

3.2.4.3. Enzyme Activity Assay

The activity of immobilized enzyme on cotton cloth was measured with 10 g/L lactose as the substrate in 0.1 M acetic acid buffer (pH 4.5) at 40 °C in 125 mL Erlenmeyer flasks in a shaker-incubator at 450 rpm. At least three samples were taken during the enzyme reaction. The reaction in the sample was stopped by adding an equal volume of 0.1 N NaOH. The glucose concentration was determined with a glucose analyzer (YSI 2700 Select, Yellow Springs, OH). One unit of immobilized enzyme activity is defined as the amount of enzyme producing 1 µmol of glucose from lactose under the defined conditions. The standard curve can be seen at Figure A 3 in Appendix A. The enzyme activity yield (%) was calculated from the activity of immobilized enzyme divided by the initial enzyme activity in the coupling solution subtracting the residual enzyme activity in the solution after immobilization.

3.2.4.4. HPLC Analysis

Concentrations of all sugars found in sample solutions (glucose, galactose, lactose, and galacto-oligosaccharides (GOS)) were determined by high performance liquid chromatography (HPLC) during the application of the cotton cloth immobilized β-galactosidase for formation of GOS. An HPLC system consisting of a pump (Waters
6000A), an autosampler (Waters WISP 710B), a carbohydrate analysis column (Phenomenex, Rezek RNM carbohydrate column, 7.8 x 150 mm), a column heater (Bio-Rad), a refractive index detector (Waters 410 differential refractometer), and a Shimadzu CLASS-VP chromatography data system (version 4.2 integrator) was used. The eluent was pre-degassed distilled water (at 85 °C) at a flow rate of 0.4 mL/min. Distilled water was degassed by first boiling and then sonication for 30 min. The column temperature was maintained at 85 °C and the detector temperature was set at 45 °C. The concentrations (w/v) of these sugars (e.g., lactose, glucose, galactose, and total oligosaccharides) are proportional to their peak areas with the same proportionality constant (Boon et al., 2000). Thus, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work.

3.3. Results and Discussion

3.3.1. Mechanism of Enzyme Immobilization

The reaction of sulfonyl chlorides, such as p-toluenesulfonyl chloride, has been proposed to follow a nucleophilic substitution mechanism (Nilsson and Mosbach, 1980, 1981). As shown in Figure 3.2, tosyl chloride reacts with primary hydroxyl groups of carrier under anhydrous condition and the amino group of the enzyme is then coupled to the carrier by replacing tosyl. Although the use of tosyl chloride for immobilization of proteins was first described by Nilsson and Moshach (1980, 1981), the exact mechanism has not been elucidated. Elemental sulfur analysis (Nilsson and Mosbach, 1980) and UV
absorbance (Nilsson and Mosbach, 1984b) were usually used to determine the level of activation by sulfonyl chlorides. However, this does not directly suggest that the enzyme follows the proposed mechanism. For example, the mechanism for tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) activation and enzyme immobilization were once proposed as the one shown in Figure 3.2 (Crossland et al., 1971), later however, a different reaction mechanism leading to sulfonate ester formation was evidenced (Demiroglou et al., 1994, Jennissen, 1995).

To verify the enzyme immobilization mechanism, the enzyme solution during the course of enzyme coupling to tosylated cotton cloth was scanned in the UV range from 300 to 200 nm. The spectra taken at 2 h and 18 h for various initial enzyme concentrations are shown in Figure 3.3. The 2-h scan was direct reading while the reading at 18 h was after 4-fold dilution due to too much tosyl content. As can be seen in this figure, the enzyme peak (at ~280 nm) at 2 h was higher for the solution initially containing more enzymes. However, at 18 h most enzyme had disappeared but with corresponding increases in tosyl content in the solution as seen from the spectrum near 260 nm. The simultaneous increase in tosyl and decrease in enzyme concentrations clearly suggested that the degree of enzyme coupling was accompanied with the displacement of tosyl, following the nucleophilic substitution mechanism for enzyme immobilization. Therefore, it can be concluded that tosyl covalently binds to hydroxyl groups and behaves as a leaving agent and is later replaced by the enzyme leading to a covalent bond as depicted in Figure 3.2. It is noted that the extent of enzyme coupling can
be monitored with the UV spectra, which has not been shown previously. It is also possible to quantify the number of tosyl displaced by enzyme by this method.

3.3.2. Effects of Mercerization with NaOH

Cellulose fibers including cotton usually have a high crystallinity (~70-75%) (Sarko, 1987). Cellulosic materials can be treated with alkali to induce swelling thereby exposing more hydroxyl groups for further reactions (Confort et al., 1989). Therefore, the effects of NaOH concentration on mercerization and subsequent tosylation and enzyme immobilization on cotton fibers were investigated. As expected, mercerization of cotton with NaOH greatly affected the level of tosylation and final activity of the immobilized enzyme (Figure 3.4). In general, tosylation increased with increasing the NaOH concentration from 0.1 to 4 N. However, comparable amounts (85-90%) of the enzyme protein were coupled to the tosylated cotton, regardless of the mercerization condition and the degree of tosylation. The effect of mercerization on the immobilized enzyme activity appeared to be complicated. Mercerization with 0.5 N NaOH gave the maximum immobilized enzyme activity and activity yield (50%), which were five to six-fold higher than those from unmercerized samples. The immobilized enzyme activity increased with increasing the NaOH concentration up to 0.5 N NaOH due to increased tosylation. Increasing NaOH concentration from 0.5 N to 2 N resulted in slight increases in tosylation and enzyme coupling efficiency, but significantly decreased the activity yield. Consequently, the compounded effect on the final immobilized enzyme activity was not significant. Further increasing NaOH concentration greatly increased mercerization and
tosylation, but resulted in a significantly lower enzyme activity due to reduced activity yield. Since the amount of the enzyme coupled to the tosylated cotton was not significantly affected by the degree of tosylation, too much tosyl on cotton might have resulted in undesirable coupling of the enzyme at multiple sites that could impose steric hindrance and lower enzyme activity (Alcantara et al., 1990, Bulew and Mosbach, 1982). It was observed that about 80% of immobilized enzyme activity was achieved within 2-4 h of coupling (see Fig. 3.7 and 3.8), yet there were a lot more tosyl acids being displaced as the incubation continued (Fig. 3.3), an indication that multiple attachments of enzyme occurred. Also, there was apparent physical change in the rigidity of the cotton. After mercerization with 4N NaOH, cotton lost its softness and became somewhat hydrophobic, also an indication of excessive tosylation. Upon drying of this cotton, it would resist absorbing water.

Native celluloses exist in the form of cellulose I structure, which is irreversibly converted to cellulose II upon mercerization (Sarko, 1987). The yield of the substitution reactions of cellulose increased dramatically when cellulose was pretreated with sodium hydroxide solution (Confort et al., 1989, Sarko, 1987). Confort et al (1989) found that 0.05 N NaOH for 3 h pretreatment barely affected the level of tresylation and protein binding, but the immobilized enzyme retained five-fold more activity. It was also observed that 1 N NaOH resulted in a physical change in the cellulose hollow fiber structure (Confort et al., 1989). Boyd and Yamazaki (1993) observed that 2.5 N NaOH treatment for 10 min increased BSA immobilization by six-fold on non-woven Rayon/Polyester blend type cloth after activation with tosyl. Since they did not use an
enzyme, what happened to activity cannot be compared although they indicated that the activated support was capable of immobilizing 25 mg protein per gram of support. In general, our results agree well with these observations. Mercerization with 0.5 \( N \) NaOH is thus recommended and was used in our subsequent studies since higher concentrations of NaOH resulted in lower enzyme activities.

### 3.3.3. Effect of Pyridine

Pyridine is an organic base with a \( pK_a \) value of 5.23 (Blanchard, 1987). It was primarily used for neutralization of HCl released during tosylation (Nilsson and Mosbach, 1987). Pyridine is also a good plasticizer, which helps opening the cellulosic structure of cotton (Sarko, 1987) and has been used as a swelling agent or catalyst in derivatization of cellulosic materials (Graham et al., 1987, West and Banks, 1987). Sarko (1987) has indicated that organic bases such as pyridine reversibly change the cellulose I or II structures into distinctly different III-I or III-II, respectively. The effects of pyridine on tosylation and immobilized enzyme activity can be seen from Figure 3.5. In general, treating cotton cloth with pyridine before and during tosylation greatly increased both tosylation and immobilized enzyme activity. Pyridine at a concentration of 16 ml per g cotton gave about 4-fold increase in tosylation and 8-fold increase in immobilized enzyme activity. It seemed that above \( \sim 12 \) ml/g, there were small changes in enzyme activity although tosylation continued to increase. Thus, it can be concluded that adding 10-12 ml pyridine per gram cotton is sufficient to give the desired effects.
Boyd and Yamazaki (1993) found that pyridine was not necessary for tosylation of rayon/polyester blend. In our case, however, there was very low tosylation and immobilized enzyme activity on cotton that was not treated with pyridine. Since pyridine was used to neutralize the HCl released during tosylation, the amount of pyridine required would be dependent on the amount of tosyl used. Tosyl (in g) to pyridine (in mL) ratio was maintained at 1:1 in many studies using agarose (Nilsson and Mosbach, 1980, Nilsson et al., 1981), corncob (Sanchez-Montero et al., 1989) as the carriers. The ratio used in our study ranged from 0.25 to 2.0, with more pyridine resulting in greater enzyme activity. Although the primary function of pyridine is to neutralize the HCl released during tosylation, the benefit of more pyridine to cotton cloth might be attributed to the plasticizing effect of pyridine on cellulosic materials (Sarko, 1987, West and Banks, 1987). The cotton became transparent or plastic upon the addition of pyridine to the solvent dried cotton. The physical change in cotton caused by pyridine appeared to be beneficial to enzyme immobilization.

It was suggested that pyridine could be of concern due to its unpleasant odor and possible toxicity (Alcantara et al., 1990, Boyd and Yamazaki, 1993). Pyridine removal could be difficult for agarose gels and other similar materials. However, there was no detectable residual pyridine after washing the tosylated cotton cloth. Any residual pyridine in the coupling solution would show up in the spectra taken for tosyl determination during the enzyme coupling step. Clearly, pyridine was effectively removed after tosylation by washing and storage in 5 mM HCl solution.
3.3.4. Effect of Tosyl

The amount of tosyl used in tosylation would affect the level of activation of cotton cloth and subsequent enzyme immobilization (Figure 3.6). As can be seen in Figure 3.6a, increasing the tosyl amount to 4 g tosyl/g cotton greatly increased tosylation and enzyme immobilization. However, further increasing the tosyl amount, without also increasing the pyridine amount, resulted in a slight increase in tosyl content but lowered the immobilized enzyme activity. Without sufficient amount of base (pyridine) to neutralize the HCl released during tosylation might have caused the reduced enzyme activity at higher tosyl concentrations (above 5 g tosyl/g cotton), which might cause partial destruction of cellulose to yield small polysaccharide fragments that were lost in the solution. In the case of agarose, excess amount of tosyl caused severe consequences and led to 40% destruction (Alcantara et al., 1990, Ballesteros et al., 1986). Therefore, it is important to maintain a proper tosyl to pyridine ratio during tosylation. When the tosyl to pyridine ratio was kept at 0.25 (g/ml), increasing the tosyl amount also increased tosylation and immobilized enzyme activity, although the effects were not dramatic. Based on these results, it is concluded that 4 g tosyl and 10 – 20 ml pyridine per gram of cotton are appropriate amounts for tosylation and enzyme immobilization.

3.3.5. Enzyme Coupling

The optimal conditions for enzyme coupling to the tosylated cotton were studied. In general, more enzymes present in the coupling solution would result in more immobilized enzyme activity, but the increase leveled off when the enzyme amount was
higher than 50 mg per g of cotton (Fig. 3.7). The coupling reaction was relatively fast for
tosylated supports (Boyd and Yamazaki, 1993, Nilsson and Mosbach, 1980, 1984b,
Nilsson et al., 1981). A short coupling time of 4 h was sufficient to complete the reaction
when the enzyme concentration was 10 mg/g cotton or lower. A longer time was
required to reach the maximum immobilized enzyme activity for higher enzyme levels
(above 25 mg/g cotton), although ~85% of the enzyme activity was achieved within 4 h.
It is noted that the coupling time is also dependent on the solution pH, which is known to
affect the reaction rate. In general, the coupling reaction is faster at pH 7 than at lower
pH values. However, even at pH 4.5, about 70% of immobilized enzyme activity was
obtained in 2 h as compared with that at 13h (Fig. 3.8).

It was suggested that tosylated supports were useful for coupling ligands at high
and 9.5 was thus usually used for coupling various enzymes onto tosyl-activated supports
However, as can be seen in Figure 3.8, the highest immobilized enzyme activity was
obtained at pH 4.5 and the level of immobilized enzyme decreased continuously with
increasing pH from 4.5 to 9. Since the enzyme was stable in a wide pH range of 4.0 - 8.0
at room temperature (Ogushi et al., 1980, Yang et al., 1994a) (Figure D.1 in Appendix D),
the lower immobilized enzyme activity indicated a lower enzyme coupling efficiency at
the higher pH. It was reported that 40% of tosyl was hydrolyzed at pH 6.0 within 30 min
due to reaction with water, and higher percentages of hydrolysis occurred at higher pHs
(Alcantara et al., 1990). As already discussed, the enzyme coupling was accompanied by
tosyl removal from the support following the reaction between the enzyme and tosyl groups on the cotton cloth (see Fig. 3.2). This suggests that tosyl can be displaced at lower pHs given longer coupling times, where more tosyl would be available for coupling due to less hydrolysis of tosyl itself. Nilsson and Mosbach (1980) also reported that enzymes containing high lysine and cysteine contents were coupled with better yields at lower pHs. There were 12 lysine residues exposed on the surface of the *A. oryzae* lactase enzyme (Naoi et al., 1984). The presence of carbohydrates (mostly aminated such as glucoseamine) as 12-15% of the molecular weight of the enzyme was also reported (Nakao et al., 1994, Ogushi et al., 1980). It is well known that thiol groups have the highest reactivity followed by amines, imidazole, and tyrosine hydroxyl groups (Nilsson and Mosbach, 1984b). Thus, some of these groups present on the enzyme may have a lower $pK_a$ value due to close association with each other than otherwise expected. These groups then may behave as nucleophiles for the enzyme coupling to the tosylated sites. Efficient enzyme coupling at a low pH value is a desirable characteristic of tosylated cloth since many of the industrially significant enzymes are more stable at low pHs.

### 3.3.6. Comparisons of Fibrous Supports

Various types of fibrous matrices were studied to evaluate their suitability for enzyme immobilization using the method developed in this work. The materials studied were non-woven polyester fabric (100% polyethylene terephthalate or PET), woven cotton towel (terry cloth), cotton wool, 100% Rayon cloth (Wonder cloth), 50% Rayon and 50% PET cloth (Sontara®), DuPont, Old Hickory, Tennessee), and 70% Rayon and
30% PET cloth (Sontara, DuPont). These materials were treated under the same condition that was close to the optimal condition as discussed before, and the results are shown in Figure 3.9. Clearly, cotton cloth was superior to all the other materials studied. For PET and PET/Rayon blends, the tosyl content after tosylation was low, less than 25 \( \mu \text{mol/g} \), and the immobilized enzyme activity was also low, indicating that PET was not readily tosylated. However, although the tosyl contents in tosylated cotton cloth, cotton wool and Wonder cloth were all at a similar level (416, 445, and 482 \( \mu \text{mol/g} \), respectively), cotton wool had only about half of the activity of cotton cloth and Wonder cloth had only about one tenth of that. Cotton cloth was the densest material compared to non-woven type light weighed rayon and rayon/PET blends. Except for rayon, low tosyl content of blends coincided with their immobilized enzyme activities. However, it was not clear why cotton cloth had better immobilization result than the other two similar materials.

