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Immobilization and characterization of enzyme for production of lactulose-based oligosaccharides

Ph.D. thesis

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Abbreviations

A_{fin}	Enzyme activities of supernatant (after immobilization)
A_{imm}	Activity of the immobilized enzyme
A_{ini}	Enzyme activities of supernatant (before immobilization)
A_{off}	Amount of enzyme offered per gram of support
A_{rec}	Recovery activity
A_{the}	Theoretical activity of the immobilized enzyme
AIC	Akaike Information Criterion
ANOVA	Analysis of variance
AOS	Arabino-oligosaccharide
BSA	Bovine serum albumin
CCD	Central composite design
CLEA	Cross-linked enzyme aggregate
COPASI	Name of a software
CT	Chitin
CTS	Chitosan
CTS-IE	Immobilized enzyme on chitosan particles carrier
DP	Degree of polymerization
FE	Free enzyme
FL	Flow rate
FO	First-order of a model
FOS	Fructooligosaccharides
Fru	Fructose
GA	Glutaraldehyde
Gal	Galactose
Glu	Glucose
GOS	Galactooligosaccharides
HPLC	High-performance liquid chromatography
IE	Immobilized enzyme
ILC	Initial lactulose concentration
LoF	Lack of fit
LSODA	A package of COPASI software
MAG	Magnetic particles carrier
MAG-CTS	Chitosan-coated magnetic particles carrier
MAG-IE	Immobilized enzyme onto magnetic particles carrier
Man	Mannose
NDO	Non-digestible oligosaccharides
OS	Oligosaccharides
OS-La	Lactose-based oligosaccharides
OS-Lu	Lactulose-based oligosaccharides
p-NPGal	*p*-nitrophenyl-β-D-galactopyranoside
PAN	Poly-aniline
PC	Protein content
PEG	Polyethylene glycol
PEI	Polyethylenimine
PEO	polyethylene oxide
PI	Prebiotic index
PQ	Pure quadratic
RID	Refractive index detector
RSM	Response surface methodology
SCFAs	Short chain fatty acids
SEM	Scanning electron microscope
SO	Second-order of a model
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TPP	Tripolyphosphate
TWI	Two-way interaction
XOS	Xylooligosaccharides
YI	Yield of immobilization in terms of expressed activity

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1 INTRODUCTION AND OUTLINE

1.1 Introduction

Oligosaccharides (OS) play an important role in biological systems such as influence the microbial composition in the gastrointestinal tract, the adhesion of micro-organisms to the epithelium, gut maturation, and cell surface glycosylation, systemic effects after intestinal absorption and association studies in humans (Moreno & Sanz, 2014). The biological functions of OS depend on their specific structures (Cardelle-Cobas *et al.*, 2011c; Moreno & Sanz, 2014). According to Crittenden and Playne (1996), food-grade OS can be classified into 12 main groups including galactooligosaccharides (GOS), lactulose (Lu), lactosucrose, fructooligosaccharides (FOS), palatinose or isomaltulose oligosaccharides, glycosyl sucrose, maltooligosaccharides, isomaltooligosaccharides, cyclodextrins, gentiooligosaccharides, soybean oligosaccharides and xylooligosaccharides (XOS). Among the OS, the group of non-digestible ones (NDO) that are not digested by enzymes of the small intestine and considered as soluble dietary fibers, after reaching the large colon stimulate the growth and/or active of one or limited number of beneficial bacteria, thus they have a beneficial effect on human health and are classified as prebiotics (Champ *et al.*, 2003). Furthermore, the NDO also have many other beneficial effects in health of the consumers including support the synthesis of K and B type vitamins, such as B1, B2, B3, B6, B9, B12 vitamin (Perugino *et al.*, 2003); stimulate and enhance absorption of minerals such as calcium and magnesium (Sako *et al.*, 1999); improve blood glucose and triglycerides level (Nakakuki, 2009); has anti-cariogenic activity (Delzenne, 1999); relief the symptoms of diabetes mellitus and lactose intolerance (Li *et al.*, 2008b); prevent colon cancer (van Dokkum *et al.*, 1999), inhibit diarrhea, protect against infection on the gastrointestinal, respiratory and urogenital tracts, reduce risk of gut cancer (Mussatto & Mancilha, 2007). With many beneficial functions, NDO are considered to be valuable materials for application in food, feed, pharmaceutical, cosmetic, etc. industries.

Among the OS, lactulose (Lu) is one of the industrial interesting compounds with the significant impact on human nutrition. Prebiotic Lu has been used in a wide variety of food as a bifidus factor or as a functional ingredient for intestinal regulation (Panesar & Kumari, 2011). In addition, prebiotic Lu is used mainly for treatment of constipation, hepatic encephalopathy, a complication of liver disease and maintenance of blood glucose and insulin level (Schumann,

2002). Unfortunately, at high doses, the Lu has laxative effects and the fact that fermentation occurs mainly in the proximal colon, which results in uncomfortable gas production (Salminen & Salminen, 1997; Tuohy *et al.*, 2002), thus the use of prebiotic Lu could be limited. Otherwise, the lactulose-based oligosaccharides (OS-Lu) are more competitive than Lu in prebiotic activity due to the higher degree of polymerization (DP). Fermentation of these OS is going slowly and their metabolism takes place more distally in the colon (Tuohy *et al.*, 2005). In other words, OS-Lu show a better anti-inflammatory profile than Lu in a model of experimental colitis (Algieri *et al.*, 2014). Thus, the studies on OS-Lu, such as production, structure, functions, as well as the application of them are currently attracting the attention of the scientific community (Cardelle-Cobas *et al.*, 2009; Cardelle-Cobas *et al.*, 2008a; Martinez-Villaluenga *et al.*, 2008).

The OS are already occurred naturally such as in human milk, bovine milk, plants, roots, fruits and vegetables, biomass, etc., and can also be synthesized by chemical methods, as well as biotechnological methods. Generally, the chemical methods have many disadvantages such as the formation of by-products, the high cost of product purification, hard to get OS with high DP, many steps required in order to achieve region-selectivity etc., thus, these methods are rarely applied in the synthesis of OS for food and pharmaceutical applications. Otherwise, due to regio- and stereo-specificities of biocatalysts, the enzymatic synthesis of OS has been preferred much because it can be done in one step with very high efficiency and without any by-products. This technology could be done by using native (also called soluble or free enzyme, FE) as well as immobilized enzymes (IE) or insoluble enzymes.

Recently, the researchers focused on IE because of its advantages over FE such as enhancement of enzyme stability, reuse or continuous use of bioreactor, convenience in separation from the reaction mixture, possible modulation of the catalytic properties, contamination prevention in the product and easier prevention of microbial contaminations. It is thus apparent that enzyme immobilization can have immediate effects on the cost of process or the quality of the products. Furthermore, it has potential tuning of properties of the enzymes through immobilization. For example, the immobilization of enzyme can change the thermal behavior of the enzyme such as it could potentially extend the operating capabilities of the process and allow the reactor to operate at even higher temperature for long time period, thus increasing the reaction rates and product yields. However, the immobilization of enzyme faces some disadvantages. One of the most common drawbacks often encountered is the partial loss of enzymatic activity. It was withered during the process of immobilization or due to

denaturation at the final immobilized enzyme conformation. In the last few years, such methods for enzyme immobilization were advised where the immobilized enzyme suffered minimal loss of activity, but commercialization of immobilized enzymes is yet to be realized on a large scale. In a literature, there are four fundamental categories of enzyme immobilization, which are non-covalent adsorption or deposition, covalent attachment or carrier binding, entrapment in a gel, matrix or membrane and cross-linking; each of them has unique advantages and disadvantages (Cao, 2006). Recently, the covalent binding of the enzyme is an important method because of minimum enzyme leakage from the matrix, compared with the other methods of enzyme immobilization. In addition, the use of very stable and ready-to-use activated support would also be very convenient (Gauisan, 2006).

1.2 Outline of the dissertation

The increase of the enzyme stability is much important in biotechnology and could be got by covalent immobilization of enzyme onto the suitable insoluble carriers. With this expectation, the main goal of this study was to develop new immobilized biocatalysts for the synthesis of lactulose-based OS. The crude enzyme (commercial Pectinex Ultra SP-L preparation) containing the β-galactosidases was used. The experimental setup of this thesis was focused on two directions of enzyme immobilization: covalent immobilization on magnetic micro- and nanoparticles, and chitosan microparticles. Another important aim was to develop a continuous system using IE preparations for the synthesis of lactulose-based OS. Detailed tasks are following:

+ preparation of carriers: activated chitosan and magnetic particles;
+ immobilization of Pectinex Ultra SP-L (β-galactosidase activities) by the covalent binding method;
+ optimization of some factors which affect the yield of enzyme immobilization;
+ characterization of the resulting IE preparations including the effect of temperature and pH on enzyme activity, thermal stability, reusability, catalytic properties for lactulose-based OS synthesis, as well as the kinetic model of the IE preparations;
+ development of the bio-system for continuous synthesis of lactulose-based OS: setting up a continuous system of bio-reactor; investigation of main factors affected (dilution rate, the initial concentration of substrate) on the yield of the OS synthesis.

2 LITERATURE REVIEW

2.1 General information of oligosaccharides

2.1.1 Chemical nature

In term of molecular size or DP of saccharides, according to IUB-IUPAC nomenclature, the OS are defined as glycosides that contain between three and ten sugar moieties. However, many disaccharides possess similar properties to the larger sugars and are often major components of food-grade oligosaccharide products. Hence, disaccharides such as lactulose, xylobiose are included as OS in this discussion (Crittenden & Playne, 1996; Vázquez *et al.*, 2000). On the other hand, based on the physiological properties, the carbohydrates can be classified as digestible or non-digestible (Mussatto & Mancilha, 2007).

Table 2.1 Typical non-digestible oligosaccharides with chemical structure
(Prapulla *et al.*, 2000)

Oligosaccharide	Chemical structure	Bond
GOS	α-D-Glu(1→4)-[*β*-D-Gal(1→6)]$_n$ (n = 2-5)	*β*(1-4), *β*(1-6), *β*(1-3)
Lactulose	*β*-D-Gal(1→4)-*β*-D-Fru	*β*(1-4)
Lactosucrose	*β*-D-Gal(1→4)-α-D-Glu-(1→2)-*β*-D-Fru	*β*(1-4)
FOS	α-D-Glu(1→2)-[*β*-D-Fru(1→2)]$_n$, n = 2-4, *β*-D-Fru(1→2)-[*β*-D-Fru(1→2)]$_n$, n = 1-9, α-D-Glu(1→2)-[*β*-D-Fru(1→2)]$_n$, n = 2-9	*β*(1-2)
Palatinose	[α-D-Glu(1→6)-D-Fru]$_n$, n = 2-4	α(1-6)
Glycosyl sucrose	α-D-GIu(1→4)-α-D-Glu(1→2)-*β*-D-Fru	α(1-4)
Malto-OS	[α-D-Glu(1→4)]$_n$, n = 2-7	α(1-4)
Isomalto-OS	[α-D-Glu(1→6)]$_n$, n = 2-5	α(1-6)
Gentio-OS	[*β*-D-Glu(1→6)]$_n$, n = 2-5	*β*(1-6)
Soybean OS	[α-D-Gal(1→6)]$_n$-α-D-Glu(1→2)-*β*-D-Fru, n = 1-2	α(1-6)
XOS	[*β*-Xy1(1→4)]$_n$, n = 2-9	*β*(1-4)

The NDOs are resistant to acid hydrolysis and to enzymes present in saliva and the digestive tract, hence they reach the colon intact. The anomeric carbon atom (C1 or C2) of the monosaccharide units of NDOs is configured such that the glycosidic bonds are resistant to human digestive enzymes (Roberfroid & Slavin, 2000). The main categories of NDOs

presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose and/or xylose. The OS show diversified structures because of the type and the number of mono-saccharides, as well as the position of glycosidic bonds (**Table 2.1**). According to Sako and co-workers (1999), over 20 different types of NDOs are available on the world market.

2.1.2 Sources

The OS of various types can be found as natural components in milk, honey, plants, roots, fruits, and vegetables, etc. For most of these sources, concentrations range between 0.3% and 6% of fresh weight; for chicory and salsify, these values are between 5% and 10% while in Jerusalem artichoke and yacon they can reach up to 20% (Voragen, 1988). Natural sources of OS were reviewed by several scientists such as Mussatto and co-workers (2007), Otles (2014) etc., and they were rephrased in **Table 2.2**.