Howlett and coworkers (1991) and Boyd and Yamazaki (1993) tested Rayon-polyester blends for enzyme immobilization. It was reported that the blend cloth after tosylation activation was capable of immobilizing 25 mg bovine serum albumin per g cloth (Boyd and Yamazaki, 1993). Tosyl activated Sepharose and Agarose have also been used successfully for enzyme immobilization, although both the coupling yield and activity yield were generally lower than what were accomplished in the present work with cotton cloth. It should be noted that tosylation of support requires anhydrous conditions to allow the hydroxyl groups to react with tosyl (Ballesteros et al., 1986). Therefore, solvent exchange of supports to water-free organic solvent-phase must be applied for various
types of matrices (Alcantara et al., 1990, Ballesteros et al., 1986, Boyd and Yamazaki, 1993, Nilsson and Mosbach, 1980, 1981). For highly cross-linked polymeric materials such as Sepharose and Agarose gel beads, solvent exchange is laborious and time consuming because of severe diffusion limitation into the gel matrix. Consequently, a large amount of solvent is usually required to dry the support before tosylation. In contrast, solvent exchange for the highly porous cotton cloth was straightforward and can be done in one quick step with a relatively small amount of solvent. In this study, acetone was directly employed after mercerization to rapidly dehydrate cotton cloth, a critical step that can be easily applied to a large-scale operation. Also, another advantage of tosylated cotton cloth is that the enzyme coupling reaction is rapid and does not produce a decline in activity due to overloading of the support, a problem commonly reported for particulate supports and gels (Confort et al., 1989, Nilsson and Mosbach, 1980, 1984b). Table 3.2 shows the comparison between various fibrous matrices used for enzyme immobilization. Apparently, tosylated cotton cloth was better than most of the other treatments reported in the literature. PEI-coated cloth (Yamazaki et al., 1984, Kamath et al., 1988) and hydrophobic cloth (Sharma and Yamazaki, 1984) also gave good immobilization results, but enzyme immobilization was not through covalent bonding and thus was not as stable. Without a covalent bond for enzyme attachment on the carrier, leaching of the enzyme during process application could be a problem.
3.3.7. Thermal Deactivation Kinetics

It is important to know if the immobilization on cotton cloth via tosyl substitution would have any effects on the enzyme in its process performance. Therefore, the reaction kinetics and the thermal stability of the immobilized enzyme were studied. There was essentially no change in the activity of immobilized enzyme at 40 °C during 15 days of continuous operation in a plug-flow reactor. As compared with the free enzyme, the thermal stability of the enzyme was increased by 25 to 28-fold after immobilization on the cotton cloth (Table 3.1). It is noted that the increase in the deactivation rate constant $k_d$ with temperature followed the Arrhenius relationship, and the activation energy $E_a$ was the same for both immobilized and free enzymes ($E_a/R = 2.8 \times 10^4$ K).

Improvement in enzyme thermal stability upon immobilization is generally observed, especially upon a covalent bond formation. As already discussed, the enzyme was most likely coupled to cotton cloth on multiple sites, which led to stronger immobilization and increased stability because it would be harder for enzyme to unfold (Ballesteros et al., 1986). The results agreed with the reported bond stability of sulfonyl chlorides (Nilsson and Mosbach, 1984b, Sturgeon, 1988). The stabilities of tosyl activated support as well as the linkage between enzyme and support are important advantages compared to other reagents such as cyanogen bromide (Confort et al., 1988, 1989, Peng, 1987).
3.3.8. Formation of Galacto-oligosaccharides (GOS)

GOS are intermediary sugars formed during lactose hydrolysis by transgalactosylation activity of lactase (Rugh, 1982). Figure 3.10 shows typical batch kinetics for lactose hydrolysis and GOS formation catalyzed by the immobilized enzyme. The data represent the weight percentages of total sugars present in the reaction mixture (w/w %). There have been reports of about 20-30 % decrease in GOS formation upon immobilization of lactase on a support, which was attributed to diffusion limitations for the larger GOS molecules (Prenosil et al., 1987a,b, Rugh, 1982, Shin et al., 1998). Changes in apparent $k_m$ values upon immobilization are generally considered as an indication for diffusion limitation, but specific product formation such as GOS would be a better index in evaluating the performance of immobilized enzyme in actual process application (Pitcher, 1980). Thus, GOS formation kinetics of immobilized and free enzymes were compared and are shown in Figure 10b. It is clear that the GOS formation kinetics was almost identical for reactions carried out with the immobilized enzyme on cotton cloth and free enzyme in solution, suggesting that enzyme immobilization on cotton cloth does not impose any diffusion limitation or alteration on GOS formation from lactose. Thus, the GOS formation ability of the enzyme was not significantly affected by the immobilization of the enzyme onto tosyl activated cotton cloth.

3.4. Conclusions

Knitted cotton terry cloth can be activated with p-Toluenesulfonyl chloride and used as a novel and inexpensive fibrous matrix for biocatalyst immobilization. The
procedure developed for cotton cloth provides simple, inexpensive, less cumbersome and industrially applicable method and support for highly active and stable immobilized biocatalyst. The optimal conditions were 0.5 $N$ NaOH for mercerization of cotton fiber, 10-20 ml pyridine and 4-8 g tosyl per g of cotton for tosylation, and pH 4.5 for enzyme coupling. Spectrophotometric evidence suggests that enzyme immobilization is assisted with substitution of tosyl from the cotton. The highly porous fibrous structure of cotton cloth and its high mechanical strength allow for high flow rates and efficient mass transfer through the matrix, which are advantageous not only during treatments for chemical activation and enzyme immobilization, but also for the application of immobilized enzyme in, e.g., GOS formation. Thermal stability of the enzyme was increased by ~25-fold upon immobilization and the immobilized enzyme has a half-life of 50 days at 50 °C and more than 1 year at 40 °C. The simple immobilization method using tosyl-activated cotton cloth and superior performance of the cotton-immobilized enzyme reactor should have many applications in industrial biocatalysis including GOS production from whey lactose.
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* Distilled water having pH ~ 7.0 was used without any adjustment.

**Table 3.1.** Experimental conditions studied for tosylation of cotton cloth and enzyme immobilization.
<table>
<thead>
<tr>
<th>Support</th>
<th>Enzyme</th>
<th>Coupling pH</th>
<th>Coupling Amount (mg/g)</th>
<th>Coupling Yield (%)</th>
<th>Activity Yield (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepharose*</td>
<td>Peroxidase</td>
<td>9.7</td>
<td>84 mg/g</td>
<td>20</td>
<td>8</td>
<td>Nilsson and Mosbach, 1980</td>
</tr>
<tr>
<td></td>
<td>Alcohol DH</td>
<td>8.5</td>
<td>112 mg/g</td>
<td>67</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Agarose*</td>
<td>Micrococcal endonuclease</td>
<td>9.0</td>
<td>8 µg/mL gel</td>
<td>100</td>
<td>50</td>
<td>Ballesteros et al., 1986</td>
</tr>
<tr>
<td>Rayon*</td>
<td>Bovine serum albumin</td>
<td>7.5</td>
<td>25 mg/g</td>
<td>-</td>
<td>-</td>
<td>Boyd and Yamazaki, 1993</td>
</tr>
<tr>
<td>Cotton cloth*</td>
<td>β-Galactosidase</td>
<td>4.5</td>
<td>45 mg/g</td>
<td>85</td>
<td>55</td>
<td>This study</td>
</tr>
<tr>
<td>PEI-coated</td>
<td>Invertase</td>
<td>-</td>
<td>-</td>
<td>1670 U/g</td>
<td>-</td>
<td>Yamazaki et al., 1984</td>
</tr>
<tr>
<td>cloth</td>
<td>Urease</td>
<td>-</td>
<td>1.2 mg/g</td>
<td>0.56</td>
<td>-</td>
<td>Kamath et al., 1988</td>
</tr>
<tr>
<td>Naphtyl</td>
<td>Bovine serum albumin</td>
<td>-</td>
<td>74 mg/g</td>
<td>-</td>
<td>-</td>
<td>Sharma and Yamazaki, 1984</td>
</tr>
<tr>
<td>cloth</td>
<td>β-Galactosidase</td>
<td>-</td>
<td>40 mg/g</td>
<td>52</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

* Supports were activated with p-toluene sulfonyl chloride

**Table 3.2.** Comparison of enzyme immobilization by various methods and fibrous matrices.
The deactivation rate constant $k_d$ was determined from experimental data which followed first-order reaction kinetic model. The enzyme half-life was calculated from $k_d$ value.

**Table 3.3.** Thermal deactivation of free and immobilized enzyme

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>Free enzyme</th>
<th>Immobilized enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_d$ (h$^{-1}$)</td>
<td>Half-life (h)</td>
</tr>
<tr>
<td>40</td>
<td>0.0017</td>
<td>399</td>
</tr>
<tr>
<td>50</td>
<td>0.0141</td>
<td>49</td>
</tr>
<tr>
<td>60</td>
<td>0.3325</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 3.1. Scanning UV spectra of p-toluene sulfonic acid between 200 and 300 nm with $\lambda_{\text{max}}$ at 261.5 nm. (a) p-toluene sulfonic acid in distilled water; (b) tosyl hydrolyzed from activated cotton cloth in 3N NaOH solution.
Figure 3.2. Proposed reaction mechanism for enzyme immobilization on cotton cloth by activation with tosyl chloride.
Figure 3.3. The scanning UV spectra of the enzyme coupling solutions taken at 2 h and 18 h after the coupling reaction. Spectra shown are for solutions with four different initial enzyme concentrations (10, 25, 50 and 100 mg/g cotton). The spectra at 2h were direct reading and the spectra at 18 h were taken after 4-fold dilution.
Figure 3.4. Effects of NaOH concentration during mercerization on tosylation of cotton cloth and enzyme immobilization. Cotton cloth without any NaOH treatment was used as control. For the dry control, cotton cloth was dried at 105 °C for 24 h.
Figure 3.5. Effects of pyridine on tosylation of cotton cloth and enzyme immobilization. Cotton cloth was treated with varying amounts of dry pyridine for 3h at room temperature in a shaker-incubator at 250 rpm.
Figure 3.6. Effects of tosyl amount on tosylation of cotton cloth and enzyme immobilization. The amount of pyridine used was 20 ml (A); the amount of pyridine was adjusted to maintain a constant tosyl (g)/pyridine (ml) ratio of 0.25 (B).
Figure 3.7. Effects of enzyme concentration and coupling time on the final immobilized enzyme activity on cotton cloth after 4 and 18 h of incubation. The initial pH of the coupling solution was ~7.0.
Figure 3.8. Effects of pH and coupling time on the final immobilized enzyme activity on cotton cloth after 2 and 13 h of incubation. The initial enzyme concentration in the coupling solution was 50 mg/g cotton. Buffers used were acetate (pH 4.0 – 6.0), phosphate (pH 6.0 – 7.0) phosphate / carbonate (pH 8.0) and carbonate (pH 9.0 – 10.0) of corresponding sodium salts at 0.1 M concentration.
Figure 3.9. Immobilization of β-galactosidase onto various tosyl-activated supports. Conditions used were: mercerization with 0.5 N NaOH for 3 h, pretreatment with 10 ml pyridine g\(^{-1}\) support for 3h, tosylation with 5 g tosyl in 10 ml dry acetone g\(^{-1}\) support for 15 h, and coupling in 60 ml enzyme solution containing 80 mg g\(^{-1}\) support for 12 h.
Figure 3.10. Kinetics of lactose hydrolysis and GOS formation catalyzed by immobilized enzyme in a recycle batch reactor at 40 °C with an initial lactose concentration of 200 g/L. (a) time course data; (b) comparison of GOS formation during lactose hydrolysis by free enzyme and immobilized enzyme.
CHAPTER 4

PRODUCTION OF GALACTO-OLIGOSACCHARIDES FROM LACTOSE BY ASPERGILLUS ORYZAE β-GALACTOSIDASE IMMOBILIZED ON COTTON CLOTH

4.1. Introduction

Galacto-oligosaccharides (GOS) selectively stimulate the growth of bifidobacteria in the lower part of human intestine (Sako et al., 1999). Increase in the growth of bifidobacteria is usually accompanied by suppression of potentially harmful bacteria such as clostridia and Bacteroides species in the gut (Fooks et al., 1999, Oku, 1996, Tanaka et al., 1983). The result of the shift in the number and/or activity of gut microflora may be reflected as a partial interchange between toxic and beneficial components (Ziemer and Gibson, 1998). The introduction of GOS into food products is thus desirable for its many health benefits (Sako et al., 1999). The commercial potential for the production and applications of GOS in food product lines is high (Playne and Crittenden, 1996, Shin et
al., 1998), but an economical production process still needs to be developed. The goal of this study was to develop an immobilized enzyme reactor for GOS production from lactose. Lactose constitutes over 70% of the total solids in whey, an abundant byproduct from cheese production. Currently, applications of lactose in food products are limited by its intolerance by some people, low solubility, and tendency to crystallize in water at low temperatures (Bourne et al., 1983, Shukla, 1975). Considering the 3% annual increase in cheese production (Foda and Lopez-Leiva, 2000), the already problematic lactose is expected to be a major concern for the dairy industry. Although there has been extensive research for better utilization of whey lactose, the dairy industry is still in need of new technologies for converting lactose into marketable products (Yang and Silva, 1995). Thus, converting lactose into a product that contains a prebiotic food ingredient and is free of problems associated with lactose is beneficial and highly desirable by the food industry (Playne and Crittenden, 1996).

GOS production from lactose has been widely studied using β-galactosidase from various sources (Yang and Bednarcik, 2001). β-Galactosidase (EC 3.2.1.23) is one of the class of oligosaccharide-hydrolyzing enzymes first isolated and purified from various organisms, and has long been used to hydrolyze lactose to glucose and galactose in the manufacture of some dairy products (Mahoney, 1998). However, under certain reaction conditions, the same enzyme also catalyzes transgalactosylation, which leads to the production of oligosaccharides (Prenosil et al., 1987b, Yang and Tang, 1988, Zarate and Lopez-Leiva, 1990). Depending on the source of the enzyme, the results for GOS production from lactose are quite different in the final product types and yields (Yang and
Bednarcik, 2001). Almost all of the previous studies for GOS production focused on improving or maximizing GOS yields from lactose. Up to 67% GOS yield from lactose has been reported (Onishi et al., 1996). However, most of the enzymes used in these studies are not approved for food use and are expensive. Furthermore, many of them were from the sources not commercially available or not available in sufficient quantities for industrial applications. In this work, commercially available *Aspergillus oryzae* β-galactosidase was used. This enzyme is already used in food products, is relatively inexpensive as compared to enzymes from other sources, and has an optimal pH of 4.5, which is close to the native pH of whey.

Production of GOS by immobilized β-galactosidase has been considered in several studies. Compared with free enzymes in solution, enzyme immobilized on a solid support provides many advantages, including enzyme reusability, continuous operation, controlled product formation, and simplified and efficient processing. Immobilized enzyme reactors also can give higher productivities and minimize downtime, enzyme costs, and reactor size, and thus generally are more economical than free enzyme reactors (Axelsson and Zacchi, 1990, Mozaffar et al., 1986a). However, GOS production from immobilized enzymes has not been addressed very well (Berger et al., 1995a, Mozaffar et al., 1987). Many of the carriers used for enzyme immobilization are some types of microparticles, such as ion exchange resins (Matsumoto et al., 1989, Mozaffar et al., 1986a), chitosan beads (Sheu et al., 1998, Shin et al., 1998), cellulose beads (Kminkova et al., 1988), and agarose beads (Berger et al., 1995b). These microparticles are expensive and difficult to scale up, which limit their applications in industry (Anspach et al., 1994,
Axelsson and Zacchi, 1990). Furthermore, the particulate supports often cause problems in packed-bed reactors, notably a high pressure drop, fouling and plugging due to particle self-compression and aggregation, which in turn limit the reactor's scalability and operational life (Howlett et al., 1991, Ichijo et al., 1985, Peng et al., 1987). It has also been observed that the immobilized enzyme in these particle carriers often resulted in 20-30% reduction in GOS yields due to diffusion limitations (Kobayashi et al., 1980, Nakanishi et al., 1983, Prenosil et al., 1987b, Rugh, 1982, Sheu et al., 1998, Shin et al., 1998).

In this work, we immobilized A. oryzae β-galactosidase on cotton cloth for GOS production from lactose. Inexpensive fibrous matrices, such as cotton cloth, with high porosity (>95%), large specific surface area, and excellent mechanical strength, have been successfully used in cell immobilization and fermentation studies (Huang and Yang, 1998, Talabardon et al., 2000, Yang et al., 1995, 1998). The reactor with cotton cloth packed in a loose spiral form has a low pressure drop, good flow rates, and little diffusion limitation. Such a reactor also would be easy to scale up. However, native cotton (cellulose) fibers are not active for enzyme immobilization and cotton cloth (or any cellulosic fibrous matrices) has rarely been used in enzyme reactors for production purposes. We have recently developed a novel, economical method to immobilize A. oryzae β-galactosidase on cotton cloth activated with tosyl chloride. In this work, the kinetics of GOS formation from lactose in the cotton-immobilized enzyme reactor was investigated in detail. The effects of lactose concentration, pH, and temperature on GOS production were studied. The thermal stability of the cotton-immobilized enzyme was also evaluated, and the feasibility of using the immobilized enzyme reactor for stable
long-term production of GOS from lactose was demonstrated. A comparison of performance with free enzyme reactions and other immobilized enzyme studies is also discussed in this study.

4.2. Materials and Methods

4.2.1. Enzyme Immobilization

Aspergillus oryzae β-galactosidase obtained from Genencor International (Rochester, New York, USA) was used in this study. Each gram of the enzyme contained 100,000 fungal lactase units. One unit is defined as the amount of enzyme that liberates one μmol o-nitrophenol from o-nitrophenyl-β-galactopyranoside (ONPG) per min at pH 4.5 and 37 °C. This commercial enzyme has been studied for its structural characterization under various process conditions (Yang et al., 1994a). The enzyme was immobilized on cotton terrycloth obtained locally. The immobilization procedure consisted of four main steps: mercerization of cotton with NaOH, pyridine pretreatment, tosyl activation of cotton, and enzyme coupling to cotton fibers. p-Toluenesulfonyl chloride (tosyl chloride, 98%) was obtained from Sigma (St. Louis, MO). Acetone (99.9%) and pyridine (100.0%) from Mallinckrodt (Paris, Kentucky) were dried over molecular sieve 4A (1/16" pellets, Advanced Specialty, S. Plainfield, NJ.) before use. For mercerization of cotton cloth, 0.5 N NaOH was applied for 3 h. The cloth was then rinsed thoroughly with distilled water to remove NaOH. The wet cotton was blotted between paper towels to remove as much water as possible. Blotted cotton was subsequently rinsed with acetone and dry acetone to remove water. The dried cotton cloth was then
bathed in 10 mL of dry pyridine per gram of cotton for 3 h. Tosyl activation of the cloth was performed with the addition of 4 g tosyl in 8 mL dry acetone to the cotton-pyridine mixture and incubated for 3 h. When tosylation was completed, the solution (reaction mixture) was removed and the cotton cloth was washed with acetone and excess amounts of 5 mM HCl solution to remove the residues. The activated cotton cloth was then rinsed with distilled water and 0.1 M acetic acid buffer (pH 4.5). Enzyme immobilization was carried out by immersing the cloth overnight in 0.1 M acetic acid buffer (pH 4.5) solution containing the enzyme. After immobilization, the cloth was rinsed with copious amounts of the same buffer and kept in it until enzyme activity was determined. All reactions during tosyl activation and enzyme immobilization were carried out in either 125- or 250-mL Erlenmeyer flasks at room temperature in a shaker-incubator at 250 rpm. Typically, for each gram of cotton cloth, 50 mg of enzyme were added in 0.1 M acetic acid buffer for immobilization, and approximately 85% coupling yield and 30% activity yield were achieved. The immobilized enzyme had about 150 U/g cloth under the defined conditions. The cotton cloth with immobilized enzyme was then packed in glass column reactors for kinetic studies and to evaluate the enzyme stability and reactor performance.

4.2.3. Immobilized Enzyme Reactor

GOS formation kinetics with immobilized enzyme was studied in a recycle batch fixed-bed reactor (Figure 4.1). A small amount of cotton cloth (~4.5 g) was placed in the glass column reactor (internal diameter of 9 mm) with a water jacket maintained at a constant temperature (40 °C, unless otherwise noted). The lactose solution in the flask
(total solution volume, 50 mL) was continuously recirculated through the immobilized enzyme reactor at a flow rate of 90 mL/min. The lactose solution was prepared by dissolving lactose (99.9% purity; from Brewster Dairy, Brewster, Ohio) in 0.1 M acetic acid buffer solution (pH 4.5, unless otherwise noted). Samples (100 μL) were taken from the flask at appropriate time intervals and analyzed for sugar content by high performance liquid chromatography (HPLC). The reaction kinetics was studied at six different initial lactose concentrations (50, 100, 200, 300, 400, and 500 g/L), three different pH values (4.5, 5.2, 6.0), and three different temperatures (30, 40, 50 °C). The effects of galactose and glucose on GOS production were also studied by adding galactose (up to 100 g/L) or glucose (up to 100 g/L) in the lactose solution.

4.2.4. Thermal Stability of Enzyme

Thermal stability of the immobilized enzyme at various temperatures (40, 50, and 60 °C) was studied in continuous single-pass reactors (Figure 4.1). Cotton cloth (~1.25 g) containing immobilized enzyme was packed in the glass column (9 mm internal diameter), and the reactor was continuously fed with a lactose solution (200 g/L in 0.1 M acetic acid buffer, pH 4.5) at a constant flow rate (1 mL/min) and temperature for a period of up to three weeks. The concentration of glucose in the reactor effluent was monitored to determine the enzyme activity in the reactor. The thermal stability of free enzyme was also studied. The enzyme was dissolved in 0.1 M acetate buffer (pH 4.5) and incubated in a constant-temperature water bath. Samples were taken at appropriate time intervals and the residual enzyme activity was assayed.
4.2.5. GOS Production in Continuous Reactor

Continuous production of GOS from lactose was studied in a single-pass reactor (Figure 4.1). Approximately 20 g of cotton cloth was placed in the column reactor (internal diameter of 15 mm). Each piece of the cotton cloth (dimension: 9 cm x 2.7 cm) was tightly rolled into a cylinder and then packed in the reactor. Eight rolls of cloth were fitted on top of each other in the reactor, with a total packed bed length of ~21 cm (the bed volume was ~37 mL). Continuous production of GOS from lactose with the reactor was studied at 40°C to evaluate the reactor long-term performance. Initially, the reactor was fed with a lactose solution (0.1 M acetic acid buffer, pH 4.5) containing 400 g/L lactose for two days at ~37 mL/h. The feed rate was adjusted so that ~50% lactose conversion and the maximum GOS content could be obtained in the product stream. The reactor was then fed with 200 g/L lactose solution (pH 4.5) for another 12 days. When the feed conditions, such as the feed lactose concentration and flow rate, were changed, at least 4-5 bed volumes were fed to allow the reactor to reach steady state. Samples from the reactor effluent were then collected at proper time intervals and analyzed by HPLC.

4.2.6. Analytical Methods

4.2.6.1. Enzyme Activity Assay

The activity of immobilized enzyme on cotton cloth was measured with 10 g/L lactose as the substrate in 0.1 M acetic acid buffer (pH 4.5) at 40 °C in 125 mL Erlenmeyer flasks in a shaker-incubator at 450 rpm. At least three samples were taken during the enzyme reaction. The reaction in the sample was stopped by adding an equal
volume of 0.1 \textit{N} NaOH. The glucose concentration was determined with a glucose analyzer (YSI 2700 Select, Yellow Springs, OH). One unit of immobilized enzyme is defined as the amount of enzyme producing 1 \mu mol of glucose from lactose under the defined conditions.

\textbf{4.2.6.2. HPLC Analysis}

Concentrations of all sugars found in sample solutions (glucose, galactose, lactose, and GOS) were determined by HPLC. An HPLC system consisting of a pump (Waters 6000A), an autosampler (Waters WISP 710B), a carbohydrate analysis column (Phenomenex, Rezek RN M carbohydrate column, 7.8 x 150 mm), a column heater (Bio-Rad), a refractive index detector (Waters 410 differential refractometer), and a Shimadzu CLASS-VP chromatography data system (version 4.2 integrator) was used. The eluent was pre-degassed distilled water (at 85 °C) at a flow rate of 0.4 mL/min. Distilled water was degassed by first boiling and then sonication for 30 min. The column temperature was maintained at 85 °C and the detector temperature was set at 45 °C. Figure 4.2 shows a typical HPLC chromatogram. The concentrations (w/v) of these sugars (e.g., lactose, glucose, galactose, and oligosaccharides including tri-, tetra-, and penta-saccharides) are proportional to their peak areas with the same proportionality constant (Boon et al., 2000). Thus, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work. It should be noted that the accuracy of this approximation was verified by checking the material balance. Different disaccharides other than lactose were not observed by the HPLC.
method. It is possible that the lactose peak shown in the chromatogram might also contain other disaccharides, although it has been reported that the extent of disaccharide formation is limited with this enzyme as compared with other lactases (Dombo et al., 1997, Toba et al., 1985).

4.3. Results and Discussion

4.3.1. Kinetics of Immobilized Enzyme Reactor

Figure 4.3 shows typical batch kinetics for lactose hydrolysis and GOS formation catalyzed by the immobilized enzyme. The data represented the weight percentages of total sugars present in the reaction mixture rather than the actual concentration (w/v % or g/L) in the solution. The concentrations of various sugars in the solution can be calculated from the weight percentage times the initial lactose concentration. Initially, a high rate of GOS formation was accompanied by a rapid decrease in lactose concentration. As the reaction continued, GOS concentration decreased while glucose and galactose concentrations continued to increase. Glucose concentration was much higher than galactose concentration at lower conversions, indicating involvement of galactose in GOS formation. It is clear that transgalactosylation dominates early in the reaction, producing GOS with a high yield, while the hydrolytic activity of the enzyme takes over as the reaction continues. When the reaction was allowed to continue, only hydrolytic products (e.g., glucose and galactose) were present in the final product solution (data not shown). The decrease in lactose hydrolysis (and GOS formation) can be attributed to competitive inhibition of the enzyme by galactose (Bakken et al., 1991, Portaccio et al., 1998).
The time courses of various sugar concentrations during the reactions depend on the enzyme activity, and are thus not convenient for comparing GOS formation under various conditions. Figure 4.4 shows that the GOS production kinetics is closely related to lactose conversion, which is defined as conversion of lactose to the other sugars (monosaccharides and oligosaccharides). As can be seen in Figure 4.4, GOS production increased with increasing lactose conversion until a maximum was reached at ~50% conversion. Before the lactose conversion reached 40%, there was a constant increase in GOS concentration as well as increases in glucose and galactose concentrations. However, at higher conversions, there was a shift in the reactions to favor hydrolysis, which resulted in increased formation of monosaccharides (glucose and galactose) and decreased amounts of GOS.

As also shown in Figure 4.4, trisaccharides dominated among various types of GOS formed in the reactions. In fact, up to 20% lactose conversion, trisaccharides were almost the only type of GOS produced in the reaction. Larger GOS, including tetra- and penta-saccharides, only appeared at higher lactose conversions. Some hexa-saccharides were also observed but the amount was at a relatively insignificant level. In general, larger GOS reached their maximum levels at higher lactose conversions. Decrease in trisaccharides was accompanied by increases in other GOS. All types of GOS decreased as the reaction continued. At 50% lactose conversion where the total GOS peaked, the proportions of tri-, tetra- and penta-oligosaccharides were approximately 70, 25 and 5 % of total GOS formed, respectively.
About 20 different types of GOS formed by β-galactosidase from A. oryzae have been reported, and they consisted of mainly three and higher unit oligosaccharides rather than disaccharides (Boon et al., 2000, Dombo et al., 1997, Matsumoto et al., 1989, Toba et al., 1985). Toba et al (1985) identified ten different oligosaccharides consisting of 3 tri-, 4 tetra-, 2 penta- and 1 hexa-oligosaccharides. Structure analysis indicated that oligosaccharides formed by A. oryzae β-galactosidase had a lactose residue at the reducing end and galactose resides were only found at non-reducing positions linked with β-like linkages. For instance, a tetrasaccharide (4OS) structure was β-Gal-(1→6)β-Gal-(1→6)β-Gal-(1→4)-Glu (Toba et al., 1985). Deya (1990) indicated that β-Gal-(1→6)-β-Gal-(1→4)-Glu (6’galactosyl lactose) constituted ~70% of total GOS formed by A. oryzae β-galactosidase, which is in good agreement with the results from the present study. Unlike other β-galactosidases from E. coli, K. lactis, and K. fragilis, which have been reported to form substantial amounts of disaccharides with various structures (Mozaffar et al., 1985, Prenosil et al., 1987a,b), A. oryzae β-galactosidase does not produce much disaccharides (Prenosil et al., 1987a). In the recent studies by Iwasaki et al (1996) and Boon et al (2000) of the same enzyme, they neither indicated nor considered disaccharide formation in their kinetic models.

It is possible that some disaccharides such as allolactose and galactobiose might also be formed by transferring galactose to glucose and galactose, respectively, by the enzyme. The molar balances on glucose and galactose over the course of the reaction were checked based on the assumption that all GOS only contained one unit of glucose with the remaining sugars as galactose (Table B.4 in Appendix B). As shown in Figure
B.1 and B.2 in Appendix B, up to 50% lactose conversion, both glucose and galactose were accountable from all sugars present in the solution with less than 3% difference from the initial condition. This finding supports the assumption and suggests that galactose is not transferred to galactose to form galactobiose by the enzyme. It is thus also very likely that the enzyme would not transfer galactose to glucose to form allolactose. However, there was increasing imbalance in galactose and glucose as lactose conversion passed 50%, where trisaccharides began to decrease (Figure B.2 in Appendix B). The molar balance on galactose indicated that there were significant amounts of galactose missing, from 5% to 10% at 70% lactose conversion. In the mean time, there were excess amounts of glucose, from 2.5% to 5% at 70% lactose conversion, showed up in the molar balance on glucose. This imbalance suggested that there might have been some galactobiose formed from the hydrolysis of trisaccharides but was not separated from lactose in HPLC. However, this imbalance also could be resulted from errors in HPLC analysis since the sensitivity of galactose was slightly lower than that of glucose by ~2.5% but this difference in sensitivity was neglected in the data analysis. It should be noted that the HPLC method was able to detect additional disaccharide peaks near the lactose peak when the GOS samples prepared from K. lactis lactase were analyzed. However, these additional disaccharide peaks were never detected with GOS samples prepared from A. oryzae β-galactosidase. Thus, the lactose data shown in Figure 3 could also contain galactobiose and other disaccharides, but the amount of these disaccharides should be small or negligible. Further experiment with gas chromatography to analyze the reaction samples from latter stages of the reaction (>50% lactose conversion) will be necessary to clarify this point.
All types and sizes of GOS including transgalactosylated disaccharides are considered non-digestible oligosaccharides due to similar physiological characteristics, although some differences and strain specificities have also been reported (Sako et al., 1999). Yanahira et al (1995) reported that oligosaccharides containing lactose (4' or 6' galactosyl lactose) unit were better utilized by human intestinal bifidobacteria compared with non-lactosyl GOS. It should be noted, however, that 6'galactosyl lactose is a natural component in human milk (Deya, 1990). Thus, in terms of prebiotic effectiveness of GOS, mainly forming 6'galactosyl lactose at 70 % of total GOS and other higher lactose-containing GOS is an advantage of A. oryzae β-galactosidase.

4.3.2. Effects of Immobilization

Enzyme reaction kinetics may change upon immobilization. Diffusion limitation plays an important role not only on the reaction rate, where diffusion of substrate towards and products away from the enzyme matter, but also the type of the products formed in the case of competing reactions with varying substrate sizes. All of these plus product inhibition can be observed in GOS formation during lactose hydrolysis by β-galactosidase where two reactions occur and compete simultaneously. The adverse effect of diffusion limitation becomes more profound due to the viscosity of high lactose concentrations (Iwasaki et al., 1996). 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. In fact, the use of immobilized enzyme was suggested
for the purpose of lactose hydrolysis based on the expected mass transfer resistance effect
where oligosaccharide production would be limited (Prenosil et al., 1987a).