Table 2.2 Natural source of typical oligosaccharides

OS	Natural source	Reference
Inulin	Onion (2–6%), garlic (9–16%), leek (3–10%), wheat (1–4%), banana (0.3–0.7%), asparagus (10–15%), Jerusalem artichokes (15–20%), chicory (13–20%).	Ötleş, 2014
Soybean OS	Extracted from soya bean (4-6%)	Švejstil *et al.*, 2015
XOS	Bamboo shoots, fruits, vegetables, milk, and honey	Vázquez *et al.*, 2000; Xiao *et al.*, 2015
GOS	Goat milk (4.8%), cow milk (1.1%)	Kim *et al.*, 2015
FOS	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye	Sangeetha *et al.*, 2005; Yun, 1996; Ziemer & Gibson, 1998
Isomaltulose	Honey, sugarcane juice	Lina *et al.*, 2002
Raffinose OS	Seeds of legumes, lentils, peas, beans, chickpeas, mallow, composite, and mustard	Johansen *et al.*, 1996; Sánchez-Mata *et al.*, 1998

Some OS could be directly extracted from plant, seed or bean (**Figure 2.1**), such as extraction of soybean OS from soybean (Švejstil *et al.*, 2015).

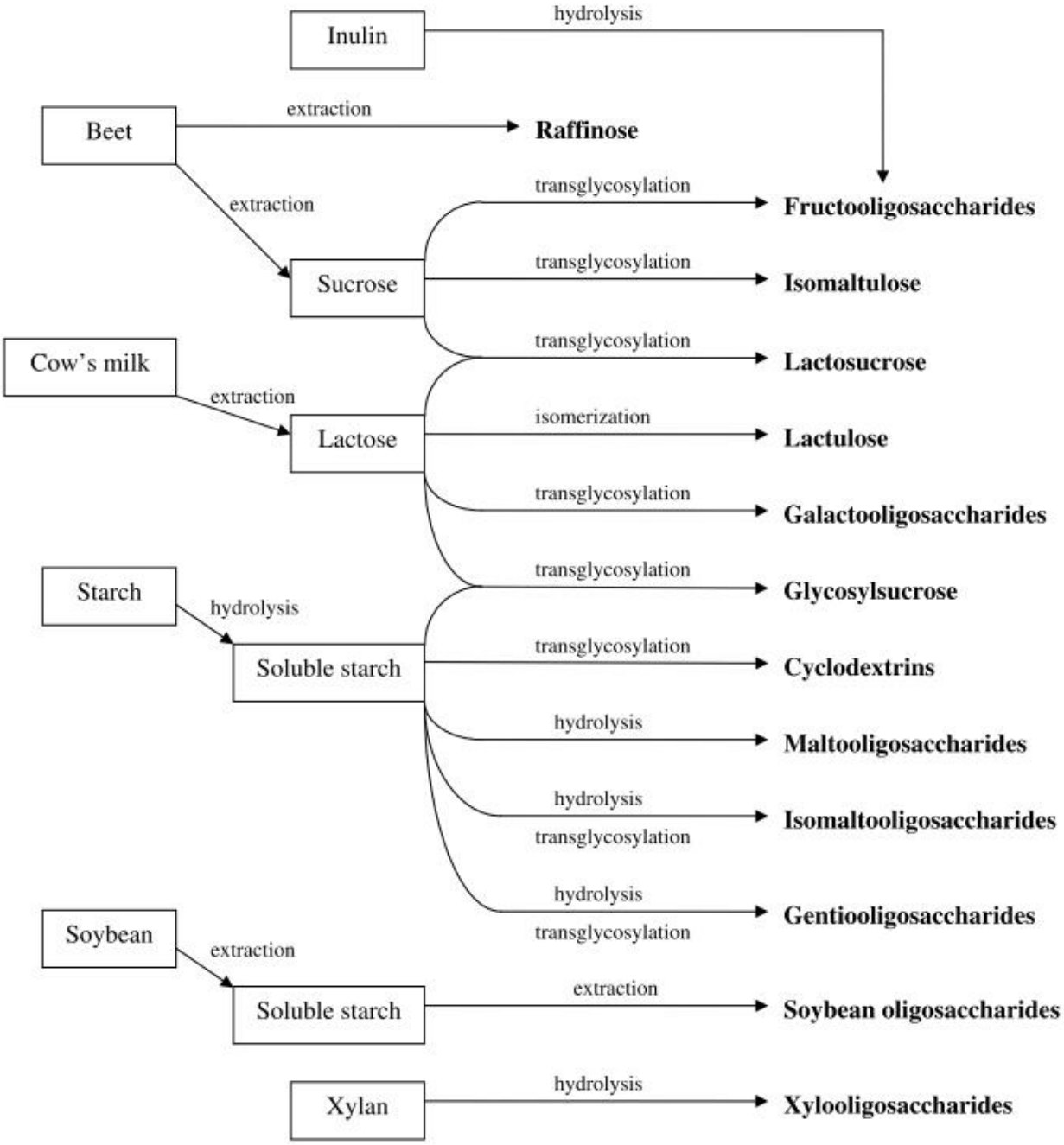

Figure 2.1 Production processes of non-digestible oligosaccharides
(Mussatto & Mancilha, 2007)

In addition to the natural source of the OS, the polysaccharides can be enzymatically hydrolyzed to form the OS (**Figure 2.1**). For instance, FOS were formed by controlled enzymatic hydrolysis of inulin with inulinase (Singh & Singh, 2010); maltooligosaccharides were produced from starch by pullulanase and isoamylase, combined with hydrolysis by

various α-amylases (Moon & Cho, 1997); or XOS from xylans by xylanases (Yang *et al.*, 2007).

Furthermore, the OS could be also synthesized by a chemical method as well as enzymatic method. Such as enzymatic synthesis of Lu and GOS from lactose (Sitanggang *et al.*, 2014; Song *et al.*, 2013); enzymatic synthesis FOS from sucrose (Santos *et al.*, 2007) etc. Additionally, some OS are produced from two raw materials (**Figure 2.1**). For example, lactosucrose is enzymatic produced using lactose and sucrose by levansucrase (Han *et al.*, 2009); or glycosylsucrose is produced using sucrose and liquid starch (Sako *et al.*, 1999). Recently, the enzymatic synthesis of OS has been studied, as an alternative method, to solve the disadvantages of a chemical method.

2.1.3 Physicochemical properties

The OS are water soluble and mildly sweet, typically 0.3 ÷ 0.6 times as sweet as sucrose. The sweetness of the OS is dependent on their chemical structure, molecular weight or DP, and the levels of mono- and disaccharides in the mixture (Crittenden & Playne, 1996). The sweetness decreases with higher DP of the OS (Roberfroid & Slavin, 2000). Their relatively low sweetness is useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavors.

Compared with mono- and disaccharides, the higher molecular weight of OS provides increased viscosity, leading to improved body and mouth-feel. They can also be used to alter the freezing temperature of frozen foods, and to control the amount of browning due to the Maillard reaction in heat-processed foods. Huebner and co-workers (2008) determined the effect of processing conditions on the prebiotic activity of commercial prebiotics using a prebiotic activity assay. The results showed that prebiotics were considered functionally stable at the food processing conditions of low pH (pH 3 ÷ 6) and Maillard reaction (up to 6 h at 85°C with 1% glycine, pH 7), while only heating at low pH (30 min at 85°C, pH 4 ÷ 7) caused a significant reduction in prebiotic activity.

In addition, the OS provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination (Crittenden & Playne, 1996). Furthermore, NDOs are indigestible but fermentable, the caloric value of them has been estimated to be 1 to 2 kcal/g (Cummings *et al.*, 1997; Roberfroid *et al.*, 1993). This is proximately 40 ÷ 50% of those of digestible carbohydrates such as sucrose (Sako

et al., 1999). For food labeling purposes, in Europe, GOS should be given a caloric value of 2 kcal/g (EC, 2008). A brief summary of physicochemical properties of some prebiotic OS are showed in **Table 2.3**. The OS have potential properties which make them be used in a variety of foods because they are chemically stable during food processing treatments, and they enhance the physical properties of foods. Additionally, the OS with a difference of chemical structure (monomer and glycosidic bonds) show different properties.

Table 2.3 Physicochemical properties of oligosaccharides (Ötleş, 2014)

OS	Physicochemical properties
Inulin	Water solubility of 28 % (w/v) at 80°C; approximately 10% sweetness of sugar/sucrose; DP of ≤ 10; melting point is 184.5 °C with 5.3% (w/w) H_2O
FOS	Highly hygroscopic; viscosity and thermal stability are higher than that of sucrose; highly stable in pH range of 4.0 ÷ 7.0; solubility, freezing and boiling points are similar to sucrose
GOS	Water solubility of about 80% (w/w); viscosity is similar to high-fructose corn syrup; stable to 37 °C at pH 2 for several months; sweetness is typically 0.3-0.6 times compared to that of sucrose
Soya-OS	Sweetness is 70% compared to sucrose; stable below 15°C; water activity is close to sucrose; lower viscosity than maltose
XOS	Sweetness is equivalent to 30% sucrose; stable over a wide range of pH (2.5 ÷ 8.0) and temperatures (100°C); water activity is similar to glucose; antifreeze activity of xylobiose on water at temperatures higher than –10°C is the same as that of xylose, but greater than that of glucose, sucrose, and maltose
Isomalto-OS	Stable in pH 2.0 ÷ 9.0, normal baking temperatures; water soluble and mildly sweet (~ 60% as sweet as sucrose); high moisture-retaining capacity
Pyrodextrins	Typically amorphous; water soluble; low intrinsic viscosity

2.1.4 Physiological properties

In addition to the OS have their own important physicochemical properties, most of the interest in their use as food ingredients is due to their many physiological properties beneficial for health.

2.1.4.1 Non-digestibility

Based on digestibility of sugars by the upper part of human digestion systems, saccharides can be divided into two main groups: digestible and non-digestible (**Table 2.4**). Most natural sugars are digestible and utilizable. However, generally, saccharides contained *β*-glycosidic linkages are unable to digest in human digestion system due to lack suitable enzymes. These OS are classified as non-digestible OS or NDO (Rivero-Urgell & Santamaria-Orleans, 2001).

Table 2.4 Classification of saccharides by digestibility (Hidaka *et al.*, 1991)

Nutrient	Small intestine	Large intestine	Example
Saccharides	Digestible	Fermentable	Glucose, fructose, maltose, lactose, starch, etc.
	Non-digestible	Fermentable	Dietary fibers (pectin, plant gums…), raffinose, stachyose, OS.
		None (or less) fermentable	Dietary fiber (agar, cellulose…)

Several *in vitro* and *in vivo* experiments have demonstrated that the OS are stable and indigestible by the digestive enzymes (Torres *et al.*, 2010). According to the consensus report from Loo and co-workers (2007), more than 90% of GOS could reach to the colon after admission. GOS with DP3 and DP4 were not hydrolyzed *in vitro* by human salivary *α*-amylase, artificial gastric juice, α-amylase of hog pancreas, and rat intestinal acetone powder. In another study, only a very small amount of 4'-galactosyllactose was digested by the homogenate of the rat small intestinal mucosa (Ohtsuka *et al.*, 1990).

Most of the *in vivo* human data regarding the indigestibility of GOS were obtained by hydrogen breath tests, a noninvasive technique. Several studies reported an increased breath hydrogen excretion when the ingested GOS amount was between 15 to 35 gram/day, indicating that GOS were not digested in digestive system, and were fermented by the colonic microbiota

(Alles *et al.*, 1999; Chonan *et al.*, 2004; Tanaka *et al.*, 1983). However, Bouhnik and co-workers (1997) showed reduced breath hydrogen after administration of a 10 gram daily dose of GOS but, at the same time, bifidobacterial numbers were increased, indicating that the GOS were fermented by colonic microbiota (Bouhnik *et al.*, 1997).

NDOs are considered to behave as soluble alimentary fibers since from a physiological point of view they fit the definition of fiber: they arrive whole to the large intestine where they are fermented by colonic flora (Hidaka *et al.*, 1991; Tsuji *et al.*, 1986). This property makes the NDOs suitable for use in sweet, low-caloric diet foods, and for consumption by individuals with diabetes (Crittenden & Playne, 1996).