Decreases in GOS content upon immobilization of the enzyme have been observed with various enzymes, supports, and methods of immobilization (Prenosil et al., 1987a,b, Rugh, 1982, Sheu et al., 1998). The highest reported declines were 20-25 % in trisaccharides and 30-35 % in tetrasaccharides as compared with the free enzyme from 200 g/L lactose (Rugh, 1982, Sheu et al., 1998). The decreases were generally attributed to mass transfer resistance imposed by immobilization. Prenosil et al (1987b) demonstrated that the level of GOS was controlled by the degree of mass transfer resistance in a capillary membrane fixed reactor.

It is noted, however, that the GOS formation kinetics was almost identical for reactions carried out with the immobilized enzyme on cotton cloth and free enzyme in solution (Figure 4.5), suggesting that enzyme immobilization on cotton cloth does not impose any limitation or alteration on GOS formation from lactose. Thus, the GOS formation ability of the enzyme was not affected by the immobilization of the enzyme onto cotton cloth. This observation may well be accounted for a limited mass transfer influence on the enzyme reaction due to the characteristics of cotton cloth.

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4.3.3. Effects of Lactose Concentration

Initial lactose concentration is by far the most significant factor affecting GOS formation (Boon et al., 2000). Figure 4.6 shows that the production of oligosaccharides increased with increasing initial lactose concentration. Also as shown, GOS yields, based on the actual amount of lactose reacted, were higher with a higher initial lactose concentration at all lactose conversion levels. It is noted that GOS yields decreased with increasing lactose conversion due to increasing dominance of hydrolysis at lower lactose concentrations. β-Galactosyl groups should have a higher probability of attaching to lactose than water as an acceptor at increasing lactose concentrations (Iwasaki et al., 1996).

It was generally observed that the hydrolysis and transgalactosylation reactions occurred simultaneously. What dominates the product profile of the reaction is largely dependent on lactose concentration. The hydrolysis reaction dominates at low lactose concentrations, while GOS formation dominates at high lactose concentrations. As the initial lactose concentration increased from 50 g/L to 500 g/L, the maximum GOS content in the product increased from 10.5% (at 35% conversion) to 27% (at 52% conversion). The maximum GOS yield from lactose reacted also increased with the initial lactose concentration, from 45% to 71%. It is noted that the effects were more prominent on larger oligosaccharides. As the initial lactose concentration increased from 50 g/L to 500 g/L, the trisaccharide content doubled (from 9.1% to 18.3%), while tetrasaccharides and pentasaccharides increased by 5.3-fold (from 1.18% to 6.2%) and 13-fold (from 0.14 % to 1.85%), respectively.
4.3.4. Effects of Temperature and pH

The reaction rates are normally affected by pH and temperature, but these factors showed minimal effects, if any, on GOS production (Iwasaki et al., 1996, Monsan and Paul, 1995). As shown in Figure 4.7, the reaction rates for lactose hydrolysis and GOS formation increased with increasing temperature from 30 to 50 °C, but GOS production at all lactose conversion levels was almost unchanged with changing temperature. Similar situations were found with changing reaction pH from 4.5 to 6.0 (Figure 4.8).

4.3.5. Effects of Galactose and Glucose

It has been shown that allolactose, a disaccharide, can be synthesized from galactose and glucose, although the product yield was only less than 10% (Ajisaka et al., 1987b, Ajisaka and Fujimoto, 1989). Galactose is known as a competitive inhibitor to the lactose hydrolysis reaction (Bakken et al., 1991, Ogushi et al., 1980, Park et al., 1979, Portaccio et al., 1998, Shukla and Chaplin, 1993, Yang and Okos, 1989). From a thermodynamics point of view, a high galactose (product) concentration could favor the reverse hydrolysis reaction and cause an equilibrium shift towards condensation, thereby increasing GOS yield. However, the effects of monosaccharides on GOS formation rate and product yield have not been well studied. Thus, the possible effect of monosaccharides on GOS formation was investigated by adding various amounts of galactose and glucose into the initial reaction lactose solution. The concentrations of galactose and glucose were chosen to simulate their actual levels near, at, and after 50% lactose conversion, and the results are shown in Figure 4.9. As expected, the reaction
proceeded increasingly slowly with increasing amounts of galactose in the solution. However, contrary to expectations, additions of galactose and/or glucose actually decreased GOS production by 10-15%. Their effect on the rate of GOS formation was much more pronounced. Glucose was not reported to be an inhibitor to *A. oryzae* lactase, however, non-competitive inhibitions of some bacterial (Shin and Yang, 1998) and yeast enzymes (Cavaille and Combes, 1995, Simova et al., 1989) were indicated in the literature. It is clear that the monosaccharides produced during GOS formation would have adverse effects on the reactions. Hansson et al (2001) reported that GOS formation with β-galactosidase was more of a kinetically controlled transgalactosylation reaction rather than reversed hydrolysis, which is under thermodynamic control. Reversed hydrolysis or condensation may occur at high concentrations of monosaccharides upon prolonged incubations, as reported for other glycosidases (Ajisaka et al., 1987a, b, Nikolov et al., 1989, Pestlin et al., 1997). However, no appreciable amount of di- or oligosaccharides could be formed from glucose and/or galactose by *A. oryzae* lactase when the monosaccharides were present as the sole substrates at 50 g/L during the course of 5 h (Appendix C). Thus, it can be concluded that free glucose and galactose are not good for GOS formation and should be removed from the reaction mixture in order to increase GOS yield and formation rate.

**4.3.6. Thermal Stability of Immobilized Enzyme**

Figure 4.10 shows the enzyme activity decay over time at various temperatures. It is clear that the immobilized enzyme was stable at 40 °C under continuous operational
conditions, and was much more stable than the free enzyme. As also shown in Figure 10, thermal deactivation of this enzyme followed first-order kinetics. The deactivation rate constants \((k_d)\) under various temperatures can be estimated from the slopes of the semi-logarithmic plots. Based on the \(k_d\) data, the half-lives of the enzyme at various temperatures were estimated and are also listed in Figure 4.10. The immobilized enzyme had an estimated half-life of more than 1 year at 40 °C and 48 days at 50 °C. In general, the thermal stability of the enzyme was increased by 25-fold by immobilization on the cotton cloth, as compared with the free enzyme. The increase in the deactivation rate constant \(k_d\) with temperature followed the Arrhenius relationship, and the activation energy \(E_a\) was the same for both immobilized and free enzymes \((E_a/R = 2.8 \times 10^4\ K)\).

4.3.7. Continuous GOS Production from Lactose

Figure 4.11 shows the production of GOS from lactose in the continuous reactor. In general, the reactor performance was very stable, although lactose conversion and GOS production varied with the lactose concentration in the feed solution and the feed rate. With 400 g/L lactose in the feed at ~37 mL/h, 50% lactose conversion was attained and the outlet product stream contained 26.6% (w/w) GOS with a reactor productivity of 106 g/L/h. The reactor productivity was calculated from the final GOS concentration (g/L) times the feed rate and divided by the reactor volume (~37 mL). With 200 g/L lactose in the feed, 50% lactose conversion was attained at the feed rate of 69 mL/h, at which the final product contained 21.7% (w/v) GOS and the reactor productivity was 80 g/L/h. As also shown in Figure 4.11, as the flow rate increased from 71 to 127 mL/h,
lactose increased sharply yet GOS content decreased a relatively small extent while GOS productivity increased from 85 to 135 g/L/h. At the operating temperature of 40 °C, there was no notable loss of enzyme activity or reactor productivity over the entire 14-day period studied. A higher operating temperature (e.g., 45 °C) may be used to increase the reactor productivity since the immobilized enzyme has good thermal stability even at 50 °C.

Table 4.1 compares the reactor productivity and the maximum GOS content in the final product achieved in various studies reported in the literature. As can be seen in Table 4.1, the productivity obtained in this study was much higher than those of the reported values. The higher productivity obtained in this study can be attributed to the higher enzyme activity immobilized on the cotton cloth and the excellent properties of cotton cloth as support matrices in the packed bed reactor. Because of the high surface area and high porosity provided by the cotton cloth, the immobilized enzyme can be thoroughly exposed to the substrate. As already shown in Figure 4.5, the immobilized enzyme in the cotton cloth worked as efficiently as free enzyme, suggesting that there was no significant mass transfer effect caused by the enzyme support matrices. Also, the reactor was operated at a relatively low lactose conversion (~50%), which also might have contributed to the higher GOS productivity since inhibition from galactose was lower at lower conversion levels.

Bacterial (Berger et al., 1995a, Shin et al., 1998) and yeast enzymes (Foda and Lopez-Leiva, 2000) usually maximize GOS at higher lactose conversions (~70 %) and
gave higher GOS contents and product yields. It is noted, however, that the higher GOS yields (above 50%) also include significant amounts of disaccharides, which are not significant in the reaction with *A. oryzae* β-galactosidase. As lactose hydrolysis continues, the reaction rate is greatly reduced due to the increase in the inhibitory monosaccharides. Consequently, with bacterial and yeast enzymes, higher GOS concentrations and yields are achieved at the expense of lower productivities (Shin et al., 1998). For economical production of GOS, a high productivity is more important than its process yield alone, since lactose is relatively inexpensive and can be found in whey, an abundant byproduct from cheese manufacturing. Thus, a low maximal GOS concentration of 25-30 % in the final product from *A. oryzae* β-galactosidase, as compared to 40-55 % from other enzymes, is compensated by the much higher reactor productivity.

A good operational stability is one of the most important criteria for industrial utilization of immobilized biocatalyst. Sheu et al (1998) used immobilized β-galactosidase from *A. oryzae* for continuous production of GOS in a reactor for 30 days at 30 °C without significant loss of enzyme activity. However, they did not employ higher temperatures. Nakanishi et al (1983) observed reversible inactivation of *B. circulans* β-galactosidase immobilized onto Duolite ES-762, Dowex MWA-1 or sintered alumina for continuous reaction in a plug-flow reactor (PFR). The authors ascribed the loss of activity in the PFR to an accumulation of oligosaccharides in the support, thereby blocking mass transfer. Thus, they could not evaluate the operational stability in the PFR. Shin et al (1998) immobilized β-galactosidase from *B. singularis* on chitosan beads and found that
~10% of the enzyme was leached from the beads at high flow rates. Consequently, they had to reduce the flow rate for long-term operation, resulting in reduced productivity. In our study, we have used both high and low flow rates and found no leaching of enzyme activity from the carrier. We also did not observe any accumulation of oligosaccharides in the reactor that could lead to a decrease in enzyme activity even at 40% lactose concentration.

It should be mentioned that we did not experience any difficulties or observed any microbial contamination even at 20% lactose feed and 40 °C for a prolonged operational period. This was probably because the high sugar concentrations used that reduced water activity and greatly delayed or even prevented any bacterial growth. It was also likely that the high flow rate and short residence time (0.5 h or lower) used with the enzyme reactor did not allow any chance for potential contaminants to attach on the cotton cloth and grow in the reactor. It is also noted that the desirable lactose concentration for GOS production is much higher than that in milk and whey. Concentrated lactose streams can be obtained by evaporation and/or reverse osmosis. It is yet to be determined as for which lactose concentration should be used for economical production of GOS from lactose.

4.4. Conclusions

GOS production from lactose by A. oryzae β-galactosidase immobilized on cotton cloth was mainly affected by the lactose concentration in the reaction media. More GOS can be produced with a higher lactose concentration feed. Temperature and pH affected
the reaction rate, but did not result in any change in GOS formation. The presence of galactose and glucose at high concentrations inhibited the reactions and reduced GOS yield by as much as 15%. The cotton cloth as the support matrix for enzyme immobilization neither imposed any significant diffusion limitation nor affected GOS formation characteristics of the enzyme. The thermal stability of the enzyme increased by 25-fold upon immobilization on cotton cloth. Cotton cloth also has many physical characteristics that are ideal for use as reactor packing. The high porosity, low pressure drop, and high mechanical strength of cotton cloth allow the enzyme reactor to be operated under a high flow rate even with a concentrated lactose feed that has a high viscosity (2.12 – 6.6 mPa). Therefore, cotton cloth is an effective and inexpensive support that suffices as a biocatalyst carrier. The immobilized enzyme on cotton cloth was stable and had a long half-life at temperatures between 40 and 50 °C. Consequently, the continuous enzyme reactor had a high productivity and stable long-term performance, and is ideal for GOS production from lactose.
<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Mode of Process&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reaction conditions</th>
<th>Max GOS&lt;sup&gt;b&lt;/sup&gt; (wt %)</th>
<th>Productivity (g/L/h)</th>
<th>Reference</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Lactose conc. (g/L)</td>
<td>T (°C)</td>
<td>pH</td>
<td></td>
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<tr>
<td>B. circulans</td>
<td>Batch (FE)</td>
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<td>40</td>
<td>6.0</td>
<td>24.0</td>
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<tr>
<td></td>
<td>Continuous (IE, CSTR)</td>
<td>45.6</td>
<td>40</td>
<td>6.0</td>
<td>40.0</td>
</tr>
<tr>
<td>B. singularis</td>
<td>Batch (IE)</td>
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<td>45</td>
<td>3.7</td>
<td>54.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Continuous (IE, PBR)</td>
<td>100</td>
<td>45</td>
<td>4.8</td>
<td>55.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>T. aquaticus</td>
<td>Batch (IE)</td>
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<td>70</td>
<td>4.6</td>
<td>34.8</td>
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<tr>
<td>K. lactis</td>
<td>Batch (FE, UF)</td>
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<td>45</td>
<td>7.0</td>
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<td>Continuous (IE, FBR)</td>
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<td>40</td>
<td>4.5</td>
<td>21.7</td>
</tr>
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<td></td>
<td></td>
<td>400</td>
<td>40</td>
<td>4.5</td>
<td>26.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> FE: free enzyme, IE: immobilized enzyme, CSTR: continuous stirred tank reactor, PBR: packed bed reactor, UF: ultrafiltration membrane reactor, FBR: fibrous bed (cotton cloth) reactor.

<sup>b</sup> Max GOS is a weight percent of GOS based on the total sugars in the reaction mixture.

<sup>c</sup> GOS content also includes disaccharides.

Table 4.1. GOS production by various β-galactosidases in batch and continuous operation.
Figure 4.1. Schematic diagram of the immobilized enzyme cotton-cloth reactor used in this study. The system was operated either as a recycle batch reactor (with recirculation) or a continuous single-path reactor (without recirculation).
Figure 4.2. A typical HPLC chromatogram showing various sugars found in lactose hydrolysis and galacto-oligosaccharide formation catalyzed by the immobilized enzyme. Elution time (min): 10.1 for hexasaccharide (6-OS), 12.2 for pentasaccharide (5-OS), 12.8 for tetrasaccharide (4-OS), 13.8 for trisaccharide (3-OS), 16.2 for lactose or disaccharide, 19.5 for glucose, and 20.5 for galactose.
Figure 4.3. Reaction kinetics of lactose hydrolysis and GOS formation catalyzed by immobilized enzyme in the recycle batch reactor at 40 °C with an initial lactose concentration of 400 g/L.
400 g/L Lactose, pH 4.5, 40 °C

Figure 4.4. Kinetics of GOS formation during lactose hydrolysis with 400 g/L initial lactose in the recycle batch reactor at 40 °C.
Figure 4.5. Comparison of GOS formation during lactose hydrolysis by free enzyme in solution and immobilized enzyme on cotton cloth.
Figure 4.6. Effect of initial lactose concentration on GOS production and yield from lactose reacted during lactose hydrolysis catalyzed by immobilized enzyme in the recycle batch reactor.
Figure 4.7. Effect of temperature on GOS production rate and yield during lactose hydrolysis catalyzed by immobilized enzyme in the recycle batch reactor.
Figure 4.8. Effect of pH on GOS production rate and yield during lactose hydrolysis catalyzed by immobilized enzyme in the recycle batch reactor.
Figure 4.9. Effect of galactose and glucose on GOS production rate and yield during lactose hydrolysis catalyzed by immobilized enzyme in the recycle batch reactor.
**Figure 4.10.** Thermal stability of free enzyme in solution and immobilized enzyme on cotton cloth at various temperatures.
Figure 4.11. Continuous production of GOS by immobilized enzyme on cotton cloth packed in a single-path reactor operated at 40 °C and various flow rates. The lactose concentration in the feed solution was 400 g/L for the first two days, and was changed to 200 g/L afterwards.
CHAPTER 5

IMMOBILIZATION OF β-GALACTOSIDASE ON FIBROUS MATRIX BY POLYETHYLENIMINE FOR PRODUCTION OF GALACTO-OLIGOSACCHARIDES FROM LACTOSE

5.1. Introduction

Biocatalyst immobilization is gaining increased attention for the synthesis of industrial bio-products ranging from neutraceuticals to chemicals. Enzyme immobilization provides many important advantages over use of enzymes in soluble form, namely, enzyme reusability, continuous operation, controlled product formation, and simplified and efficient processing. The main challenges in enzyme immobilization include not only containment of large amount of enzyme to be immobilized while retaining most of its initial activity, but also the performance of immobilized enzyme in actual production processes in industrial type reactors. Thus, the success of immobilized enzyme is not only driven by its applications, but also relies on a number of factors,
including enzyme, support, chemical reagent, and reactor. The enzyme support is generally considered as the most important component contributing to the performance of the immobilized biocatalyst reactor. In addition to being a very inexpensive and widely available fibrous material, cotton cloth provides a number of desirable characteristics, including high porosity (>$95\%$), large specific surface area, and excellent mechanical strength. Cotton cloth has been successfully used in cell immobilization and fermentation studies (Huang and Yang, 1998, Talabardon et al., 2000, Yang et al., 1998, Yang et al., 1995). Cotton cloth immobilized enzyme placed in a loose spiral shape in a plug-flow type reactor provides good flow rates, low pressure drop and negligible mass transfer resistance. These characteristics are also highly desirable for industrial enzyme application. Thus, cotton fabric also can be used for the development of an industrially applicable fibrous bed enzyme bioreactor where the immobilized enzyme functions as good as soluble enzyme.

Although enzymes can be immobilized on cotton cloth activated with tosyl chloride, the method is somewhat tedious and involves the use of organic chemicals (Albayrak and Yang, 2001). The goal of this research was to develop a novel enzyme immobilization method using polyethylenimine (PEI) for various fibrous matrices including cotton cloth so as to lead the development of an effective fibrous-bed biocatalyst reactor. PEI, an extremely branched cationic chain polymer (Zemek et al., 1982), has found for many applications in biochemistry with regards to its electrostatic interaction with negatively charged species (Jendrisak, 1987). PEI has been an essential ingredient of many enzyme immobilization procedures where it serves to coat an inert
support such as porous glass microbeads (Wasserman and Hultin, 1980) or charged insoluble carriers (Emneus and Gorton, 1993, Yang et al., 1994a). Cotton cloth coated with PEI has been used as a support for immobilization of several enzymes, including glucose oxidase (Kumar et al., 1997), urease (Kamath et al., 1988, Das and Kayastha, 1998) and invertase (Yamazaki et al., 1984), and yeast cells (D’Souza and Kamath, 1988). In these applications, PEI was adsorbed on the cotton cloth and then excess PEI was washed away with water or buffer solution. The remaining PEI was then crosslinked with glutaraldehyde (GA) before (Kamath et al., 1988, Das and Kayastha, 1998) and/or after enzyme coupling (Yamazaki et al., 1984, D’Souza and Kamath, 1988, Kamath et al., 1988, Das and Kayastha, 1998). Although these studies used the desirable characteristics of cotton cloth as the fibrous matrix for enzyme immobilization, the amount of enzyme immobilized was rather low and need to be improved.

Further goal of this study was to evaluate the performance of cotton cloth immobilized enzyme for galacto-oligosaccharides (GOS) production from lactose. While lactose found in cheese whey is an abundant byproduct from dairy industry, GOS is a prebiotic functional food ingredient, which selectively stimulate the growth of bifidobacteria in the lower part of human intestine (Sako et al., 1999). The introduction of GOS into food products is thus desirable for its many health benefits (Sako et al., 1999). Commercial potential for the production and applications of GOS in food product lines is high (Playne and Crittenden, 1996, Shin et al., 1998), but an economical production process still needs to be developed. There has been a steady 3% annual increase in cheese production (Foda and Lopez-Leiva, 2000). The already problematic lactose is thus
expected to be a major concern for the dairy industry. Although there have been extensive research for better utilization of whey lactose, the dairy industry is still in need of new technologies for converting lactose into marketable products (Yang and Silva, 1995). Thus, converting lactose into a valuable food ingredient that is free of problems associated with lactose is of benefit and highly desirable by the food industry (Playne and Crittenden, 1996).

Production of GOS by immobilized β-galactosidase has been considered in several studies. However, GOS production from immobilized enzymes has not been addressed very well (Berger et al., 1995a, Mozaffar et al., 1987). Many of the carriers used for immobilization of β-galactosidases applied in GOS production are some types of microparticles, such as ion exchange resins (Matsumoto et al., 1989, Mozaffar et al., 1986a), chitosan beads (Sheu et al., 1998, Shin et al., 1998), cellulose beads (Kminkova et al., 1988), and agarose beads (Berger et al., 1995b). In addition to operational (back pressure, aggregation, clogging) and economical (expensive) disadvantages, commonly noted diffusion limitations in these immobilized systems not only reduce the reaction rate in general, but also affect the product spectrum and specifically reduce GOS formation. For example, 20 to 30 % decreases in the GOS formation has been reported with immobilized enzymes due to introduction of mass transfer resistance in the system (Prenosil et al., 1987a, b, Pederson et al., 1985, Rugh, 1982, Prenosil and Heider, 1985).

In this research, the development of a novel enzyme immobilization technique for fibrous support such as cotton cloth involving the use of PEI for β-galactosidase from A.
oryzae, and application of the immobilized enzyme in GOS production were addressed. In this work, PEI was used in such a way that the exterior surface of the cotton fibrils in the knitted form was coated with large PEI-enzyme aggregates of high activity while preserving the desirable features of cotton cloth such as mechanical strength and high porosity. Various factors affecting PEI-enzyme aggregate formation in solution and growth of aggregates on cotton fibrils leading to the development of a ‘multilayer’ immobilization of the enzyme were investigated. Cotton cloth immobilized enzyme was evaluated in GOS production from lactose in a packed-bed reactor. The effects of enzyme loading, pH, and temperature on the kinetics of GOS formation of the immobilized enzyme were also studied. Comparisons for various fibrous supports used for enzyme immobilization and for performance of GOS production with free enzyme reactions and other immobilized enzyme studies are also discussed here.

5.2. Materials and Methods

5.2.1. Enzyme and Reagents

β-galactosidase from *Aspergillus oryzae* (Fungal lactase activity 100,000 U/g) was obtained from Genencor International (Rochester, New York, USA). Each gram of the enzyme contained 100,000 fungal lactose units (FLU). One unit is defined as the amount of enzyme that liberates one μmol o-nitrophenol from o-nitrophenyl-β-galactopyranoside (ONPG) per min at pH 4.5 and 37 °C (Genencor). Lactose (99.9%) was donated by Brewster Dairy (Ohio, USA). Polyethylenimine [PEI; (C₂H₅Nₙ)] 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). Glacial acetic acid (Fisher) and sodium acetate trihydrate (J.T. Baker, Phillipsburg, NJ.) were used to prepare acetic acid buffer. Cotton terry cloth and nonwoven polyethylene terephthalate (PET) fabrics were 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. PEI – Enzyme Aggregate Formation

The procedures to form PEI-enzyme complex/aggregate by mixing PEI and enzyme in solution are illustrated in Figure 5.1. Various amounts of PEI (0.15 mg - 0.60 mg in 0.1 mL solution) were mixed with 1 ml of 5 mg/mL enzyme solution in microcentrifuge tubes to study the effect of PEI concentration (or the ratio of PEI to enzyme) on the formation of PEI-enzyme aggregates. After ~5 min, 0.1 ml of 0.2% GA solution was added to the mixture. The mixture containing PEI-enzyme aggregates was centrifuged at 10,000 rpm for 1 min. Initial enzyme activities associated with the PEI-enzyme slurry (containing GA) and supernatant was determined and compared with free enzyme (containing neither PEI nor GA). The morphology of PEI-enzyme aggregates in cloudy turbid slurry was analyzed with a light microscope.

5.2.3. Enzyme Immobilization on Cotton Cloth

The procedures for PEI-enzyme immobilization on cotton cloth are illustrated in Figure 5.2. 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 crosslinking of PEI-enzyme aggregates coated on the cotton. The crosslinked cottons were washed extensively with distilled water and then with acetic acid buffer (0.1 M, pH 4.5). The solutions were kept cold on ice right until use. The treated cotton cloth with immobilized enzyme was stored in the buffer (0.1 M, pH 4.5) and refrigerated until use. All procedures were carried out in 125 mL Erlenmeyer flaks, and incubations were performed in a shaker-incubator (Lab-Line) at 150 rpm at room temperature. It is noted that two procedures were developed in this study. The first one also involved washing after PEI adsorption and thus presumably produced “monolayer” enzyme immobilization on the cotton fibrils. The second procedure did not wash after PEI coating and thus presumably produced “multilayer” enzyme immobilization.

5.2.3.1. Monolayer

The method was a modification of the procedure developed by Yamazaki et al (1984) for invertase (Canadian Patent Number 1203187). A large volume of PEI solution (50 ml per gram cotton cloth) was allowed to adsorb to cotton cloth for 2 h. After adsorption, cotton cloth was extensively washed under running distilled water to remove excess PEI from the cotton. The washed cloth was blotted between paper towels and was soaked in enzyme solution for 2 h. Enzyme adsorbed cotton was then crosslinked with 2% GA for 2 h.
5.2.3.2. Multilayer

Unless otherwise noted, 1 mL of PEI solution (pH 8.0) containing 2.2 mg of PEI was added to each 0.2 g piece of cotton cloth. 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. Upon the addition of enzyme to PEI adsorbed cotton; a ‘milky’ turbid solution was formed. The flasks were put into a shaker incubator for 5-10 min. Within 5 min, the white turbidity disappeared and the coupling solution was completely clarified. The clarified coupling solution was slowly decanted and PEI-enzyme coated cottons were immersed in a cold GA solution (0.2 % (w/v), pH 7.0) for crosslinking for 5 min. The crosslinked cottons were washed extensively with distilled water and then acetic acid buffer (0.1 M, pH 4.5). It is important to note that there was no washing step until the completion of GA crosslinking.

5.2.4. Reaction Kinetics

GOS formation kinetics with immobilized enzyme was studied in a recycle batch packed-bed reactor (Figure 5.3). A small piece of cotton cloth (~ 0.4 g) was placed in the glass column reactor (internal diameter of 9 mm) with a water jacket maintained at a constant temperature (40 °C, unless otherwise noted). The lactose solution in the flask (total solution volume, ~ 85 ml) was continuously recirculated through the immobilized enzyme reactor at a high flow rate (90 ml/min). The lactose solution was prepared by dissolving lactose in 0.1 M acetic acid buffer solution (pH 4.5, unless otherwise noted).
Samples (100 μL) were taken from the flask at appropriate time intervals and analyzed for sugar contents by high performance liquid chromatography (HPLC). The reaction kinetics was studied at 400 g/L lactose solution for three different levels of enzyme loading (35, 130, 240 mg enzyme /g cotton), two different pH values (4.5, 6.0), and two temperatures (40, 50 °C).

GOS formation kinetics of PEI-enzyme aggregates and free enzyme was also investigated under similar conditions. In order to prepare aggregates in solution, PEI solution (1 mL, 0.22% w/v, pH 8.0) was mixed with 10 ml enzyme solution (5 mg/mL) and incubated for 10 min. After incubation, 1 mL GA solution (0.2% w/v) was added and the incubation was continued for 5 min. The solution (~12 mL) containing PEI-enzyme aggregates was added to 50 mL of lactose solution (440 g/L in 0.1M acetic acid buffer, pH 4.5) in 125 mL Erlenmeyer flaks and the reaction was carried out at 40 °C, 250 rpm in a shaker-incubator. For control, free enzyme solution just contained distilled water, instead of PEI and GA solutions, and same conditions were used for GOS formation. Samples (100 μL) were drawn from the reaction mixtures at appropriate time intervals and added to 900 μL distilled water at 95 °C to stop the enzyme activity. The sugar contents were analyzed by HPLC.

5.2.5. Stability of Immobilized Enzyme

The thermal stabilities of PEI-immobilized enzyme in 0.1 M acetate buffer (pH 4.5) at 50 and 60 °C were studied in a constant-temperature water bath. During
incubation, the cloth was removed at appropriate time intervals and the residual enzyme activity was assayed. Operational stability of PEI-immobilized enzyme on cotton cloth was also studied in a single-pass continuous reactor (see Fig 5.3) at 40 °C. Cotton cloth immobilized at the level of 150 mg/g cotton as described above was used in this study. The reactor packed with cotton cloth was continuously fed with a lactose solution (100 g/L in 0.1 M acetic acid buffer, pH 4.5) at a constant flow rate (100 ml/h) and temperature for a period of 155 hours. Samples from the reactor effluent were collected at proper time intervals and analyzed by HPLC.

5.2.6. Scanning Electron Microscopy (SEM)

Fibrous matrix samples were dried in a critical point dryer. After being sputter-coated with gold/palladium, the samples were examined using a scanning electron microscope (Philips XL-30).

5.2.7. Analytical Methods

5.2.7.1. Enzyme Assays

The activity of cotton cloth immobilized enzyme was measured with 100 g/L lactose as the substrate in 0.1 M acetic acid buffer (pH 4.5) at 40 °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 taken and mixed at one to one ratio with 0.1 N NaOH to inactivate possible free enzyme activity leached during activity determination. The
glucose concentration in the sample was determined with a glucose analyzer (YSI 2700 Select, Yellow Spring, OH). The activity of immobilized enzyme was determined by direct comparison of reading from the free enzyme activity curve [(mg/ml)· min vs. g/L glucose] and then used to estimate the amount of active enzyme (mg/g cotton) and immobilization yield (%).

5.2.7.2. HPLC Analysis

The concentrations of sugars in sample solutions (glucose, galactose, lactose, and galacto-oligosaccharides) were determined by HPLC. An HPLC system consisting of a pump (Waters 6000A), an autosampler (Waters WISP 710B), a carbohydrate analysis column (Phenomenex, Rezek RNM Carbohydrate column, 7.8 x 150 mm), a column heater (BioRad), a refractive index detector (Waters 410 Differential Refractometer), and a Shimadzu CLASS-VP Chromatography Data System (version 4.2 integrator) was used. The eluent was pre-degassed distilled water (at 85 °C) at a flow rate of 0.4 ml/min. Distilled water was degassed by first boiling then sonication for 30 min. The column temperature was maintained at 85 °C and the detector temperature was set at 45 °C. A typical HPLC chromatogram is shown in Fig 5.4. The concentrations (w/v) of these sugars (e.g., lactose, glucose, galactose, and oligosaccharides including tri-, tetra-, and penta-saccharides) are proportional to their peak areas with the same proportionality constant (Boon et al., 2000). Thus, the normalized sugar concentrations, presented as weight percentages of total sugars, were determined from peak heights.
5.3. Results and Discussion

5.3.1. PEI – Enzyme Aggregate Formation

It has been long known that highly branched and positively charged PEI molecules formed electrostatic complexes with negatively charged species such as proteins and nucleic acids (Jendrisak, 1987, Dissing and Mattiasson, 1996, Onda et al., 1999). We have also observed that when a clear enzyme solution in distilled water was mixed with a PEI solution, cloudy, turbid or ‘milky’ slurry of PEI-enzyme aggregates were formed instantaneously. It was also observed that within a few minutes the initial homogenous milky solution led to larger particles that eventually precipitated upon standing. A proposed mechanism for the PEI-enzyme association leading to the formation of aggregates is shown in Figure 5.1A, which also reflects the relative sizes of PEI (MW. 750,000) and enzyme (MW. 110,000). Figure 5.1B shows the morphology of PEI-enzyme aggregates observed under a light microscope. These PEI-enzyme aggregates were approximately 10-50 micron in diameter.