2.1.4.2 Prebiotic properties

A prebiotic can be defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health" (Gibson, 2004; Gibson *et al.*, 2004; Gibson & Roberfroid, 1995). The gut microbiota is now perceived as a key player in health and well-being with a composition in which potentially health-promoting dominant microorganisms (especially the saccharolytic genera/species, such as bifidobacteria) are elevated and/or more active than the potentially harmful ones (especially the proteolytic/putrefactive genera/species) (Roberfroid *et al.*, 2010). The composition and activity of the intestinal microbiota can influence health and disease through its involvement in nutrition, host physiological functions, and pathogenesis of certain disease conditions (Ringel & Carroll, 2009; Roberfroid, 2008). A large number of human intervention studies that have been performed show that prebiotic can result in statistically significant changes in the composition of the gut microbiota, especially an increase of faecal concentrations of beneficial microbiota (e.g. bifidobacteria), in line with the prebiotic concept (Andersen *et al.*, 2011; Maccaferri *et al.*, 2012; Shimizu *et al.*, 2013; Toward *et al.*, 2012)

Some NDO are known as prebiotics because they are potential functional components in food and pharmaceutical field as well (Rycroft *et al.*, 2001; Torres *et al.*, 2010; Tuohy *et al.*, 2002), and these have been selected for their health benefits on the host. Gibson and co-workers (1995) presented the popularity of inulin, FOS, and GOS as health benefit substrates. In human studies, the addition to the bread of 7 g of FOS has been shown to beneficially affect the dominant bifidobacteria as compared to common bread. The authors clearly proved that the use

of FOS exerted a profound effect upon bifidobacteria (Gibson *et al.*, 2004). Drakoularakou and co-workers (2010) stated that the consumption of the tested GOS mixture showed significant potential in the preventing the incidence and symptoms of travellers' diarrhea. The data from Moro and co-workers (2002) indicated that supplementation of a term infant's formula with a mixture of GOS and FOS has a dose-dependent stimulating effect on the growth of bifidobacteria and *Lactobacilli* in the intestine (Moro *et al.*, 2002). A similar study also showed that at a low level of GOS (0.24 g/100 mL) in infant's formula can improve stool frequency, decrease fecal pH, and stimulate intestinal bifidobacteria and lactic acid bacteria as in those fed with human milk (Ben *et al.*, 2008). Other research on elderly illustrated that GOS can be a useful dietary candidate for the enhancement of gastrointestinal health and immune function in elderly (Vulevic *et al.*, 2008).

Among the various properties of NDOs, the prebiotic potential has attracted attention (Meyer *et al.*, 2015). The main physiological effects of OS are related to their impact on the composition and activities of the intestinal microbiota. The human intestinal tract harbors a complex community of bacteria, eukaryotic microorganisms, archaea, viruses, and bacteriophages, collectively referred to as the intestinal microbiota. The majority of the members of the colonic microbiota are obligate anaerobic genera, including *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus* and *Ruminococcus* (McCartney & Gibson, 2006). A prebiotic NDO must neither be hydrolyzed nor absorbed in the upper part of the gastrointestinal tract; and must be selectively fermented, assimilated by one or by a limited number of potentially beneficial microorganisms residing in the colon (Collins & Gibson, 1999). To improve colonic function, live microorganisms can be administered in adequate amounts, being known as probiotics; and to be used in food, these organisms must be able to survive passage through the gut; to proliferate and to colonize the digestive tract; and must be safe and effective (Rioux *et al.*, 2005; Roberfroid, 2008). The selective properties of prebiotics are supposed to relate to the growth of bifidobacteria and lactobacilli at the expense of other groups of bacteria in the gut (Macfarlane *et al.*, 2006). Along with inulin, FOS and Lu, GOS are also food ingredients that have been consistently established as prebiotic ingredients from several studies conducted *in vitro* and *in vivo* (Gibson *et al.*, 2004; Rastall, 2006; Roberfroid, 2007; Tzortzis & Vulevic, 2009).

The fermentation and prebiotic properties of NDOs depend on their chemical structure, such as number and type of monomers; type, position, and conformation of the glycosidic

linkages. The differences in DP and structures of the OS present in OS mixtures, which occur mainly due to the conditions and source of enzymes used for their production (Otieno, 2010), are expected to be important when it comes to OS assimilation by bifidobacteria in the colonic microbiota (Tzortzis & Vulevic, 2009). For example, the administration of a GOS mixture (3.6 grams per day) containing mainly β(1-3), as well as β(1-4) and β(1-6) linkages, proved to have a better bifidogenic effect than a GOS mixture (4.9 grams per day) containing mainly β(1-4), as well as β(1-6), after 1 week of intake by healthy humans (Depeint *et al.*, 2008). Two important factors must be taken into account for the evaluation of suitable prebiotic carbohydrates, probiotic microorganisms or possible synbiotic combinations. One of them is the rate at which a microorganism can grow on a particular carbon source, because it will influence its ability to compete with other bacteria in the colon; the other one is the extent to which the substrate is converted into bacterial mass, as cell numbers will affect the degree of prebiotic or probiotic activity (Hopkins *et al.*, 1998). Studies on GOS utilization by bacteria have proved that they possess different ability to ferment GOS and individual strains have specific substrate preferences (Barboza *et al.*, 2009; Hopkins *et al.*, 1998; Huebner *et al.*, 2007; Ignatova *et al.*, 2009; Smart *et al.*, 2009). The first study on the effect of OS-Lu on pure cultures growth by Cardelle-Cobas and co-workers (2011) showed that twelve strains belonging to genera *Lactobacillus*, *Streptococcus* and *Bifidobacterium* were able to utilize lactulose and different trisaccharides derived from lactose and lactulose as carbon sources. These data indicated that the structure (glycosidic linkage and/or the monosaccharide composition) of the trisaccharides affected the growth of individual strains, most probably as the result of the different β-galactosidases specificity that the microorganisms possess. The data showed general preference of the strains towards β-galactosyl residues having β(1-6) and β(1-1) linkages over those of β(1-4). In addition, similar or higher cell densities and speed of growth were achieved on 6'-galactosyl-lactulose than on 6'-galactosyl-lactose (Cardelle-Cobas *et al.*, 2011b). A range of disaccharides (glucose-, galactose-, fructose- and mannose-containing disaccharides), prebiotic, non-prebiotic and unknown activity, were investigated to determine structure – prebiotic function relationships by Sanz and co-workers (2005) via *in vitro* fermentation method. It was also confirmed that disaccharides with linkages of 1-2, 1-4, and 1-6 generated a high PI score than that of others. Apart from 6α-mannobiose, mannose-containing disaccharides gave a low PI due to low numbers of bifidobacteria and lactobacilli and an increase in bacteroides (Sanz *et al.*, 2005). More results were summarized in **Table 2.5**.

Table 2.5 Summary of the structure effect on relative probiotic index among the tested disaccharides (Sanz *et al.*, 2005)

Structure	**Disaccharides**	**Prebiotic index (PI)**
Effect of linkage	*α*-glucobioses	*α*(1−2) > *α*(1−6) > *α*(1−3) > *α*(1−4) > *α*(1−1)
	β-glucobioses	*β*(1−2) > *β*(1−4) > *β*(1−3) > *β*(1−6) > *β*(1−1)
	α-galactobioses, melibiose	*α*(1−6) > *α*(1−3)
	β-galactobioses, lactose	*β*(1−4) > *β*(1−6)
	α-mannobioses	*α*(1−6) > *α*(1−3) > *α*(1−4) > *α*(1−2)
	α-fructobioses	*α*(1−2) > *α*(1−4) > *α*(1−6) > *α*(1−5) > *α*(1−1) > *α*(1−3)
Effect of monosaccha-ride composition	*α*-1−2 linkage	Glu-glu > Glu-fru > Man-man
	α-1−3 linkage	Glu-glu > Gal-gal > Glu-fru > Man-man
	α-1−4 linkage	Glu-fru > Glu-glu > Man-man
	β-1−4 linkage	Gal-gal > Glu-glu > Gal-glu > Gal-fru
	α-1−6 linkage	Glu-glu > Man-man > Gal-glu > Glu-fru
	β-1−6 linkage	Gal-gal > Glu-glu
	1−1 linkage	*α*, *β* Glu-glu > *α* Glu-fru > *α*, *α* Glu-glu > *β*, *β* Glu-glu

The influence of structure on the fermentation of maltose based OS by human gut bacteria was investigated by Sanz and co-workers (2006) by using anaerobic *in vitro* fermentation method. Their results illustrated that carbohydrates of DP3 showed the highest selectivity towards bifidobacteria; however, oligosaccharides with a higher molecular weight (DP6 ÷ DP7) also resulted in a selective fermentation. Oligosaccharides with DPs above seven did not promote the growth of "beneficial" bacteria (Sanz *et al.*, 2006).

The two basic types of fermentations taking place in the gut are saccharolytic fermentation and proteolytic fermentation. The main end products of carbohydrate metabolism are the short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (Ötleş, 2014). Most of the prophylactic health effects proposed for GOS arise from their selective consumption by bifidobacteria and lactobacilli, as a fermentative substrate. These reported health effects have been recently reviewed by other researchers (Macfarlane *et al.*, 2008; Playne & Crittenden, 2009; Tzortzis & Vulevic, 2009), and include protection against enteric

infections; increased mineral absorption; immunomodulation for the prevention of allergies and gut inflammatory conditions; trophic effects of SCFAs on the colonic epithelium; fecal bulking; and reduced toxigenic microbial metabolism that may reduce risk factors for colon cancer (Conway, 2001; Roberfroid & Slavin, 2000).

Among the OS, Lu is one of industrial interest compound because of the significant impacts on human digestion. Lu has been used in a wide variety of food as a prebiotic (Panesar & Kumari, 2011). The prebiotic property of Lu on the colonic motility pattern as well as its ability to promote the selective growth of beneficial intestinal bacteria located in human gut has been reported by numerous publications (De Preter *et al.*, 2006; Schumann, 2002; Tuohy *et al.*, 2002). Furthermore, in the pharmaceutical field, Lu is used mainly for treatment of constipation, hepatic encephalopathy, a complication of liver disease and maintenance of blood glucose and insulin level (Schumann, 2002). Unfortunately, at high doses, the Lu has laxative effects and the fact that fermentation occurs mainly in the proximal colon, which results in uncomfortable gas production (Salminen & Salminen, 1997; Tuohy *et al.*, 2002), this thus makes a limitation in use of Lu. Moreover, the prebiotic index of lactulose-based oligosaccharides (OS-Lu) may be higher than that of the Lu due to the higher degree of polymerization. Fermentation of these OS is going slowly and their metabolism takes place more distally in the colon (Tuohy *et al.*, 2005). In addition, OS-Lu showed a better anti-inflammatory profile than Lu in a model of experimental colitis (Algieri *et al.*, 2014). In that Algieri and co-workers (2014) presented their study to test the preventative effects of OS-Lu with prebiotic properties in the trinitrobenzene sulfonic acid model of rat colitis and compared them with those of lactulose. The results showed that both treatments modified bacterial profile in intestinal contents, by increasing the bifidobacteria and lactobacilli counts and up-regulating the production of SCFAs, although OS-Lu generated a larger amount of SCFAs. OS-Lu also inhibited to greater extent different pro-inflammatory markers such as interleukins. However, both prebiotics equally restored colonic epithelial integrity, evaluated both with a histological score and by measuring several key proteins of the mucosal barrier. In conclusion, OS-Lu showed a better anti-inflammatory profile than Lu in this model of experimental colitis. Furthermore, López-Sanz and co-workers (2015) studied the physicochemical modifications of OS-Lu during the processing of milk and apple juice. The results presented herein demonstrate that OS from lactulose can be used as prebiotic ingredients in a wide range of functional foods, including those intended for diabetics and lactose intolerant individuals. The OS-Lu was stable in milk heated at temperatures up to 100 °C for 30 min and in apple juice heated up to 90 °C

for 15 min. The sensory properties of juice with OS-Lu were acceptable and similar to those of apple juice with commercial GOS (López-Sanz *et al.*, 2015). Thus synthesis and functional property, as well as applications of OS-Lu are currently attracting the attention of the scientific community (Cardelle-Cobas *et al.*, 2009; Cardelle-Cobas et al., 2008a; Martinez-Villaluenga *et al.*, 2008).

2.1.5 Applications

The health benefits of functional OS are well known and far reaching. The OS are applied in diverse industrial applications, such as in nutraceutical, pharmaceutical, prebiotics, cosmetics, animal feed and agriculture sector.

2.1.5.1 Food applications

These NDOs are used in a variety of foods not only for their prebiotic effect but also because they are non-cariogenic, they represent less sweet sweeteners and they enhance the physical properties of foods (Sako *et al.*, 1999). As their novel properties, prebiotic OS can be served as functional food ingredients because they satisfied two important requirements, including safety and chemical stability during food processing treatments. The specific physicochemical and physiological properties of food-grade OS products vary depending on the type of mixture purchased. Accordingly, the most appropriate OS for food application will also vary. However, some properties are common to almost all OS products (Crittenden & Playne, 1996). The OS provide some manufacturing and health benefits, which make their use as food ingredients particularly attractive (Crittenden & Playne, 1996). In food biotechnology, prebiotics are increasingly used in the development of new food products (Gibson & Roberfroid, 1995). Prebiotic OS can be incorporated into many foodstuffs as agents to improve or maintain a balanced intestinal microflora to enhance health and well-being; they can also significantly improve organoleptic characteristics, upgrading both taste and mouth-feel. These applications are illustrated in **Table 2.6**. Because of their stability, in addition to infant foods, OS can also be incorporated into a wide variety of other foods. They have been used in beverages (such as fruit juices), meal replacers, fermented milk, flavored milk and confectionery products. Bread is a suitable candidate for OS incorporation because, during the fermentation and baking processes, OS molecules are not cleaved or consumed. Furthermore, due to the high moisture retaining the capacity of OS, excessive product drying is prevented

conferring this bread a better taste and texture. Specialized foods for the elderly and hospitalized people are also promising fields of application of GOS (Sako *et al.,* 1999). The NDOs have a pleasant taste and can increase the texture and mouth-feel of foods providing bulk properties as sucrose. OS are resistant to salivary degradation and are not used by the oral microbiota and can, therefore, be used as low-cariogenic sugar substitutes. Being indigestible they have a negligent impact on blood glucose (Prapulla *et al.,* 2000). GOS are mainly used in infant milk formula, follow-on formula, and infant foods (Playne & Crittenden, 2009). Supplemented infant formulas usually contain 6.0 to 7.2 $g.L^{-1}$ GOS together with 0.6 to 0.8 $g.L^{-1}$ FOS (Rastall, 2006).

Table 2.6 Applications of oligosaccharides in food biotechnology (Ötleş, 2014)

Applications	Functional properties
Yogurts and desserts	Sugar replacement, texture and mouth-feel, fiber, and prebiotics
Beverages and drinks	Sugar replacement, mouth-feel, foam stabilization, and prebiotics
Bread and fillings	Fat or sugar replacement, texture, fiber, and prebiotics
Meat products	Fat replacement, texture, stability, and fiber
Dietetic products	Fat or sugar replacement, fiber, and prebiotics
Cake and biscuits	Sugar replacement, moisture retention, fiber, and prebiotics
Chocolate	Sugar replacement, heat resistance, and fiber
Sugar confectionery	Sugar replacement, fiber, and prebiotics
Soups and sauces	Sugar replacement, and prebiotics
Baby food	Texture, body and mouth-feel, fiber, stability, and prebiotics

It was noted that the too high dosage of prebiotic OS showed negative effects on the gut system, causing diarrhea and consequently decreasing growth performance (Biggs, 2007). Spiegel and co-workers (1994) reported that the minimum dose of FOS causing diarrhea is 40 – 50 grams per day. Furthermore, the European Commission confirmed that FOS and inulin could be used in foods targeted towards infants older than six months of age at a concentration of 0.8 gram per day (Rao, 2002). The effective bifidogenic dose of OS is generally less than 15 grams per day (Tomomatsu, 1994).

2.1.5.2 Non-food applications

Besides the food field, other areas, such as the cosmetic and pharmaceutical industries, can also exploit the physicochemical and physiological properties of OS (Torres *et al.*, 2010). There are many of the promising OS drugs in development interfere with adhesion events between different cell types (cell-cell), and between antibodies and cells (antibody-cell). For example, the OS are competitive inhibition of adhesion: glycoconjugates (as glycoproteins and glycolipids), which are expressed on cell surfaces, participate in many cell-cell and other direct molecular recognition and binding processes, in health and in disease, and present a multitude of opportunities for therapeutic intervention. These processes generally proceed through the carbohydrate portion of these glycol-conjugates. The simplest approach to the disruption of recognition and binding processes is direct competitive inhibition of adhesion of pathogens or their toxins to host cells, antibodies to their carbohydrate antigens, lectins to their respective saccharide ligands and of adhesion between autologous cells (e.g. in inflammation and metastasis). Three examples of competitive inhibition of recognition and adhesion events of relevance to disease states were examined: adhesion of bacteria to epithelia, adhesion of leukocytes to endothelial cells and adhesion of antibodies to endothelial cells. An abundance of therapeutic opportunities for complex carbohydrates, the earliest diseases being targeted involve cell-surface adhesion events in inflammation, infectious diseases, and xenotransplantation, in which OS compete with natural carbohydrate ligands and inhibit the adhesion events involving leukocytes, endothelial cells, microorganisms, and antibodies. This marked the emergence of a period that will see the greater integration of complex carbohydrates into the repertoire of therapeutic, diagnostic and nutritional agents. Furthermore, complex carbohydrate-based agents are being developed for use in metabolic and cardiovascular diseases, as cancer vaccines and in drug delivery (Simon, 1996).

Otherwise, in the cosmetics sector, the prebiotic GOS can selectively stimulate "beneficial" bacteria on the human skin and some formulations for that purpose have already been developed (Krutmann, 2009).

In addition, for the purpose of improving health and growth, improving gut microbial ecology, minimizing the use of antibiotics, preventing early mortality; and reducing fecal odor, the application of NDOs in the livestock feed and pet food industries is also increasing. For example, GOS are finding increased use in the poultry such as a study on young chicken (Biggs *et al.*, 2007), pig (Modesto *et al.*, 2011; Tzortzis *et al.*, 2005), and aquaculture, as health protective agents for fishes (Defoirdt *et al.*, 2011) industries.

2.2 Production of oligosaccharides

The OS possess great structural diversity and have a wide range of roles. The large scale application of functional OS in foods, feed, pharmaceutical, cosmetics and agrochemistry industries, make it imperative to develop scale-up synthesis methods (Patel & Goyal, 2011). In terms of their production, OS can be obtained by (*i*) extraction from natural sources, or (*ii*) depolymerisation or synthesis by physical, chemical as well as biotechnological methods (Courtois, 2009; Ötleş, 2014). Only few OS are naturally produced, so strategies have been developed to obtain them by latter one, which is commonly employed (Courtois, 2009; Mussatto and Mancilha, 2007). Furthermore, the OS can be obtained by extraction from yeast biomass (Bychkov *et al.*, 2010). Prebiotics OS can be formulated either as a powder or syrup and marketed as supplements or incorporated into food products (Heller, 2001).

2.2.1 Chemical methods

Commercial and chemical synthesis and synthetic strategies have been well developed (Garegg, 1990). Prebiotic NDOs can be obtained by either chemical hydrolysis (degradation) or chemical conversion (reversion) methods. These methods have their own advantages and limitations.

A chemical method using diluted or concentrated acids (to 2 M) such as HCl, H_2SO_4, TFA, formic, or nitrous acid under heating (between 50 and 90°C), can be applied to polymers containing essentially neutral sugars as fucans, carrageenans, pectins and galactans (Delattre *et al.*, 2005). Polysaccharide depolymerization by hydroxyl free radicals is a complex mechanism where the Fenton-like reaction of transition metals as copper or iron in the presence of H_2O_2 generates hydroxyl free radicals. Then, the polymer is attacked by free hydroxyl radicals. The molecular mass decrease indicates that radical attacks occur at the glycosidic linkage (Sarbu *et al.*, 2003). For example, XOS are produced from xylan-containing lignocellulosic materials by autohydrolysis with water or steam or in media catalyzed with externally added mineral acids (Moure *et al., 2006*)

This method is not the best one as the cleavage of glycosidic linkages is not really specific, and a wide diversity of oligomers can be obtained. Ovalle and co-workers (2001) proposed that some degradations may happen in the sugar ring and that recombination reactions

between the various radicals occur. In fact, by controlling the degradation conditions (temperature and duration), very few ring degradations are observed (Sarbu *et al.*, 2003). As for the depolymerization under acid conditions, a random cleavage is observed.

Otherwise, it is well known that OS can be formed from monosaccharides by the action of mineral acids. This process, known as "reversion" explains the production of OS during acidic hydrolysis of lactose, first observed in the 1950s (Aronson, 1952). The conditions suitable for OS production during acidic hydrolysis of lactose and the resulting OS structures formed have been well studied (Huh *et al.*, 1990; 1991). It was reported that there is the formation of a complex mixture of disaccharides and trisaccharides, with a variety of linkages with α- and *β*-anomeric configurations and anhydrosugars, as a result of this chemical process (Huh *et al.*, 1991). Probably due to the lack of product specificity and extreme conditions applied during acidic hydrolysis of lactose, this GOS production process is not used on a large scale.

In addition, alkaline catalysis is applied for the conversion of lactose to lactulose. Prebiotic Lu is produced from lactose by an alkaline isomerization process, which is used to convert the glucose moiety in lactose to a fructose residue. Alkaline-catalyzed isomerization of glucose to fructose is simple but gives rise to serious lactic acid and color by-product formation (Crittenden & Playne, 1996). However, product yields of about 87% are higher than those obtained through enzymatic synthesis of up to 45% (Wang *et al.*, 2013).

2.2.2 Biotechnological methods

The biotechnological methods use either enzymes or microorganisms as biocatalysts to produce OS by hydrolysis (of polysaccharides), reverse hydrolysis, as well as transglycosylation reactions. Practically, the enzymes are usually obtained from microorganisms (so-called microbial enzymes). They could be exo-enzyme (extracellular enzyme) or endo-enzyme (intracellular enzyme). The exo-enzymes are secreted by a microbial cell and usually, involved in the breakdown of larger molecules. Microorganisms are popular sources for industrial enzymes due to easy availability and fast growth rate.

Enzymatic production of OS has been implemented as an alternative to the chemical methods, to avoid costly and cumbersome downstream processing. There is a great deal of interest in developing methodologies for the enzymatic synthesis of these OS (Ichikawa *et al.*, 1992; Rastall & Bucke, 1992; Toone *et al.*, 1989). These methods possess the advantages such

as highly region-selective, stereo-selective, efficiency and so on. However, they meet the limitations such as the price of commercial enzyme preparation, the yield of bio-reaction, and the need of specific sugar, etc. There are two main classes of enzymes currently being used for the *in vitro* synthesis of OS including glycosidases and glycosyltransferases (such as fructosyltransferase, galactosyltransferase, etc.). The enzymes that selectively build or break down glycosidic bonds, therefore reign over one of the most diverse sets of molecules of biology (Henrissat *et al.*, 2008; Prapulla *et al.*, 2000). In other words, the enzyme can selectively catalyze for either degradation of polysaccharides or conversion of mono- as well as disaccharides forming OS.

2.2.2.1 Biodegradation

The OS are industrially produced using the catalytic activity of glycoside hydrolases. These enzymes are more readily available than glycosyltransferases, but they are generally less stereo-selective (Tzortzis & Vulevic, 2009). Enzymatic hydrolysis of the glycosidic bond is performed by two catalytic residues of the enzyme acting as a general acid and a nucleophile/base, respectively. Depending on the spatial position of these catalytic residues, hydrolysis can occur with 1 of 2 possible stereochemical outcomes: inversion of anomeric configuration, if the average distance between the 2 catalytic residues is approximately 10 A˚; or retention of anomeric configuration, if the average distance between the 2 catalytic residues is about 5.5 A˚ (Koshland, 1953).

The OS can be produced by controlled enzymatic hydrolysis of such polysaccharides as starch, inulin, xylan and so on (Prapulla *et al.*, 2000). Enzymatic degradation (arabino hydrolases from *Chrysosporium lucknowense*) of sugar beet arabinan with a mixture of the arabino hydrolases releases the main degradation products arabinose and arabinose, but as well produces various unknown arabino-oligosaccharides (AOS), then AOS was isolated from the mixture (Westphal *et al.*, 2010). XOS can be produced from xylan-containing lignocellulosic materials by either chemical methods (by auto-hydrolysis with water or steam or in media catalyzed with externally added mineral acids) or enzymatic hydrolysis of a susceptible substrate (Katapodis *et al.*, 2002), or even a combination of chemical and enzymatic treatments (Yuan *et al.*, 2004).