PEI-enzyme association and precipitation seemed to be the driving force of enzyme immobilization on cotton cloth. Therefore, the effects of various factors, such as PEI to enzyme ratio, pH, and presence of buffer, on the activity of PEI-enzyme aggregates and remaining activity in the supernatant after centrifugation of the cloudy solution were investigated. As shown in Figure 5.5, the concentration of PEI did not affect the enzyme activity at all ratios studied when the initial pH of PEI solution was adjusted to ~8.0. PEI-enzyme aggregate formation (cloudy solution) does not necessarily
yield precipitation. The highest amount of enzyme precipitate was obtained at the PEI to enzyme weight (mg/mg) ratio of 1/20-25, while at higher or lower ratios of PEI to enzyme yielded ineffective particle formation that stayed in solution. The PEI to enzyme ratio of 1/50 produced lightly turbid solution but no precipitation even after centrifugation, while 1/100 produced no turbidity and no precipitation whatsoever (data not shown).

Although PEI to enzyme ratio was found to be the most critical factor regarding PEI-enzyme aggregate formation and the activity of the complex, pH and presence of especially negatively charged salt ions in the buffer solution were of no less importance. It was observed that the optimum pH range was between 6 and 8 where similar precipitation and activity were obtained. As the pH of PEI enzyme slurry was lowered to below 5, especially below 4, the turbid solution became clear and no precipitation occurred. At pHs above 8, aggregation and precipitation were not affected but enzyme lost its activity. It was also observed that when enzyme solution was prepared in acetate or phosphate buffer (0.1 M), regardless of the pH, most of the enzyme, ~ 90-95%, stayed in the solution. Thus, PEI enzyme aggregate formation was totally reversible. The aggregates can be dissociated upon lowering the pH, and the enzyme in the PEI-enzyme complex can be replaced by small negatively charged species.
5.3.2. Enzyme Immobilization on Cotton Cloth

5.3.2.1. Monolayer

PEI has been used in many enzyme immobilization procedures with many different types of support and enzyme. In most procedures, excess PEI was removed by generally washing with distilled water (Yamazaki et al., 1984, Dekker, 1989, Emneus and Gorton, 1993, Bardeletti, 1997, Mateo et al., 2000, Isgrove et al., 2001). The enzyme immobilization procedure using PEI and cotton cloth developed by Yamazaki et al (1984) was employed for immobilization of β-galactosidase from *A. oryzae* in this study. In this method, excess PEI solution was removed after adsorption onto cotton by washing with distilled water. Therefore, only a monolayer of PEI molecules is expected to stay on the cotton fibrils because individual PEI molecules would repel each other. Thus, this monolayer of PEI molecules can only interact with enzyme and other species carrying the opposite charges in the solution. The effects of several variables including PEI concentration (0.01-2% w/v), PEI adsorption time (10-120 min), enzyme concentration (10-300 mg/g), coupling time (0.8-22 h), and GA crosslinking concentration (0.01-2% w/v) and time (5-120 min) were tested. However, only the initial concentration of enzyme affected the level of immobilization. As shown in Figure 5.6, the amount of immobilized enzyme on cotton increased with increasing initial enzyme amount from 10 to 30 mg with a high immobilization yield of 95%. However, at higher initial enzyme concentrations, the level of immobilized enzyme stayed almost unchanged and thus the immobilization yield went down. Thus, a maximum capacity of 25-30 mg enzyme per
gram of cotton can be adsorbed on the monolayer PEI-cotton cloth. When the enzyme solution was prepared in 0.1 M phosphate or acetate buffer, only 10-12 or 20-25% of immobilized enzyme was obtained, respectively, compared with that obtained in distilled water. Obviously, small and negatively charged buffer molecules out-competed the enzyme for adsorption to PEI and greatly reduced the immobilization efficiency.

5.3.2.2. Multilayer

For multilayer enzyme immobilization on cotton cloth, the most critical factor affecting the level of immobilized enzyme should be the PEI to enzyme ratio. A range of PEI to enzyme ratios from 1/5.7 to 1/80 was investigated. As shown in Figure 5.6, increasing the PEI amount from 11 mg to 44 mg for 250 mg enzyme per gram of cotton resulted in a decrease in the immobilized enzyme amount from 218 to 138 mg/g. It was found that the maximum enzyme immobilization could be achieved when the PEI to enzyme ratio was near 1/20-25. This ratio was consistent with the optimal ratio found for the formation of PEI-enzyme aggregates. As also shown in Figure 5.6, the amount of immobilized enzyme increased almost proportionally with the initial amount of enzyme in solution up to 350 mg/g cotton at the ratio of 1/22-25. Under this condition, a relatively constant immobilization yield of 80-90% was obtained. Further increasing the enzyme amount above 350 mg/g cotton reduced the immobilization yield. Thus, PEI to enzyme ratio of 1/22 was kept constant at 250 mg enzyme per gram of cotton cloth for the rest of this study.
PEI-multilayer enzyme immobilization on cotton cloth was also dependent on pH and temperature (Fig 5.7). The pH of PEI-enzyme aggregate was driven by the initial pH and the concentrations of reactants since no buffer was used in the preparation of both PEI and enzyme solutions. The solution of 0.22% PEI had a pH value of about 9.5-10. When no pH adjustment was done to the PEI solution, the final pH of the PEI-enzyme cloudy solution was about 8.2-8.4. When the pH of PEI solution was adjusted to between 6.0-8.0 and the enzyme was dissolved in distilled water (pH 6.6), insignificant differences were observed in the immobilization yield. When the pH of PEI enzyme cloudy solution was reduced to 3.5, the solution lost the cloudy appearance and very little enzyme was immobilized. The PEI solution was normally prepared by using distilled water. When the PEI solution was prepared in 0.05 M phosphate buffer, very low yield of enzyme immobilization was achieved (Fig 5.7). Clearly, phosphate bearing negative charges competed with enzyme for interacting with PEI and essentially blocked the formation of PEI-enzyme aggregates. Ions with positive charges, on the other hand, would cover the enzyme, and consequently, PEI would not be able to reach or would be repelled by the enzyme. Since ionized buffer species are small compared with the enzyme, the immobilization capacity of PEI would be reduced. Therefore, no buffer should be used and the solution pH should be kept in the range of 6-8 during the PEI-enzyme coupling reaction.

The temperature for PEI enzyme aggregate immobilization also affected the activity of immobilized enzyme. The enzyme solution was kept on ice until added but the temperature was not controlled during enzyme coupling. Cold enzyme solution (between
0 and 4°C) produced a higher immobilized enzyme activity yield and more rapid enzyme immobilization (Fig 5.7). The cloudiness of enzyme-PEI mixture was cleared within 5 min, and over 95% of the activity associated with the initial enzyme solution was retained on the cotton cloth. Moreover, more reproducible results were obtained when cold enzyme solution was used.

The final and a crucial step of the PEI enzyme immobilization was GA crosslinking. If no crosslinking was performed, most of the enzyme would be leached out from the aggregates during enzyme activity determination (due to the presence of acetate buffer). For instance, the presence of phosphate buffer (0.05 M) during GA crosslinking reduced the yield of immobilization to 10%. Once PEI-enzyme aggregates were coated on the cotton cloth, the enzyme solution was decanted and GA solution was added to permanently glue the aggregates on the support. Similar to the PEI-enzyme coupling reaction, the result of the cross-linking reaction seemed to be also affected by the temperature. Using a cold GA solution tended to produce a higher final enzyme activity and more reproducible results. However, variations in the concentration (0.05 to 0.2 %, w/v) and pH (6 to 8) of the GA solution and the reaction time (5 to 120 min) did not show any significant effect on the final activity of immobilized enzyme. Thus, 0.1% GA for 5 min was applied in the rest of this study.

It is noted that the color of the cotton cloth coated with PEI enzyme aggregates remained white as normal but changed to light yellow after GA crosslinking. During GA crosslinking, a light yellow color was developed within 3-5 min and there was no further
change in color upon prolonged incubation. The strength of the color (darkness) seemed to be directly associated with the concentrations of GA, PEI and enzyme. The higher the concentrations of GA, PEI and enzyme, the darker the color was. It appeared that once the color was completely developed, the GA cross-linking reaction was also completed and there was no further change (decrease or increase) in the final enzyme activity.

5.3.3. GOS Formation Kinetics

GOS formation kinetics from lactose with the multilayered PEI immobilized enzyme was studied in packed-bed reactors. Figure 5.8 shows typical reaction kinetics for lactose hydrolysis and GOS formation. In general, a high rate of initial GOS formation was accompanied with rapid decrease in lactose concentration. As reactions continued, GOS formation leveled off and then decreased while glucose and galactose continued to increase. The amount of galactose produced from lactose hydrolysis was less than that of glucose because galactose was also used to form GOS. Figure 5.9 shows that the GOS production kinetics as affected by lactose conversion, defined as conversion of lactose to the other sugars. As seen, a maximum GOS production was obtained at ~50% lactose conversion. As also shown in Figure 5.9, the GOS produced from the reaction was primarily composed of trisaccharides (3-OS). Larger GOS such as tetra- and penta-saccharides were produced at lower levels and their production peaked at higher lactose conversions, suggesting successive conversions to higher oligosaccharides (from 3-OS to 4-OS and then to 5-OS, etc.). At 50% lactose conversion where the total
GOS peaked, the proportions of tri-, tetra- and penta-oligosaccharides were approximately 70, 25 and 5% of total GOS formed, respectively.

5.3.3.1. Effect of PEI-Enzyme Aggregate

Although the activity of the enzyme was not impaired by the relatively large PEI-enzyme aggregates (10-50 micron in diameter), as shown in Figure 5.5, the conditions used for GOS production might encounter severe mass transfer limitation with the larger GOS molecules. The high lactose concentrations (~400 g/L) and lactose conversion (~50%) used to favor GOS formation might not work as well with the large enzyme aggregates since there would be some internal mass transfer resistance introduced upon aggregation of the enzyme with PEI. Due to the viscosity of the lactose solution (Iwasaki et al., 1996), formation of GOS products larger in size and simultaneous release of small monosaccharides known to be inhibitory to the enzyme, mass transfer limitations would cause a reduction in GOS formation. However, as shown in Figure 5.10A, the reaction kinetics of PEI-enzyme aggregates was unchanged as compared to the soluble enzyme reaction. Almost identical curves for lactose hydrolysis and GOS formation were observed. Not only the catalytic activity was preserved but also was the GOS formation characteristics, indicating that the PEI-enzyme aggregates were highly porous and permeable, and did not impose any adverse effect caused by diffusion.
5.3.3.2. Effects of Enzyme Loading

The possible effect of mass transfer limitation on GOS formation kinetics was also studied for the multilayered PEI immobilized enzyme. As can be seen in Figure 5.10B, the GOS formation rate did not seem to be affected by the different enzyme loadings. However, the level of total GOS content seemed to decrease slightly with the immobilized enzyme, especially at higher enzyme loadings (Fig 5.11). Compared with the free enzyme, the GOS content at 50% lactose conversion decreased by about 0.5, 1.0 and 1.5% for the immobilized enzyme with an enzyme loading of 35, 130, and 240 mg/g cotton, respectively. It was noticed that the small difference disappeared beyond 55% lactose conversion. The decline in GOS content, nevertheless small, accompanied with increasing the enzyme loading, suggested that there might be some diffusion limitation imposed on GOS in the system when the reaction rate was high.

GOS yields based on lactose reacted and productivities from these three loading levels are shown in Figure 5.12. Lower enzyme loadings at low lactose conversions seemed to give slightly higher GOS yields. It should be noted that 30% lactose conversion level was obtained in the first sample drawn after 5 min, thus there was no sample available at low lactose conversions with high enzyme loadings. Very high GOS yields (~65-70%) obtained at low lactose conversions were accompanied with high productivities. As the lactose conversion increased, the yields and productivities declined due to increasing inhibitory effect of monosaccharides (mainly galactose). The GOS productivities at about 40% and 50% lactose conversions are also shown in Figure 5.12B. As the enzyme loading increased, the GOS productivities also increased proportionally.
Thus, the small decrease in GOS formation is well compensated by the large increase in GOS productivity as the enzyme loading increased. It is important to point out that one gram of cotton cloth occupies only 2-5 mL reactor volume, depending on the packing density. Therefore, with the cotton cloth immobilized enzyme, a working enzyme concentration of more than 100 mg/mL (240 mg/g cotton in 2 – 5 mL reactor volume) can be achieved, which is 100-fold higher than a free enzyme concentration of, for example, 1 mg/mL. It is needless to underline that the volumetric productivity is the major factor that determines the production cost.

5.3.3.3. Effects of Temperature and pH

Although temperature and pH normally affects the reaction rate, they have been found to have negligible effects on GOS content as were reported with various β-galactosidases (Iwasaki et al., 1996, Monsan and Paul, 1995, Yang and Bednarcik, 2001). Nevertheless, PEI-enzyme immobilization involves in electrostatic complex formation with negatively charged enzyme and positively charged PEI. Thus, the optimum pH of enzyme is likely to change. The immobilized enzyme within the PEI matrix is more likely to respond to changes in the pH as compared with other enzyme immobilization methods that do not involve charges such as covalent bond formation (Albayrak and Yang, 2001). Since the most likely substrates for industrial GOS production are whey and whey permeate, the effects of pH (4.5 and 6.0) and temperature (40 and 50 °C) on GOS formation were investigated. As shown in Figure 5.13A, a higher rate of GOS formation was obtained at pH 4.5 and 50 °C, which was consistent with expectation. However, there
were significant variations in the GOS content produced under different pH and temperature conditions (Fig. 5.13B). It was noticed that the decrease in the GOS production level seemed to be related to the reaction rate. A lower GOS production level was associated with the condition that gave a faster reaction. This was consistent with the observation that diffusion limitation reduced GOS formation, which would be more likely to happen when the reaction rate was higher.

5.3.4. Thermal Stability

Thermal deactivation of the PEI-immobilized enzyme followed the first-order kinetics. The deactivation rate constants ($k_d$) at 40, 50, and 60 °C were estimated from the experimental data (not shown) and are given in Table 5.1. The half-lives of the enzyme at various temperatures were estimated from the $k_d$ values and are also listed in Table 5.1. PEI immobilized enzyme had an estimated half-life of close to 1 year at 40 °C, which was 20-fold more stable than that of soluble enzyme. Tosyl immobilized enzyme, which was by covalent binding, and PEI-immobilized enzyme had similar stabilities at 40 °C. However, at 50 and 60 °C, the PEI immobilized enzyme showed shorter half-lives compared with tosyl immobilized enzyme. This could be due to repeated activity determinations performed for PEI immobilized enzyme at these temperatures. The same PEI immobilized enzyme was used for activity determination for as many as ten times.

The stabilization effect of enzyme immobilization on PEI composites may be attributed to several mechanisms. (I) The motion of protein chain segments is restricted
through attachment to PEI and individual contact of enzymes is restricted (Bryjak, 1995; Bryjak and Noworyta, 1995, Onda et al., 1999). (II) Due to charges of enzyme and PEI, the immobilized enzyme is well hydrated (Margolin et al., 1981, 1985), and protein denaturing segmental collisions are unlikely (Fernandez-Lafuente et al., 1998a, b). (III) Since enzyme is embedded in PEI, access by proteases is blocked. (IV) Access of hydrophobic molecules is restricted from the aggregate due to hydrophilicity of the system. However, the stabilization effect of the ionic immobilization of enzyme varies with the type of enzymes. For instance, glucose oxidase and lipase were immobilized by the same method using PEI yet the latter was stabilized quite more (Mateo et al., 2000).

5.3.5. Continuous Reactor

Figure 5.14 shows the operational stability of cotton cloth immobilized enzyme in the continuous reactor at 40 °C. In general, the reactor performance was stable and there was no apparent decrease in the level of GOS or lactose conversion throughout the entire 173-h continuous run. The reactor was operated at about 70% lactose conversion, which was higher than the 40% conversion level for achieving the maximum GOS with 100 g/L lactose feed. However, the level of GOS was not different from that of free enzyme at the same lactose conversion level. With 10% lactose feed, the maximum GOS content of 15% can be obtained if the feed rate is increased to maintain the lactose conversion level at ~40%. Higher GOS production also can be obtained with a higher lactose concentration feed. The reactor productivity for continuous GOS production from 400
g/L lactose feed at 40°C was found to be about 5 kg/L/h with an enzyme loading of 245 mg/g cotton when the reactor was maintained at 50% lactose conversion.

5.3.6. Factors Affecting PEI-Enzyme Immobilization

PEI forms ionic complexes with macromolecules containing acidic domains leading to water soluble and insoluble complexes, and this behavior is affected by salt concentration, pH, and the concentration of precipitable components (Jendrisak, 1987, Dissing and Mattiasson, 1996, Onda et al., 1999). Khan (1998) indicated that variation among different enzymes should be expected. In order to enhance effective complex formation with PEI, a polyaspartic acid tails were fused to glucoamylase (Suominen et al., 1993) and β-galactosidase (Parker et al., 1990, Zhao et al., 1990). The more negatively charged the enzyme is, the less amount of PEI is necessary for complex formation (Suominen et al., 1993). Caruso and Schuler (2000) studied the effect of enzyme complexation on its activity in solution and found that glucose oxidase or peroxidase that was precomplexed with oppositely charged polyelectrolyte (enzyme-to-polymer mass ratio of 1:10) in solution had 60-70% less activity than the corresponding free enzymes. In our case, although large macroscopic sizes of PEI-enzyme aggregates were formed, the activity of the enzyme was not impaired. Intact catalytic activity even after GA crosslinking suggested that the PEI enzyme aggregates were highly porous and permeable to lactose and GOS.
In previous studies, PEI was first adsorbed on the cotton cloth and then the excess PEI was washed away with water or buffer solution, resulting in a low efficiency for enzyme immobilization. It is important to note that cotton cloth lacks any specific adsorption capacity for PEI except a rough surface and high porosity. Since positively charged PEI would strongly repel each other, only a ‘monolayer’ of PEI on the fibrils of cotton cloth is expected after PEI adsorption. When washed with water, especially with buffer, the numbers of PEI molecules are greatly reduced. The washed cloth was usually crosslinked with GA to activate for enzyme coupling. It was indicated that once GA treated, GA active aldehydes were fairly well removed from the PEI polymer backbone (Bahulekar et al., 1991), thus almost only GA aldehydes were available for enzyme immobilization. Thus, with these methods not only is electrostatic enzyme attraction to support severely restricted but also few reactive groups are available for actual enzyme immobilization, which surely limit the amount of enzyme immobilized, and more importantly, can make a just lightly bound enzyme susceptible for detaching from the carrier.

In this work, multilayered enzyme immobilization procedure was developed by eliminating the washing step after PEI adsorption on fibers. Besides the cotton cloth in the knitted form, various types of fibrous materials with different physical (e.g., knitted, nonwoven) and chemical characteristics, including polyethylene terephthalate (PET) and rayon (restructured cellulose) were also investigated following the same procedure described before for cotton cloth. It was found that the enzyme immobilization yields achieved were similar to that of cotton cloth (Table 5.2). For instance, similar to cotton
cloth, the optimum PEI to enzyme ratio of 1/22 was obtained using nonwoven PET fabric with 77% immobilization yield (220 mg/g PET). However, when PEI coated PET fabric was washed (monolayer method) before enzyme addition, almost no immobilization of enzyme was achieved. With cotton, on the other hand, about 25-30 mg/g was obtained when cotton was washed after PEI adsorption. This may indicated that cotton retained more PEI, or adsorbed PEI more strongly than PET, which could be attributed to the smoothness and hydrophobicity of PET surface compared with cotton. Similarly, Isgrove et al (2001) reported that nylon having hydrophobic and smooth surface was not good for enzyme immobilization and they thus applied an acid hydrolysis to increase surface roughness before PEI adsorption.

Surface characteristics of multilayer immobilized enzyme on cotton cloth and PET fabrics were studied under a light microscope and by scanning electron microscopy. As can be seen in Figure 5.15, the fibril surfaces of both cotton and PET fibers treated with the multilayered PEI enzyme immobilization were heavily (entirely) coated with layer(s) of PEI-enzyme aggregates. It is noted that the surface characteristics of PET and cotton fibers are quite different as can be seen from the images of the untreated fibers. PET fibers had a smooth surface, and were thicker in diameter and round shaped, while cotton cloth had rough surface with a flat twisted ribbon shape. It should be noted that the cracks or flacks seen especially in the coat of PET fiber were due to drying applied prior to SEM imaging. SEM imaging of PEI-monolayer immobilized enzyme on cotton cloth was no different from the untreated control samples. It is noted that the phenomenon of multilayered PEI enzyme immobilization relies more on the three-dimensional
association of aggregates leading to growth and ultimately coating on the fibril surfaces of the fibrous matrix rather than just a formation of PEI-enzyme aggregate in solution only. The driving force of the growth of aggregates appears to be dependent on a critical ratio of PEI to enzyme, yet the actual course of events is rather difficult to elucidate. Although the multilayered PEI enzyme immobilization method developed here worked well with various types of materials, further experiments with other enzymes of similar and different chemical and surface characteristics should be carried out.

5.3.7. Comparisons to Other Studies

Table 5.2 shows the comparison between various fibrous matrices used for immobilization of several enzymes. Kamath et al (1988) found that optimum enzyme (urease) loading was about 20 mg/g cotton flannel cloth. The activity yield was 43% when the PEI cloth adsorbed enzyme crosslinked with 1,1-carbonyldiimidazole while only 7% activity was obtained when GA was used. Vol’f et al (1984) used several different types of fibers and enzymes for therapeutic applications. Most of these procedures required several steps for activation or modification of the fiber before immobilization. It was found that the results of enzyme immobilization depended on the type of the fibrous supports and ranged from 10 to 90% immobilization yield. Apparently, multilayer enzyme immobilization produced higher activities and shorter immobilization time than most of the other methods reported. It should be noted that there was no prior activation necessary for this method. Recently, Kawai et al (2001) described a novel multilayered immobilization procedure for aminoacylase in porous hollow-fiber support. The method was based on grafting of polymer chains containing
epoxy group on hollow-fiber membrane by radiation-induced graft polymerization. An amount of 200 mg enzyme per gram of hollow fiber was introduced at 95% coupling yield (5% of the immobilized enzyme leached after GA crosslinking). The multilayer was composed of about 15 layers of enzyme. The yield they indicated was based on protein efficiency while the activity of immobilized enzyme was not described.

Table 5.3 compares the reactor productivity and the maximum GOS content in the final product achieved in various studies reported in the literature. The productivity obtained from multilayered PEI immobilized enzyme was much higher (50-100 fold) than those of the reported values. β-Galactosidase is one of the most commonly used enzymes, and is widely available in large quantities. Axelsson and Zacchi (1990) performed economic evaluation of lactose hydrolysis by \textit{A. oryzae} β-galactosidase. It was found that when considering the use of free enzyme, the cost for the enzyme increases with increasing enzyme concentration in reaction mixture. The cost for soluble enzyme constitutes about 30% of the total cost at the optimal loading of 60 mg/L. When the enzyme was immobilized in alginate beads with carbodiimide crosslinking [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide], the cost was about 10% of the total cost for the immobilized enzyme at the optimal loading of 5000 mg/L in a plug-flow tubular reactor (PFTR). The effectiveness factor was the lowest on PFTR compared to batch or controlled stirred tank reactor (CSTR). This is probably due to diffusion effect in alginate beads where enzyme was encapsulated. In our case, 100-200 mg enzyme can be included per mL of the reactor volume, which means 2 to 4-fold more enzyme loading can be introduced with a linear increase in productivity. Also, with the use of cotton fabric, the
immobilized enzyme functions as well as the free enzyme. Thus, much higher productivities and in turn much lower product cost can be realized.

5.4. Conclusions

Using PEI, multilayered enzyme immobilization on fibrous matrix for the development of novel fibrous type enzyme reactor was developed. Large surface area of cotton cloth immobilized in such a way that every fibrils in the fabric was coated with layers of immobilized enzyme; thus a large amount of enzyme were introduced in an active way. Knitted cotton fabric offers not only large amount of surface available for coating of PEI-enzyme aggregates but also the mechanical strength that holds the aggregates while exposing to surrounding solution where the activity of immobilized enzymes are fully involved in operation. Fibrous support offers great advantages over particulate resins or porous beads in industrial scale enzyme immobilization. The method of multilayered enzyme immobilization should also be applied for other enzymes. By comparing large body of enzyme immobilization systems available, multilayer enzyme immobilization on cotton cloth can be considered as one of the cheapest, safest, fastest and most successful system. The method is simple and straightforward, and requires no sophisticated expertise, which is unlike many other enzyme immobilization methods. No need for prior activation of carriers or using any special apparatus, which is a great burden for large and small-scale industrial applications. FDA has approved that PEI can be used in production of food ingredients. With the high enzyme loading of 150-250 mg/g cotton cloth and 95% immobilization yield, the multilayered PEI method is among
the most successful ones ever reported in the literature. More importantly, the high enzyme loading gave proportionately increased reactor productivity and did not significantly alter the GOS formation kinetics. This immobilized enzyme technology should have important application in GOS production from lactose.
Table 5.1. Comparison of thermal stabilities of free and immobilized enzymes*.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Free enzyme(^b)</th>
<th>Immobilized Enzyme on Cotton Cloth**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k(_d) (h(^{-1}))</td>
<td>half life (h)</td>
</tr>
<tr>
<td>40</td>
<td>0.0017</td>
<td>399</td>
</tr>
<tr>
<td>50</td>
<td>0.0141</td>
<td>49</td>
</tr>
<tr>
<td>60</td>
<td>0.3325</td>
<td>2</td>
</tr>
</tbody>
</table>

* The deactivation rate constant k\(_d\) was determined from experimental data which followed first-order reaction kinetic model. The enzyme half-life was calculated from k\(_d\) value.

** Immobilized enzyme in packed-bed reactor

\(^b\) Enzyme immobilization on tosylated cotton cloth (Albayrak and Yang, 2001).

\(^b\) Incubated in pH 4.5 acetate buffer and activity was determined at various intervals.
<table>
<thead>
<tr>
<th>Fibrous matrix</th>
<th>Means of Activation and/or Immobilization</th>
<th>Enzyme Coupling</th>
<th>Immobilization Yield (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphoteric polycaproamide fiber</td>
<td>Glutaraldehyde (GA)</td>
<td>Papain</td>
<td>24 h 30 mg/g 23%</td>
<td>Vol'f et al., 1984</td>
</tr>
<tr>
<td>Aldehyde containing polyvinyl alcohol</td>
<td>GA</td>
<td></td>
<td>3 h 77 mg/g 88%</td>
<td></td>
</tr>
<tr>
<td>Regenerated cellulose fibers</td>
<td>Dye direct white</td>
<td></td>
<td>0.4 h 40 mg/g 84.5%</td>
<td></td>
</tr>
<tr>
<td>Porous hollow fiber membrane</td>
<td>Radiation-induced graft polymerization / GA</td>
<td>Aminoacylase 24 h 200 95 (protein yield)</td>
<td>Kawai et al., 2001</td>
<td></td>
</tr>
<tr>
<td>Nonwoven Rayon/polyester blend (30%/70%)</td>
<td>1,1-carbonyldiimidazole</td>
<td>Bovine serum albumin 25 95%</td>
<td>Howlett et al., 1991</td>
<td></td>
</tr>
<tr>
<td>Cotton terry cloth</td>
<td>Tosyl Chloride</td>
<td>β-galactosidase 12 h 50 mg/g 45%</td>
<td>Albayrak and Yang, 2001</td>
<td></td>
</tr>
<tr>
<td>Cotton flannel cloth</td>
<td>PEI / Dimethyl suberimidate</td>
<td>Jack bean urease 4 h 20 mg/g 43%</td>
<td>Kamath et al., 1988</td>
<td></td>
</tr>
<tr>
<td>Cotton cloth</td>
<td>monolayer PEI / GA</td>
<td>Invertase 6 h 0.1% 1670 U/g</td>
<td>Yamazaki et al., 1984</td>
<td></td>
</tr>
<tr>
<td>Cotton terry cloth</td>
<td>monolayer PEI / GA</td>
<td>β-galactosidase 0.2 h 30 mg/g 95.6</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Cotton terry cloth</td>
<td>multilayer PEI / GA</td>
<td>β-galactosidase 0.2 h 250 mg/g 92%</td>
<td></td>
<td></td>
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<tr>
<td>Nonwoven PET</td>
<td></td>
<td></td>
<td>87%</td>
<td></td>
</tr>
<tr>
<td>Nonwoven Rayon (100%)</td>
<td></td>
<td></td>
<td>82%</td>
<td></td>
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</table>

Table 5.2. Comparison of various types of fibrous matrices and enzyme immobilization methods.
<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Mode of Process&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lactose Conc. (g/L)</th>
<th>T (°C)</th>
<th>pH</th>
<th>Max GOS&lt;sup&gt;b&lt;/sup&gt; (wt %)</th>
<th>Productivity (g/L/h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. circulans</em></td>
<td>Batch (FE)</td>
<td>45.6</td>
<td>40</td>
<td>6.0</td>
<td>24.0</td>
<td>2.2</td>
<td>Muzaffer et al., 1984</td>
</tr>
<tr>
<td></td>
<td>Continuous (IE, CSTR)</td>
<td>45.6</td>
<td>40</td>
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<sup>a</sup> FE: free enzyme, IE: immobilized enzyme, CSTR: continuous stirred tank reactor, PBR: packed bed reactor, UF: ultrafiltration membrane reactor, FBR: fibrous bed (cotton cloth) reactor.
<sup>b</sup> Max GOS is a weight percent of GOS based on the total sugars in the reaction mixture.
<sup>c</sup> GOS content also includes disaccharides.