2.2.2.2 Biosynthesis

2.2.2.2.1 Reverse hydrolytic activity

OS can be also synthesized by glycosidase in reverse (equilibrium synthesis) (Crout & Vic, 1998). Two suggested approaches for OS synthesis using glycosidase were described (Rastall & Bucke, 1992), which are the equilibrium and the kinetic approach.

The equilibrium approach is based on the fact that all enzyme reactions are reversible. The reversal of the normal glycoside hydrolysis reaction is achieved by increasing the concentration of the products of the forward reaction and reducing the concentration of reactants, but at such high concentrations the reaction rates are slow; the temperature of the reaction is therefore increased to allow equilibrium to be reached within a reasonable time period (Rastall & Bucke, 1992). So, this is done by incubating the enzyme in a highly concentrated solution of monosaccharides. The limitation of this method is that equilibrium yields are low. However, no hydrolytic side-products are obtained from this reaction, and when combined with an effective separation process, a hypothetical 100% yield is possible (Bruins *et al.*, 2003).

The kinetic approach takes advantage of the fact that the hydrolysis of glycosidic bonds proceeds through a two-stage process. The 1st irreversible stage involves the formation of a covalently linked glycosyl-enzyme intermediate, followed by the exit of the "leaving group". This covalent bond is subsequently hydrolyzed, again with inversion, completing the reaction with retention of configuration via a general acid-base catalytic mechanism. In a well-known variant of the 2nd step, the glycosidase enzyme may be intercepted by nucleophiles other than water, potentially any sugar (as an acceptor molecule) in solution, to form trans-glycosidic products (GOS) (Prapulla *et al.*, 2000). For the synthesis of an OS, a reactive donor substrate, which promotes rapid glycosylation of the enzyme, and a glycoside acceptor are required to be prepared. Glycosidases are generally nonspecific for acceptor sugars, so hetero-oligosaccharides can be synthesized by co-condensation of two monosaccharides or a monosaccharide and an OS. The production of hetero-oligosaccharides can be promoted over the production of homo-oligosaccharides by increasing the percentage of acceptor sugar in the mixture, but this leads to a decrease in the total yield of OS products (Rastall & Bucke, 1992). Compared to glycosyltransferases, glycosidases are more stable, easier to be purified and not required in completely pure form (Rastall & Bucke, 1992). However, the disadvantage of these

enzymes is that regulation of the proportions of the various OS in the final product is quite difficult.

2.2.2.2.2 Glysosyltrasferation

Many glycosyltransferases, such as galactosyl-, fructosyl-, and glucosyl-transferase, have been purified and applied to *in vitro* synthesis of oligosaccharides (Ichikawa *et al.*, 1992). For preparing complex and highly pure OS, application of microbial enzyme galactosyltransferases and fructosyl-transferases are currently recognized very effective. The first stage in the synthesis involves the formation of sugar-1-phosphates by a kinase enzyme followed by transfer of the sugar residue to a nucleoside triphosphate by a nucleoside transferase. The second stage involves the transfer of sugar units from the sugar nucleotides to sugar acceptors, catalyzed by glycosyltransferases (Rastall & Bucke, 1992). Therefore, converting sugar into OS by *β*-galactosidases is a kinetically controlled reaction, by means of the competition between hydrolysis and trans-galactosylation. Specifically, during this conversion, the thermodynamically favored hydrolysis of lactose, which generates D-galactose and D-glucose, competes with the transferase activity that generates a complex mixture of various galactose-based di- and oligosaccharides of different structures (Tzortzis & Vulevic, 2009). Hence, knowledge of the reaction time course is required to determine the point of maximum yield of the desired product. Transgalactosylation involves both intermolecular and intramolecular reactions. In the case of lactose conversion into GOS, intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose. Otherwise, intermolecular or indirect trans-galactosylation is the route by which disaccharides, trisaccharides, and tetra-saccharides and eventually longer GOS, are produced from lactose (Huber *et al.*, 1976).

In the catalysis of fructosyl-transferase (*β*-fructosyltransferase, EC 3.2.1.26 or *β*-D-fructosyltranferase, EC 2.4.1.9), FOS are synthesized from sucrose. The fructosyl-translation of sucrose takes place via the cleavage of the *β*-2,1-glycosidic bond and the transfer of the fructosyl moiety onto any acceptor other than water, such as sucrose or a fructooligosaccharide (Vega & Zuniga-Hansen, 2014). This synthesis is a complex process in which several reactions occur simultaneously, both in parallel and in series, because FOS are also potential substrates of these enzymes (Garegg, 1990). In other words, the substrate is acting simultaneously as a donor and acceptor for the fructosyl moiety (Alvarado-Huallanco & Maugeri Filho, 2011; Duan *et al.*, 1994; Guio *et al.*, 2012). Because this set of trans-

fructosylation chain reactions has two reactants, the Michaelis-Menten mechanism is not applicable. Several kinetic models (Alvarado-Huallanco & Maugeri Filho, 2011; Duan *et al.*, 1994; Guio *et al.*, 2012) have been developed to predict the reaction progress for the synthesis of FOS from sucrose, and the models are of great interest for defining strategies that allow the optimization and industrial scale-up of these bioprocesses (Suzuki *et al.*, 2002).

Table 2.7. Enzymatic synthesis of lactulose-based oligosaccharides by *β*-galactosidase

Enzyme source	Enzyme form	Method of immobil-ization	Results	Refs.
Pectinex Ultra SP-L derived from *Aspergillus aculeatus*	Soluble	-	Product of trisaccharide 6′-galactosyl-lactulose, and other GOS Optimal pH 6.5 and 60°C	Cardelle-Cobas *et al.*, 2008a
β-galactosidase from *Kluyveromyces lactis*	Soluble	-	Two products are OS-Lu. The 1st one has a galactose unit linked to C-6 of galactose moiety, the other one has a galactose unit linked to C-1 of the fructose moiety.	Martinez-Villaluenga *et al.*, 2008
β-galactosidase from *Aspergillus oryzae*, *A. aculeatus*, *K. lactis*	Soluble	-	The degree of polymerization of OS-Lu is up to 6, 5, 4, respectively.	Hernández-Hernández *et al.*, 2011
β-galactosidase from *A. oryzae*, *K. lactis*, *B. cierculans*	Soluble	-	The enzyme produced the highest yield and specific productivity of synthesis, being selected for optimization study. The optimal yield was reached at 70 °C and 60% w/w Lu concentration.	Guerrero *et al.*, 2013
β-Galactosidase from *A. oryzae*	Soluble and immobi-lized	Covalent immobil-ization onto agarose support	Optimal conditions with both FE and IE for OS-Lu synthesis were 50 °C, pH 6.5, 456 g/L of Lu, and 8 U/mL of the enzyme. Immobilization increased the enzymatic stability to temperature changes and allowed to reuse the enzyme.	Cardelle-Cobas *et al.*, 2016

The primary advantage of glycosyltransferases is their great specificity, allowing for precise and sequential construction of OS. However, this requires the presence of a very wide range of enzymes; thus, the availability of purified enzymes is a major limiting factor. Another major problem with the use of glycosyltransferases as synthetic reagents is that these enzymes are generally unstable in solution.

Because of attractive properties of OS-Lu, the synthesis of this sugar is reviewed below in more details. Technically, OS-Lu can be produced by trans-galactosylation reactions catalyzed by *β*-galactosidases from different fungal sources, including *Aspergillus aculeatus* (Cardelle-Cobas *et al.*, 2008a; Guerrero *et al.*, 2013), *Kluyveromyces lactis* (Guerrero *et al.*, 2013; Martinez-Villaluenga *et al.*, 2008) and *Aspergillus oryzae* (Guerrero *et al.*, 2013). Enzyme *β*-galactosidases (*β*-D-galactoside-galactohydrolase, EC 3.2.1.23) are important biocatalysts for the industry, exhibiting both hydrolytic and trans-galactosylation activities (Huber *et al.*, 1976; Panesar *et al.*, 2010). They have been used to reduce the lactose content in foods via their hydrolysis activity (Szczodrak, 2000), furthermore, as catalysts for trans-galactosylation reactions leading to the synthesis of potential prebiotics such as lactose-based galactooligosaccharide (OS-La) (Cardelle-Cobas *et al.*, 2011b; Padila *et al.*, 2012), lactulose-based galactooligosaccharide (OS-Lu) (Cardelle-Cobas *et al.*, 2011a; Cardelle-Cobas *et al.*, 2008a; Guerrero *et al.*, 2015; Guerrero *et al.*, 2013; Martinez-Villaluenga *et al.*, 2008), Lu (Song *et al.*, 2013), lactosucrose (Li *et al.*, 2009), etc. In the enzymatic synthesis process of OS-Lu, prebiotic substrate Lu plays both the roles of donor and acceptor of the galactosyl residue. The galactose unit (from a donor by enzymatic hydrolysis reaction) can link to C-6 of the galactose moiety or C-1 of the fructose moiety (Martinez-Villaluenga *et al.*, 2008). The degree of polymerization of OS-Lu may depend on the source of enzyme. Hernández-Hernández and co-workers (2011) reported that *β*-galactosidases from *A. aculeatus, K. lactis,* and *A. oryzae* were able to synthesize OS-Lu with DP up to DP5, DP4, and DP6, respectively. **Table 2.7** summarizes the enzymatic synthesis of OS-Lu. The table presented source and form of enzyme, main results, as well as the method of enzyme immobilization (in case of using the immobilized enzyme).

2.2.2.2.3 Microbial production

Microbial production of OS has been extensively reviewed by Prapulla and co-workers (2000). As above mentioned, the enzymes could be either intracellular or extracellular which

are usually produced by the microorganisms (yeast, fungi, etc.), so-called microbial enzymes. Hence, the OS can be synthesis by either enzyme preparations (above section) or mycelium (Chien *et al.*, 2001; van Balken *et al.*, 1991), or homogenized cell suspension (Kumar *et al.*, 2011), or crude enzyme/culture filtrate (Lopez-Leiva & Guzman, 1995; Park *et al.*, 2001).

Moreover, in the purpose of high OS content production, the microbial methods have been developing with several techniques. Technique 1 – elimination of inhibitor components. The mixed enzyme systems, using a commercial enzyme (e.g. glucose oxidase and catalase), and mycelia (e.g. of *Aspergillus japonicas* and *Aspergillus niger* with *β*-fructofuranosidase activity) to produce higher yields of FOS than that of individual applications, was carried out. In that, glucose an inhibitor of *β*-fructofuranosidase, produced was converted by glucose oxidase to gluconic acid, which was then precipitated by a slurry of $CaCO_3$ to calcium gluconate in solution. The system produced more than 90 % (w/w) FOS on a dry weight basis (Sheu *et al.*, 2001). Other examples, Sheu and co-workers (2002) have reported a complex biocatalyst system with a bioreactor equipped with a microfiltration module to produce high-content FOS in a continuous process initiated by a batch process. The system used mycelia of *Aspergillus japonicas* or *Aureobasidium pullulans* with *β*-fructofuranosidase activity and *Gluconobacter oxydans* with glucose dehydrogenase activity. Technique 2 – use of mineral salts. The mineral salts in the fermentation media have been found to improve FOS production by Vigants and co-workers (2000). It has been reported that 0.6 M NaCl concentration led to an increase of FOS production by 3.5-fold by *Zymomonas mobilis* during fermentation in a medium containing 10 % of sucrose.

In addition, like immobilized enzymes, the use of immobilized cells has led to the development of effective and economic methods for large – scale and continuous production of OS. For example, a method for the continuous production of FOS by immobilized cells was studied by Chien and co-workers (2001), when the mycelia were immobilized on gluten particles and then packed into a column reactor.

2.3 Immobilization of biocatalysts and bioreactor engineering

Recently, the researchers focused on IE because of its advantages over FE in biotechnological applications. Immobilization facilitates the efficient recovery and reuse of costly enzymes (Sheldon, 2007). Additionally, advantages include enhanced stability, great operational control e.g. choosing an immobilization method that can improve the thermal

behaviour of an enzyme could potentially extend the operating capabilities of a process and allow a reactor to operate at higher temperatures, hence increasing the reaction rates and product yields (Bernal *et al.*, 2012; Chalkias, 2007), flexibility of reactor design, and easy separation from catalyst and unreacted substrate contamination. However, enzymatic immobilization has some disadvantages. One of the most common drawbacks often encountered is the partial loss of enzymatic activity during the process of immobilization or due to denaturation at the final immobilized enzyme conformation. Even though in the last few decades the field of enzyme immobilization has made significant advances. Methods of immobilization were advised where the immobilized enzyme suffered minimal loss of enzymatic activity when compared to its native soluble form, but commercialization of immobilized enzymes is yet to be realized on a large scale and only a few examples exist.