**Table 5.3.** GOS production by various β-galactosidases in batch and continuous operations
Figure 5.1. Proposed mechanism for PEI-enzyme aggregate formation (A) and morphology of PEI-enzyme aggregates in solution seen under a light microscope (B).
Figure 5.2. The procedure and proposed mechanisms for PEI-monolayer (A) and multilayer (B) enzyme immobilization on cotton cloth.
Figure 5.3. Schematic diagram of the immobilized enzyme cotton-cloth reactor used in this study. The system was operated either as a recycle batch reactor (with recirculation) or a continuous single-path reactor (without recirculation). The scanning electron micrograph shows a string of cotton cloth immobilized enzyme.
Figure 5.4. A typical HPLC chromatogram showing various sugars found in lactose hydrolysis and galacto-oligosaccharides formation catalyzed by the immobilized enzyme. Elution time (min): 10.1 for hexasaccharide (6-OS), 12.2 for pentasaccharide (5-OS), 12.8 for tetrasaccharide (4-OS), 13.8 for trisaccharide (3-OS), 16.2 for lactose or disaccharide, 19.5 for glucose, and 20.5 for galactose.
Figure 5.5. Effect of PEI to enzyme ratio on enzyme activities of PEI-enzyme aggregates in solution and in supernatant after centrifugation at 10,000 rpm for 1 min.
Figure 5.6. Effect of enzyme concentration on immobilization yields for multilayer and monolayer enzyme immobilization on cotton cloth. While 20 mg PEI was used for monolayer, PEI to enzyme ratio of 1/22-25 was used for multilayer procedure.
Figure 5.7. Effects of pH of PEI solution and enzyme coupling temperature during multilayer enzyme immobilization on cotton cloth. 250 mg/g enzymes were added at the beginning.
Figure 5.8. Reaction kinetics of lactose hydrolysis and GOS formation catalyzed by PEI-immobilized enzyme in the recycle batch reactor at 40 °C with an initial lactose concentration of 400 g/L.
**Figure 5.9.** Kinetics of GOS formation as affected by lactose conversion catalyzed by PEI-immobilized enzyme in the recycle batch reactor at 40 °C with an initial lactose concentration of 400 g/L.
Figure 5.10. Comparison of GOS formation kinetics during lactose hydrolysis between free enzyme and PEI-enzyme aggregates in solution (A) and PEI-immobilized enzymes with various enzyme loadings in the recycle batch reactor (B).
Figure 5.11. Comparisons of GOS formation during lactose hydrolysis catalyzed by free enzyme, PEI-enzyme aggregates in solution and PEI-immobilized enzymes on cotton cloth with various enzyme loadings in the recycle batch reactor.
**Figure 5.12.** Effects of enzyme loading on GOS productivities and yields during lactose hydrolysis catalyzed by PEI-immobilized enzyme in the recycle batch reactor.
Figure 5.13. Effects of pH (130 mg/g enzyme loading) and temperature (170 mg/g enzyme loading) on GOS production during lactose hydrolysis catalyzed by PEI-immobilized enzyme in the recycle batch reactor.
Figure 5.14. Continuous production of GOS by PEI-immobilized enzyme on cotton cloth packed in a single-pass reactor operated at 40 °C with 100 g/L lactose in the feed solution.
Figure 5.15. Fibril micrographs of knitted cotton cloth and nonwoven polyethylene terephthalate (PET) fabrics containing PEI-multilayer immobilized enzyme (250 mg enzyme and 12 mg PEI per gram of fabric) seen under a light (A) and scanning electron microscope (SEM) (C) as compared with SEM image for control fibrils (B).
CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

Since the turn of the century, various enzyme immobilization systems, including method, reagent and support, have been evaluated and applied to many enzymes. *Aspergillus oryzae* β-galactosidase is one of the most commonly immobilized model enzymes. Although use of immobilized enzyme for several applications including lactose hydrolysis was found quite feasible compared with free enzyme, enzyme immobilization technology has only been appreciated by a few industrial scale applications. The success in enzyme immobilization is of course measured by coupling and activity yields that is the more enzyme protein and higher the activity the better. Regardless of the level of enzyme immobilization, most commonly cited problems are cost (enzyme, chemicals, carrier, etc.), toxicity of the chemicals and complicated procedures requiring expertise. Also, the problems associated with performance of immobilized enzyme in reactor conditions such as short half-lives and possible microbial contamination, diffusion resistance, reactor clogging, and high hydrodynamic resistance can not be
overemphasized. In the case of food ingredients, a necessary approval of production system by Food and Drug Administration is also a burden for use of immobilized enzyme.

Enzyme immobilization was addressed in this study especially from the support point of view. Knitted cotton terry cloth as a novel and inexpensive fibrous matrix was evaluated for the development of fibrous bed biocatalyst reactor. Cotton cloth was chemically activated by using p-toluenesulfonyl chloride. Enzyme immobilization on tosylated cloth followed the substitution mechanism. Approximately 50 mg enzyme per gram of cloth was coupled with an immobilization yield of 55%. Greater tosylation of cotton cloth or addition of more enzyme did not increase the level of immobilized enzyme activity. Immobilized enzyme was quite stable in continuous reactor and no apparent loss of activity was observed. Enzyme thermal stability also increased compared with free enzyme. In conclusions, the procedure developed for cotton cloth provides simple, inexpensive, less cumbersome and industrially applicable method and support for highly active and stable immobilized biocatalyst. The procedure can also be applied to other enzymes.

While the immobilization on tosylated cloth was a covalent type of enzyme immobilization, a different enzyme immobilization approach involving electrostatic attraction of enzyme to the support was undertaken to improve the level of immobilized enzyme on cotton cloth. By applying polyethylenimine (PEI), a novel enzyme immobilization technique was developed. The technique is dependent on PEI-enzyme
aggregate formation and coating of aggregates on the fibrils of cotton cloth. The level of immobilized enzyme obtained with tosylation was increased about 10-fold by this PEI-enzyme immobilization method. The method also offers several advantages over other enzyme immobilization techniques, including use of inexpensive chemicals (PEI and glutaraldehyde) at a very low amount and completion in a short time. Immobilized enzyme activity under optimized condition reached 90-95% of free enzyme at a loading of 250 mg/g. Furthermore, increase in enzyme loading was accompanied with proportional increase in GOS productivity, indicating GOS formation functionality of the immobilized enzyme was unaltered.

*Aspergillus oryzae* β-galactosidase is a useful enzyme for production of galacto-oligosaccharides. GOS formation was only affected by lactose concentration while reaction pH, temperature, and ionic strength did not affect GOS formation, although they affected the reaction rate. The enzyme forms approximately 25-26% (w/w) GOS at 50% lactose conversion from 400 g/L initial lactose solution, which appears to be the optimal concentration. Increasing the lactose concentration beyond 400 g/L does not result in much increase in the GOS content. Beyond 400 g/L, viscosity starts to limit enzyme reaction and GOS formation. The role of monosaccharides in GOS formation was elucidated. Enzyme was unable to convert free glucose and/or galactose for formation of disaccharides or GOS to appreciable amounts, if any. When added to the initial lactose solution, monosaccharides severely delayed GOS formation and decreased 10-15% in the total GOS content. Thus, detrimental effects of monosaccharides on GOS formation were noticed.
Except for cotton cloth, tosylation and enzyme immobilization levels for either fibrous supports investigated were quite low, while similar enzyme immobilization levels were achieved with PEI enzyme immobilization method. A stable long-term continuous operation was demonstrated with both types of immobilized enzyme. Increases in the thermal stability for both enzymes were observed although tosyl immobilized enzyme showed greater stability. Overall, high porosity (>95%) and low pressure drop of cotton cloth allowed the reactor to be operated without any difficulty. Cotton cloth immobilized enzymes showed no reduction in GOS formation, and did not impose any significant diffusion limitation nor affected the GOS formation characteristics of the enzyme as compared to free enzyme. Compare to other reported studies, the GOS productivity was increased by 5-10 times using the tosyl activated cotton cloth immobilized enzyme, and about 100-fold higher using PEI immobilized enzyme. The high GOS productivities should allow the development of an economical process for the production of GOS from lactose using the cotton-cloth immobilized β-galactosidase.
6.2. Recommendations

Tosyl activation technique on cotton cloth involved the use of pyridine as a neutralizing and plasticizing agent. Pyridine does not enter the tosylation reaction and complete removal from the cloth by washing after activation was observed. Nevertheless, using pyridine requires working in a hood during activation reaction and also raises caution flag due to possible toxicity and bad odor. Therefore, its complete removal from cotton cloth should be checked by elemental nitrogen analysis after each washing to determine the necessary washing duration. Replacement of pyridine with another non-toxic organic base should be investigated. Moreover, elemental sulfur analysis of cotton cloth during enzyme immobilization can provide further quantitative evidence for the nucleophilic substitution mechanism.

PEI immobilized enzyme was primarily dependent on the characteristics of PEI and its interaction with negatively charged enzyme. Although there is no concern for the safety of PEI, an approved secondary food additive, toxicity of GA, however, may be in question. The procedure involves extensive washing of cotton cloth after GA crosslinking and the cloth should be free of unbound or unreacted aldehydes. However, it is necessary to investigate possible presence of free GA in the product mixture. Further experiments should be carried out to scale up the PEI enzyme immobilization for especially immobilization in place. PEI enzyme immobilization method was developed using Aspergillus oryzae β-galactosidase. In order to establish the technique for general use, applicability of this novel method to other types of enzymes should be investigated.
Pure lactose was used in this study for GOS formation. However, the more likely substrate for GOS production would be concentrated whey permeate. GOS production from whey permeate feed is of industrial significance and should be investigated. The whey permeate with high salt and residual protein content may constitute a challenge for immobilized enzyme reactor. However, no problem is anticipated, such as reactor clogging or decrease in enzyme activity, due to the relatively open structure of cotton fabric immobilized enzyme, which should allow small particulates to flow freely through the fibrous bed.

An efficient separation technique for recovering GOS from the reaction medium is needed. *Aspergillus oryzae* β-galactosidase forms approximately 25% (w/w) GOS at 50% lactose conversion from 400 g/L initial lactose solution. The GOS product at 50% lactose conversion from 400 g/L initial lactose can be conveniently concentrated to 60-70% sugar by evaporation and may be used as food ingredient. However, the composition remains the same. It may be desirable to increase the concentration or purity of GOS in the final product for some applications. Also, when the maximum GOS content in the reaction was obtained, half of the lactose left unreacted, which is of a concern. Thus, further studies are needed in two areas: 1) how to utilize the remaining 50% lactose, and 2) how to increase GOS content from 25% to a higher level. A separation system should be investigated although there is no question that the task is challenging due to close sizes and similar chemical characteristics of the sugar molecules.
Sterigmatomyces elviae CBS8119 was reported as one of the best microorganisms to produce GOS (Onishi et al., 1995). When cell suspensions were used in 300 g/L lactose solution, total GOS reached its max 75 g/L (25% of the total sugar) at ~ 50% lactose conversion. Further hydrolysis of lactose proceeded at a very slow rate so did the GOS hydrolysis. The effect of glucose on GOS formation was studied and found that 90 g/L glucose addition to 360 g/L lactose decreased the GOS formation by 64%. When an fermentation experiment was designed in which the glucose was subjected to metabolic utilization by growing cells as lactose hydrolyzed, there was a linear decrease in the hydrolysis of lactose and a linear increase in the production of GOS to 64% of total sugar at 85% lactose conversion. It can be concluded from this study that 64% GOS was a direct consequence of the effect of removing one of the inhibitor during the GOS formation reaction rather than the enzymes' ability to produce higher yields of GOS. There is no specific reason to suggest that this yield cannot be achieved with other enzymes, such as Aspergillus oryzae β-galactosidase.

Based partially on our experimental data about the detrimental effect of monosaccharides, it can be hypothesized that continuous removal of monosaccharides (galactose and glucose) during GOS formation would bring about several desirable effects. First, it is assumed that GOS, as intermediary products, are more of a kinetically controlled reaction. Therefore, inhibitors of the enzyme are responsible for not only the reduction of the reaction rate of the enzyme but also lower GOS yields. Thus, their specific removal would facilitate higher GOS yields (up to 40-50%) along with much higher GOS productivity, thereby lowering the content of unutilized lactose in the final
product. Therefore, it is recommended that further studies should be conducted to increase GOS formation by designing novel systems where monosaccharides are continuously removed from the sugar solution.

Removal of monosaccharides can be facilitated by a nanofiltration unit with appropriate molecular weight cut-off to separate monosaccharides from other sugars. The retentate should be partially mixed with the lactose feed until desirable GOS yield was achieved. The goal of continuous removal of monosaccharides can also be achieved by biochemical means such as simultaneous reaction using glucose and galactose oxidase to hydrolyze glucose and galactose, respectively. Both approaches, however, require extensive analysis and a thorough investigation. Chromatographic separations involving ion exchange resins are commonly used to separate glucose from fructose in the production of high fructose corn syrup. It can be recommended that similar ion-exchange resins be investigated for separation of monosaccharides from oligosaccharides.
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A. STANDARD CURVES

**Figure A.1.** Protein Standard Curve with enzyme solution performed according to ‘Bio-Rad Standard Assay’ procedure. 5 ml of diluted dye (Comassive Brilliant Blue) was mixed with 0.1 mL of enzyme solution and incubated for 5 min at room temperature. After development of color, absorbance values were obtained at 595 nm. Control sample contained distilled water instead of enzyme solution.
Figure A.2. Protein Standard Curve with enzyme solution performed according to 'Bio-Rad Micro Assay' procedure. 0.2 ml of concentrated dye (Comassive Brilliant Blue) was mixed with 0.8 mL of enzyme solution and incubated for 5 min at room temperature. After development of color, absorbance values were obtained at 595 nm. Control sample contained distilled water instead of enzyme solution.
Figure A.3. Enzyme activity standard curve constructed by mixing 9 to 1 ratio of substrate to enzyme solution. The reaction mixture contained 10 g/L lactose in 0.1 M acetic acid buffer pH 4.5 at 40 °C. Under the defined conditions, 1 mg enzyme had 26.7 U of activity.
Figure A.4. Enzyme activity standard curve constructed by mixing 9 to 1 ratio of substrate to enzyme solution. The reaction mixture contained 10 g/L lactose in 0.1 M acetic acid buffer pH 4.5 at 40 °C.
Figure A.5. Enzyme activity standard curve constructed by mixing 9 to 1 ratio of substrate to enzyme solution. The reaction mixture contained 100 g/L lactose in 0.1 M acetic acid buffer pH 4.5 at 40 °C.
B. CALCULATIONS

Table B 1. HPLC Peak heights of sugars obtained during lactose hydrolysis by immobilized enzyme in differential reactor at 400 g/L lactose at 40 °C pH 4.5.

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Table B 3. Molar Sugar concentrations obtained during lactose hydrolysis by immobilized enzyme in differential reactor at 400 g/L lactose at 40 °C pH 4.5. Molecular weights (MW) of tri-, tetra-, penta- and hexa- oligosaccharides were assumed as 504, 666, 828 and 990, respectively.

<table>
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<th>Time (h)</th>
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<th>[4OS]</th>
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<th>Total [GOS]</th>
<th>[Lac]</th>
<th>[Glu]</th>
<th>[Gal]</th>
<th>Mono [Gal +Glu]</th>
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<td>0.005</td>
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<td>0.011</td>
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Table B 4. Material balance equations used to check the assumption made upon HPLC analysis of sugars. Molar Sugar concentrations calculated in Table B 3 was used.


Material Imbalance [IB] = \{ [MONO]_{time=0} - [MONO]_{time} \}

<table>
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<th>Time (h)</th>
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<th>[GOS] + [GB]</th>
<th>[GLU]</th>
<th>[Lac] - [GLU]</th>
<th>[MONO]</th>
<th>[GAL]</th>
<th>[GAL] + [GB]</th>
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Figure B.1. Graphical representation of tabulated material balance data (Table B4) during lactose hydrolysis and GOS formation catalyzed by immobilized enzyme in the recycle batch reactor at 40 °C with an initial lactose concentration of 400 g/L.
Figure B.2. GOS and GB formations as affected by lactose conversions catalyzed by immobilized enzyme in the recycle batch reactor at 40 °C with an initial lactose concentration of 50, 100, 200, 300 g/L (A) and 300, 400 and 500 g/L (B). Similar to Table B 3 and B 4, GB amounts were obtained from material balances (Data not shown).
C. DISACCHARIDE FORMATION

Figure C.1. Kinetics for Formation of probably disaccharides (shown by their retention times on HPLC) from glucose at 50 g/L initial concentration. The reaction was catalyzed by immobilized enzyme in the recycle batch reactor at 40 °C pH 4.5 in 0.1 M acetate buffer.
Figure C.2. Disaccharides formation kinetics (shown by their retention times on HPLC) from galactose at 50 g/L initial concentration. The reaction was catalyzed by immobilized enzyme in the recycle batch reactor at 40 °C, pH 4.5 in 0.1 M acetate buffer.
Figure C.3. Disaccharides formation kinetics (shown by their retention times on HPLC) from glucose and galactose each at 50 g/L initial concentration. The reaction was catalyzed by immobilized enzyme in the recycle batch reactor at 40 °C, pH 4.5 in 0.1 M acetate buffer.
**Figure D. 1.** The effect of pH on the activity of soluble enzyme. The initial enzyme activities were determined using substrate solutions prepared with acetate (pH 4.0 – 6.0), phosphate (pH 6.0 – 7.0) phosphate / carbonate (pH 8.0) and carbonate (pH 9.0 – 10.0) buffers.
**Figure D. 2.** Effect of pH on the stability of soluble enzyme. The initial enzyme concentrations of 10 mg/L were prepared in acetate (pH 4.0 – 6.0), phosphate (pH 6.0 – 7.0) phosphate / carbonate (pH 8.0) and carbonate (pH 9.0 – 10.0) buffers and incubated for 24 h at room temperature. Initial activities determined at pH 4.5.