In a literature, there are four main categories of enzyme immobilization, which are non-covalent adsorption or deposition, covalent attachment or carrier binding, entrapment in a gel, matrix or membrane and cross-linking (Cao, 2006); each of them with unique advantages and disadvantages (**Table 2.8**).

Although the basic methods of enzyme immobilization can be categorized into four main categories only, hundreds of variations have been developed by combining the original methods (Katzbauer *et al.*, 1995). Correspondingly, many carriers of different physical and chemical nature or different occurrence have been designed for a variety of enzyme immobilizations and separations (Bickerstaff, 1992; Gemeiner, 1992). A rational combination of these enzyme immobilization techniques with a great number of polymeric supports and feasible coupling chemistries leaves virtually no enzyme without a feasible immobilization route (Akgöl *et al.*, 2001).

It has recently been increasingly demonstrated that rational combination of methods can often solve a problem that cannot be solved by an individual one. For example, the drawbacks of an enzyme immobilization by physical entrapment in a gel matrix, such as serious diffusion constraints, and lower stability than that for other immobilized enzymes, can be solved by the rational combination of different methods (Cao, 2006). For instance, higher stability can be achieved by means of the so-called pre-immobilization stabilization strategy (Akgöl *et al.*, 2001) or post-immobilization strategy (Wilson *et al.*, 2004).

Table 2.8 Methods of enzyme immobilization and their characteristics

Method	Advantage	Disadvantage
Physical absorption	Little or no conformational change of the enzyme or destruction of its active center. Simple and cheap	Desorption of the protein resulting from changes in pH, temperature, ionic strength. Non-specific
Ionic binding	Little changes in the conformation and active site of the enzyme. High activities in most cases	Leakage of enzymes from the carrier may occur in substrate solution of high ionic strength or upon variation of pH
Covalent binding (between enzyme molecules and insoluble carrier via functional group)	Usually thought to be the stable method by enzyme carrier bond, which prevents elution of protein into the production stream. The wide range of choices is possible by selecting carrier materials and binding method. This allows a great deal of flexibility in designing an immobilized enzyme with specific physical and chemical properties, such as charge distribution, hydrophobicity/hydrophilicity ration, spacer arm separation, partitioning capabilities, etc.	They are relatively expensive and complicated in procedures. Also, activity yields may be low due to exposure of the enzyme to harsh environments or toxic reagent. Active site may be modified through the chemical reactions used to create covalent binding
Entrapping enzymes	Simple technique. Could be combined with different methods easily	Easy leakage. Serious diffusion constraints. Lower stability than that for other methods
Cross-linking (covalent binding between enzyme molecules)	Very little desorption (enzyme strongly bound). It is the best used in conjunction with one of the other methods. It is used mostly as a means of stabilizing adsorbed enzymes and also for preventing leakage	Cross-linking may cause significant changes in the active site of enzymes, and also sever diffusion limitation may lead to significant loss of activity. Loss of enzyme activity during preparation

In the former case, the enzyme, for instance, can be first cross-linked to form stabilized enzyme preparations e.g. CLEA. Subsequent entrapment endows the CLEA with a suitable particle size and high mechanical stability (Akgöl *et al.*, 2001). Stabilization can be also achieved by chemical modification (Wilson *et al.*, 2004). For instance, chemical modification of the soluble enzyme with a hydrophilic polymer often stabilizes the enzyme because of the

introduction of a favorable hydrophilic microenvironment. Thus, the subsequent entrapment of the stabilized enzyme often leads to the formation of the more stable enzyme, compared with the entrapped native enzymes (Bille *et al.*, 1989; Mohapatra & Hsu, 2000; Wilson *et al.*, 2004).

In the latter case, the entrapped enzyme can be further cross-linked, with the aim of enhancing the stability or avoidance of enzyme leakage. For instance, α-amylase from *Bacillus megaterium* immobilized in BSA gel matrix and subsequently covalently cross-linked was fourteen time more thermally stable than the native enzyme (Ray *et al.*, 1994).

Because of these possibilities, a rational combination of the available methods will facilitate the design of robust immobilized enzymes that can suit various applications. There is, nevertheless, still a significant lack of systematic analysis of the methods available. Most enzyme immobilization has been performed without any knowledge of structural information, and the relationship between the performance of the immobilized enzyme and the method selected for immobilization has, so far, rarely been defined or identified. Thus, a central task in the future development of immobilization techniques is probably not to develop new methods of immobilization but to establish guidelines linking the method selected with the performance expected.

The factors that influence the choice of the method to be used for the enzyme immobilization are generally found to be empirical. Few general rules exist, although some more obvious considerations such as a requirement for a specific physical form of the immobilized enzyme may rule out certain methods. The choice of methods for the immobilization of enzyme normally arises from a screening of the different methods available (Kennedy *et al.*, 1989). It must, therefore, be expected that choice of the method of immobilization is mainly dictated by the specific conditions and requirements of each application, which should selectively employ the positive attributives of the method selected. In this sense, the diversity of enzyme immobilization techniques could be a powerful asset in the design of robust immobilized enzymes, because changes in the peculiarities of the applications often require the design of new immobilized enzymes which fit the new applications (Cao, 2006).

Analysis of all the methods of immobilization currently available has led to the proposal of a rational general approach to enzyme immobilization based on three stages, selection of enzymes, selection of carriers, and selection of conditions and post-treatments, as shown in **Figure 2.2**.

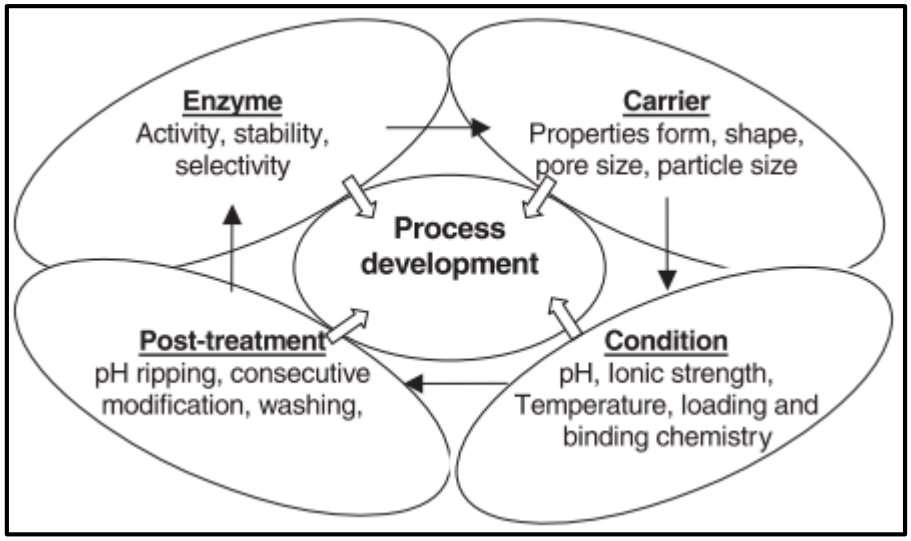

Figure 2.2 General procedures for enzyme immobilization (Cao, 2005)

Covalent immobilization of enzyme has flourished since the 1950s, and is now an important method because of minimum enzyme leakage from the matrix, compared with the other types of enzyme immobilization method. The covalent bonds usually provide the strongest linkages between enzyme and carrier over the other types of enzyme immobilization method (Zaborsky, 1973). Generally, covalent binding of an enzyme to a carrier is based on the chemical reaction between the active amino acid residues located on the enzyme surface and active functionalities that are attached to the carrier surface or vice versa. To achieve efficient linkage, the functionality of the carrier and/or the enzyme must be activated before immobilization. Often, carriers are activated before their use for binding enzymes (Gemeiner, 1992). In addition, the use of very stable and ready-to-use activated support would also be very convenient (Gauisan, 2006).

It has been increasingly appreciated that the physical and chemical nature of the selected carrier also strongly dictates the performance of the immobilized enzymes, for example, activity, selectivity, and stability. In general, covalent binding of enzymes to carriers belongs to the category radical method of enzyme immobilization. This is reflected not only by the irreversibility of the binding, freezing of the enzyme conformation (because of the multipoint attachment), alteration of the chemical entity (because of the chemical modification) but also by the fact that enzyme performance, such as activity, selectivity and stability can be radically improved in comparison not only with other methods of enzyme immobilization but also with the native enzyme. The covalently immobilized enzymes on carriers can be regarded

as a composite consisting of the components carrier, spacer, linkage and enzyme. Thus, the following properties are expected to affect the performance of a covalently carrier-bound immobilized enzyme (Cao, 2006):

- Physical nature of the carrier (e.g. pore size, particle size, porosity, shape, etc.);
- The chemical nature of the carrier (chemical composition of the backbone; active functionality; other non-active functionality);
- The nature of the linkage or binding chemistry;
- The conformation of the enzyme at the moment it is immobilized or after immobilization;
- Enzyme orientation;
- The nature and length of the spacer;
- The properties of the medium used for binding the enzymes;
- The number of bonds formed between the enzyme and the carrier;
- Enzyme distribution on or within the carriers.

It has been found that the performance of a number of carrier-bound immobilized enzymes depends not only on the chemical nature of the carriers but also very much on the physical nature of the carrier such as the shape, size, pore size, porosity and mode of pore distribution (Aparicio & Sinisterra, 1993). Thus, it is obviously beneficial to know the requirements of the physical nature of the carrier, to enable the design of a desired immobilized enzyme with high enzyme loading and higher retention of activity. The important dimension-related properties of the covalently immobilized enzyme are the surface, which is closely related to enzyme loading, as well as the density of binding functionality, which has a strong connection with enzyme stability or activity retention. So it is expected that the criteria for selecting these physical properties might differ for enzyme to enzyme, from carrier to carrier, even from application to application (Mujawar *et al.*, 1999)

The chemical nature of a carrier can be divided into several essential components including chemical nature of the backbone and the surface-tethered functional groups which can be principally placed into two sub-groups: carrier-bound active groups that take part directly in the binding and carrier-bound inert group that do not directly take part in the binding. Although carrier-bound active group (group functionality) will participate in the binding of the enzyme to the carrier, carrier-bound inert group (non-active functionality) do not form any covalent linkage with the enzyme but can also exert great influence on the performance of the

immobilized enzymes such as activity retention, binding efficiency, stability and selectivity (Cao, 2006).

Apart from these pendant groups, the spacers that link the carrier-bound inert group or carrier-bound active group to the backbone of the carrier are also important factors influencing enzyme performance (Cao, 2006). Usually, spacers can affect the binding of biomolecules to the carriers and the performance of the resulting immobilized systems (Hipwell *et al.*, 1974; Steers *et al.*, 1971). It was expected that the properties of spacers such as length (Hayashi & Ikada, 1991; Manecke & Polakowski, 1981), hydrophilicity/hydrophobicity and charged/neutral character can exert a conspicuous influence on binding capability, retention of activity, stability and catalytic performance (Hayashi & Ikada, 1991). In general, hydrophobic spacers have a more profound effect on non-specific binding capacity than hydrophilic spacers (Gemeiner, 1992).

In general, the spacer can be characterized by the several properties: length and size, structure, shape (linear or globular), hydrophobicity/hydrophilicity, positively charged/negatively charged/neutral. Many compounds, varying from small molecules to macromolecules, can be used as spacers:

+ Various bifunctional compounds, for example, bifunctional crosslinkers such as glutaraldehyde (GA) (Martin *et al.*, 2002);
+ Linear polymers such as PEG-diamine (Nouaimi *et al*., 2001) or PEO acid (Shiroya *et al*., 1995), dextran (Penzol *et al*., 1998) and PEI (Anzai *et al*., 1989);
+ Functionalized polysaccharides such as aldehyde dextran, amino dextran, and proteins such as bovine serum albumin (BSA);
+ Polyether (Cao *et al.*, 2007).

Among these spacer-arms, GA is one of the most popular candidates to activate the insoluble carrier and to covalently immobilize enzymes (Betancor *et al.*, 2006; Tukel & Alptekin, 2004)

The diversity in enzyme nature and application often complicates the establishment of such universally applicable rules for the selection of carriers and the method of enzyme immobilization. On the other hand, it is always possible to find a feasible method of immobilization for the selected enzyme because of the diversity and broad availability of the carrier. Thus, it is not surprising that there is no universal carrier for enzyme immobilization and a good carrier for one enzyme applied in a specific process might be not the proper carrier for another enzyme or the same enzyme applied in a different process (Messing, 1975).

Consequently, the change of application, reaction type (hydrolysis, or condensation), reaction medium (aqueous or organic solvents) or the enzyme and substrates often necessitates the design or screening of new carriers or immobilization method.

According to the functionality of the enzymes involved in binding to the carriers and the functionality of the carriers to be used for binding enzyme molecules, it has been established that the coupling reaction can be one of the nine groups (diazotization, peptide bond, alkylation and arylation, Schiff base formation, Ugi reaction, amidination, thiol-disulfide exchange reaction, mercury-enzyme interaction, and oxidation). It has been observed that each type of binding chemistry requires specific functionality between the carriers and the enzymes (Taylor & Swaisgood, 1972). It is also very difficult to predict which carrier-bound active binding group is best-suited for a given enzyme, because other factors such as nature of the backbone also dictate the activity of the binding functionality and the activity of the resulting immobilized enzyme, and in particular the immobilization conditions, as has been demonstrated in many studies (Chikere *et al.*, 2001; Taylor, 1985)

Among a lot of solid carriers for covalent immobilization of enzyme, chitosan (CTS) is considered to be an excellent carrier (Krajewska, 2004; Kumar, 2000; Yazdani-Pedram *et al.*, 2000), because of many interesting properties such as a low cost, renewable, biodegradable natural product, which has very good biocompatibility, low toxicity, biocompatible and chemical stability (Dwevedi & Kayastha, 2009; Mendes *et al.*, 2011). Furthermore, there is a wide diversity of its form which is applied in enzyme immobilization, such as enzyme can be carried out by entrapment into chitosan beads (Freeman & Dror, 1994) or by covalent binding to transparent chitosan films (Akkuş Çetinus & Öztop, 2000) or by chitosan microparticles (Nguyen *et al.*, 2011), etc. Enzyme immobilization can be performed simply and highly after chitosan activated by glutaraldehyde (GA), which is one of the spacer-arm compounds (Kumar, 1999).

The CTS is the partial deacetylated form of chitin (CT), the second most abundant polymer in nature after cellulose. When the degree of acetylation is lower than 50 molar %, the product is named chitosan and becomes soluble in acidic aqueous solutions (Roberts, 1992). The deacetylation could be conducted either by chemical or by enzymatic methods. The former one was preferred to apply due to their low cost and suitability for mass production (No & Meyers, 1995).

From a chemical point of view, either acids or alkalis can be used to deacetylate chitin. During this process, acetyl groups were removed but also depolymerization reaction occurs,

indicated by changes in CTS molecular weight. The *N*-de-acetylation of chitin could be performed heterogeneously or homogeneously (Chang *et al*., 1997). In this work, the heterogeneous deacetylation was conducted on chitin particles by acidic hydrolysis according to reported previously (Nguyen *et al.*, 2011). Consequently, resulted CTS was both insoluble particles and soluble form as well because of the depolymerization. However, only the insoluble part was gained and further used for covalent enzyme immobilization after activated. According to Einbu and Vårum (2007), it is assumed that the acid catalyzed the cleavage of the *N*-acetyl linkage is an SN2 reaction where the rate limiting step is the addition of water to the carbonate ion. The mechanism of the reaction was depicted in **Figure 2.3**.

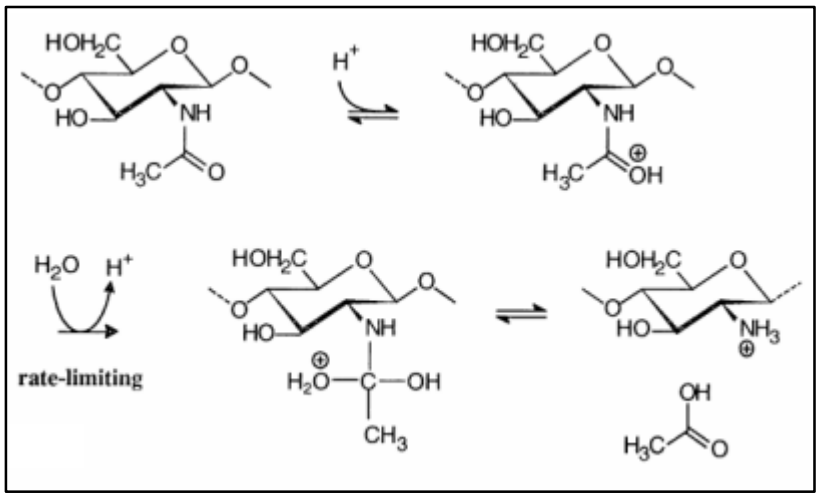

Figure 2.3 The proposed reaction mechanism for the acid-catalyzed hydrolysis of the *N*-acetyl linkage (S_N2 reaction) (Einbu & Vårum, 2007)

Otherwise, immobilization of enzyme on nanostructured materials has been recognized as a promising approach to enhance enzyme stability, capability and engineering performances; and allow the creation of a microenvironment surrounding the enzyme catalysts for maximal reaction efficiencies. Furthermore, enzyme immobilization using these carriers can significantly increase life cycles of the biocatalyst (Misson *et al.*, 2015). With large surface areas, nanostructure materials allow a higher enzyme loading and reduce mass transfer resistance for the substrate. Compare with other nanostructured materials, magnetic Fe_3O_4 particles (MAG) are an excellent carrier for immobilization of enzymes because of their magnetic property, besides the advantages of the nanostructure. Immobilized enzyme onto MAG can be separated easily from the mixture of reaction by applying a magnet.

Till now, MAG have been used for immobilization of many kind of enzymes, such as lipase (Kuo *et al.*, 2012; Liu *et al.*, 2011; Osuna *et al.*, 2015; Wang *et al.*, 2015), protease (Wang *et al.*, 2014), glucoamylase (Zhao *et al.*, 2011; Zhao *et al.*, 2012), amylase (Eslamipour and Hejazi, 2015; Talekar *et al.*, 2012), α-galactosidase (Corchero *et al.*, 2012), *β*-galactosidase (Liu *et al.*, 2012; Neri *et al.*, 2011; Pan *et al.*, 2009) and so on. But, MAG particles tend to aggregate in liquid media due to the strong magnetic dipole-dipole attractions between MAG (Denkbaş *et al.*, 2002; Li *et al.*, 2008a). In addition, many proteins undergo conformational changes during direct adsorption on metals, leading to loss of enzyme activity (Rospendowski *et al.*, 1991). Thus, some biocompatible and biodegradable polymers such as chitosan (CTS) supported by tripolyphosphate (TPP), polyaniline (PAN), etc. have been used as a stabilizer to modify and increase the stability of the carrier (Matsuno *et al.*, 2004). The CTS is considered to be one of the most widely distributed biopolymers. Furthermore, it is cheap, non-toxic, biodegradable and biocompatible polyelectrolyte. Therefore, it has been extensively investigated for potential applications in food, pharmaceutical, cosmetics and biotechnological field (Hu *et al.*, 2008; Rinaudo, 2006).

In addition to the advantages of immobilized enzyme, in both laboratory and industrial scale, generally, the synthesis processes including GOS synthesis can be carried out in several ways such as batch, continuous or semi-continuous. Each method has its own characteristics. However, the continuous processes have the following advantages over a batch process (Malhotra, 2005), which improve the profitability of system:

+ Production of a narrow specification product, i.e. higher and consistent product quality
+ Reduced manufacturing cost
+ Improved asset utilization
+ Increases productivity – more units completed in less time
+ Reduced waste

As above mentioned, with several advantages over native enzyme, covalently immobilized enzymes do not only increase the haft-life time and the operational (pH and temperature) stability but also exhibit convenient separation of IE and sugars solutions. These benefits of IE are useful for making a continuous system of bioconversion.

3 MATERIALS AND METHODS

3.1 Materials

Pectinex Ultra SP-L commercial enzyme preparation from *Aspergillus aculeatus* containing *β*-galactosidase was purchased from Novozymes A/S (Denmark). Chitosan (low molecular weight), chitin particles (No. C7170), and sodium tripolyphosphate were from Sigma – Aldrich (Hungary). Lactulose was from Panreact Applichem (Germany). All the other chemicals and reagents were of analytical grade and purchased from either Sigma-Aldrich, Reanal (Hungary) or VWR (Hungary).

3.2 Preparation of activated solid carriers

3.2.1 Magnetic nano-, micro-particles

Preparation of MAG particles: Magnetic Fe_3O_4 nano-, microparticles were prepared by co-precipitating of Fe^{2+} and Fe^{3+} ions, followed by chemical co-precipitation method with some modifications (Pan *et al.*, 2009; Qin *et al.*, 2015; Saravanakumar *et al.*, 2014). While 50 mL of 0.3M ferric and ferrous (molar ratio 1:1) solution in deionized water was vigorous mechanical stirring, 10 mL of ammonium hydroxide solution (25%) was added to. Then 10M NaOH solution was slowly adding, during the processing pH and temperature was maintained at about 10 and 70 °C, respectively. After 30 min, the suspension, which was black color, was cooled to room temperature with mechanical stirring. Finally, the resulting magnetic Fe_3O_4 particles were collected magnetically by a permanent magnet and washed several times with deionized water to remove the excess base till the pH reached 7. The reaction involved was as shown in equation (3.1)

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O \qquad (3.1)$$

Coating magnetic Fe_3O_4 particles with CTS (called as MAG-CTS): The MAG particles were dispersed in 100 mL of CTS solution (4 mg/mL of 1% acetic solution). Then, 50 mL of 0.5 mg/mL TPP solution was added simultaneously. The suspension was treated with Ultrasound for 30 min at room temperature. The resulting MAG-CTS were collected from the mixture by placing on the magnetic field, washed several times with deionized water. The performance of coating was not determined, and it was considered as a delimitation.

Activation of MAG-CTS: 50 mg of MAG-CTS particles were re-dispersed in 30 mL of 4 % (v/v) GA solution at pH 4 with a mild shaking at room temperature. Then activated MAG-CTS particles were separated by a permanent magnet and washed several times with de-ionized water. The size of activated MAG-CTS particles was measured by scanning electron microscope (SEM) method.

3.2.2 Chitosan microparticles

The CTS particles were formed by heterogeneous deacetylation of chitin according to the procedure of Nguyen and co-workers (2011) with some modifications. The 25 g of CT particles was added in a 1000 ml beaker, then 600 ml of 0.5N HCl was added. After 3 h of continuous stirring at room temperature, the chitosan was collected from the mixture by vacuum filtering and washed with distilled water till the pH reached 7. Then CTS particles were dried overnight at 60 °C and stored at room temperature for later use.

On the next step, the CTS particles were activated with GA spacer-arm. 20 g of CTS particles was scaled into a glass containing 500 ml of 0.1 M McIlvaine buffer solution (pH 5.5). Subsequently, 10 ml of 25% GA solution was added and mixed gently for 1 h at 4 °C. The activated chitosan was vacuum filtered and washed with the same buffer solution several times. Then activated CTS particles were stored in the same buffer solution at 4 °C for later use. The size of activated CTS particles was measured by scanning electron microscope (SEM) method.

3.3 Immobilization of enzyme

3.3.1 Covalent immobilization of *β*-galactosidase onto activated chitosan carrier

Immobilization of Pectinex Ultra SP-L was done according to the method published by Nguyen and co-workers (2011) with some modifications. 2.2 mL of Pectinex Ultra SP-L (25 mg of protein) was dissolved in 50 mL of 0.1N McIlvaine buffer solution at pH 5. The 5 g of activated CTS was added to this solution (after taking out 5 mL for assay of protein content as well as *β*-galactosidase activities of initial solution). The mixture was stirred overnight at 4 °C. The immobilized enzyme (CTS-IE) was collected by filtration and washed with the same buffer solution several times. Also, the supernatant was gained for determination of *β*-galactosidase activities and protein content, as well.

3.3.2 Covalent immobilization of *β*-galactosidase onto activated magnetic carrier

Enzyme immobilization procedure described by Pan and co-authors (2009) with some modifications was applied. Three main factor including protein content, time of immobilization, and pH of buffers solution, were optimized by response surface methodology (RSM) to get maximum yield of enzyme immobilization, which was considered as a response. A set of 20 experiments, obtained from Central Composite Design of the experiment, was conducted at different values of these factors. Twenty test tubes with cap contained the same amount of activated carrier (MAG-CTS-GA) were filled with 5 mL (for each tube) of enzyme solutions, which were different concentration and prepared by dissolved Pectinex Ultra SP-L in the McIlvaine buffer solution at different pH. These mixtures were mild shaken at 4 °C for different times. Finally, immobilized enzymes (MAG-IE) were collected by the permanent magnet and washed 5 times (5 mL for each time) with the McIlvaine buffer solution (pH 5). The supernatant solutions were collected for determination of protein content, and enzyme activity as well.

3.4 Yield of enzyme immobilization

Technically, the yield of enzyme immobilization can be determined in term of expressed activity or protein content. Here, the former one was selected for calculation of recovery activity, which is important for the evaluation of immobilization performance. Immobilization yield was determined in term of expressed activity (YI) according to equation (3.2), where A_{ini} and A_{fin} were enzyme activities (U/mL) in the supernatant (soluble enzyme) before and after immobilization, respectively (Silva *et al.*, 2012).

$$YI = \frac{A_{ini} - A_{fin}}{A_{ini}} x100 \qquad (3.2)$$

As covalent immobilization of enzyme is conducted by covalent binding of enzyme and the activated carrier, the physical and/or chemical properties of the enzyme could be changed after immobilization. Thus, it is necessary to measure enzyme activity recovery (A_{rec}). The A_{rec} was calculated using equation (3.3), where A_{imm} is the activity of the IE (U/g support). The theoretical activity (A_{the}, U/g support) of IE on the activated CTS support could be calculated using the amount of enzyme offered per gram of support (A_{off}) and the YI (Silva *et al.*, 2012).

$$A_{rec} = \frac{A_{imm}}{A_{the}} x100 = \frac{A_{imm}}{IY.A_{off}} x100 \quad (3.3)$$

3.5 Optimization of enzyme immobilization

The CCD was applied for optimization of enzyme immobilization in the case of activated magnetic carrier. The series of 20 experimental runs, involving three independent variables including protein concentration (PC), pH of enzyme solution, and time of immobilization, even in the presence of complex interactions at three levels (-1, 0, 1) were carried out (**Table 4.2**). The independent variable and their ranges were selected based on preliminary experiments which were performed by Pan and co-workers (2009), where the effect of the factors was investigated particularly, without interactions of the factors. The yield of enzyme immobilization was a response (dependent variable). Both of first- and second-order models were given and compared.

Statistical analysis, including the design of the experiment, multiple regressions, ANOVA, and Stepwise Algorithm model selection by Akaike Information Criterion (AIC) method as well, were conducted by using R statistical software version 3.1.3 (www.r-project.org). The fit of regression models was analyzed by the determination coefficient (R-square), adjusted R-square coefficient, Lack of fit (LoF) and AIC as well. Three-dimensional surface plots were drawn to the interaction effects of the independent variables on the dependent variable, being described by a polynomial equation, which was fitted to the experimental data.

3.6 Effect of pH and temperature on enzyme activity

Basically, a procedure of investigation pH and temperature effect on enzyme activity was applied for both MAG-IE and CTS-IE. Firstly, the temperature was constant of 60 °C, which is the optimum temperature got from published scientific papers of the same enzyme branch (Cardelle-Cobas *et al.*, 2008a), the pH of solutions was ranged from 3 to 6.5 with a step of 0.5 unit. The enzyme activity was determined under conditions of constant temperature, the amount of IE, and ILC, but various pH values. The optimal pH refers to the maximum enzyme activity at the defined reaction conditions. Secondly, the enzyme activity was determined under

conditions of constant pH (which was got from the previous step), the amount of IE, and ILC, but various temperature, which was changing from 40 to 75 °C by 5 °C of a step.

3.7 Stability of free and immobilized enzyme

Thermal stability of both FE and IE preparations were determined with standard procedure. Briefly, about 100 U of these enzyme preparations were transferred into the test tubes and then 5 ml 0.1M McIlvaine buffer (pH 5.5 for FE and pH 5.0 for IE, respectively) were added. The test tubes were placed in the water incubator pre-set at optimal temperature (60 ^{o}C). Samples were periodically taken every day and their hydrolytic activities were assayed by using *p*-NPGal as a substrate according to the protocol described below.

Storage stability of the enzyme preparations was determined by the same way of operational stability determination but at 5 °C.

3.8 Reusability of immobilized enzyme preparations

The MAG-IE was applied on the batch bioconversion with Lu substrate at optimal pH 4.5 and temperature 60 ºC for 4 h. The immobilized enzyme was recovered by the permanent magnet, then washed five times with McIlvaine buffer solution, then reused with a new batch at the same way and conditions. The supernatants were collected and homogenized, then put in the boiling water for 10 min. The supernatants and substrate solution were kept in the freezer for sequence determining of sugars contents by HPLC by protocol presented below. Each batch with identified conditions was considered to one time of use of the immobilized enzyme. Relative GOS formation yields were calculated and compared to that of beginning.

The CTS-IE was repeatedly used for several cycles in batch mode. All cycles were done in the same conditions (≈ 0.5g support, 1.5 mL of 20% of Lu substrate solution, temperature 60 ºC, and 12 h of reaction for each batch). At the end of each cycle, the yield of OS-Lu was determined by protocol presented below. Then the IE was washed for several times with the same buffer solution and the new substrate was fed for next cycle.

3.9 Lactulose-based oligosaccharides synthesis and enzyme kinetic model

OS-Lu was synthesized from Lu under catalysis of resulted IE preparations in batch reactions with a volume of 2 mL. The prebiotic Lu substrate was dissolved in McIlvaine buffer

pH 4.5 at different initial Lu concentration (ILC: 20 – 80 % w/v). In that, 0.8% is the saturating concentration of lactulose at 60 ºC (Oosten, 1967). The temperature was set and kept at 60 °C by using an incubator. The samples were taken at regular interval time of reaction after applying a permanent magnet, released enzyme (if there was any) was deactivated by heating the samples in boiling water for 10 minutes, then stored in the freezer for sequence analysis by HPLC.

The kinetic model of OS-Lu synthesis reactions was considered based on the results of HPLC analysis of OS-Lu formation, and the model which was given by Rodriguez-Fernandez and co-workers (2011) with some modification. Several chemical models included either the Lu hydrolysis or the trans-galactosylation reactions, with an assumption of negligible enzyme inhibition by fructose, were supposed. In that, *β*-galactosidase can accept Lu as donor and acceptor of trans galactosylated galactose, leading to the synthesis of OS-Lu. The synthesis of OS-Lu, which is made with fructose terminal (instead of glucose or galactose) also called as fructosyl-galactooligosaccharides (Martinez-Villaluenga *et al.*, 2008). The products including monosugars (fructose and galactose), OS-Lu (DP3 and DP4) were presented in both ways of incorporated into single species (Palai *et al.*, 2012) or separately.

Consequently, several sets of several nonlinear ordinary differential equations that define the rate of reactants change on time course including the constant rates of reactions, which were considered to the kinetic parameters of this model or this kinetic mechanism. A better approach for kinetic parameter estimation is the use of multiresponse nonlinear regression for the whole set of experiments (Bates & Watts, 1988), which has been used in this work, by using the LSODA solver of COPASI software version 4.16 (Hoops *et al.*, 2006). Then the best model was chosen by the minimal root mean square error.

3.10 Development of continuous system

The system including the main components of IE packed column, with a jacket of water flow for incubating, a pump which is able to change flow rate, water incubator, big and small tubes (**Figure 3.1**). The column with the dimensions of 40 cm height, 6 cm outer diameter and 1.9 cm inner diameter has been filled up with the chitosan-immobilized enzyme. Two pieces of filter paper were used at two ends of the column for keeping the IE inside the column. The water incubator was connected to the column via big tubes while the pump was using the small one for feeding substrate solution. Firstly, the McIlvaine buffer at pH 5.5 was pumping for

adjusting the position of IE in the column. Next, the flow rate was set, and after being constant, the substrate solution was conducted.

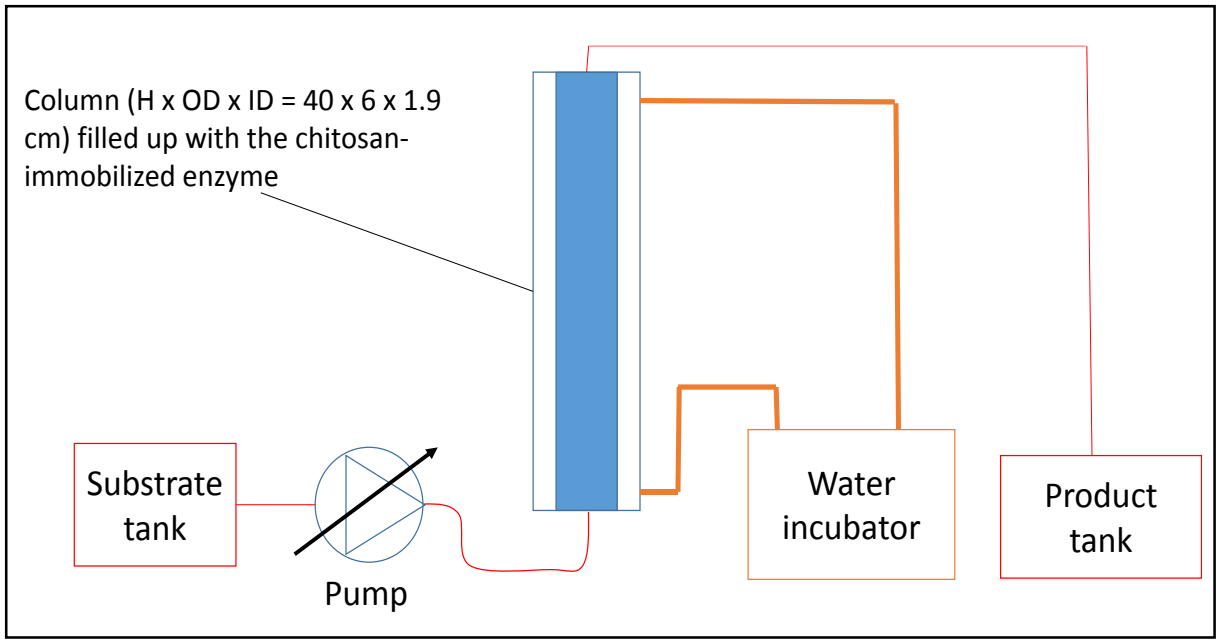

Figure 3.1 Diagram of a continuous bioconversion for production of the galactooligosaccharides

After setting up the system, effects of the initial substrate concentration as well as the flow rate or dilution rate consequently, on the yield of GOS formation has been investigated.

Effect of the dilution rate on the yield of GOS synthesis. The initial concentration of substrate (20 %) and temperature (50 °C) were kept constantly, while the flow rate was set and changed at three levels 16, 27 and 60 mL.h^{-1}, consequently, the dilution rates were 0.14, 0.24 and 0.53 h^{-1}, respectively. The samples were collected at time periods and put in the boiling water for 10 minutes for deactivation. Then they were kept in a freezer for further sequence analysis by using the HPLC.

Effect of the initial concentration of substrate on the yield of GOS formation. The temperature and the dilution rate of the substrate solution have been set constantly to 50 ^{o}C and 0.24 h^{-1}, respectively. The substrate solutions (substrate dissolved in 0.1M McIlvaine buffer pH 4.5) has been set and changed to 4 %, 20 % and 30 % (w/v) of initial lactulose concentration after the end of every reaction. The samples were taken at different time intervals and kept in the freezer for further sequence analysis by using HPLC, after deactivating enzyme activity (if there was any) by putting in the boiling water for 10 minutes.

3.11 Analytical methods

3.11.1 Determination of saccharides

Qualitative analysis: Thin layer chromatography (TLC) was applied for qualitative analysis of carbohydrates. This method shows the advantages such as simple, quick and low cost. Stationary phase was silica gel 60 pre-coated aluminum plate (20 x 20 cm). The mobile phase was a mixture of chloroform – acetic acid – water at ratio 30 – 35 – 5 of volume. The staining solution consisted of 0.3 % (w/v) *N*-(1-naphtyl) ethylenediamine dihydrochloride, 5 % (w/v) H_2SO_4 and 94.7 % methanol. Two microliters of the 10 times diluted samples and the standards solutions were loaded onto the TLC baseline. After 2 h of running, the working TLC plates were dried, then dipping into the staining solution in seconds. After that, they were heated at 110 ºC for 10 min.

Quantitative analysis: The carbohydrates were analyzed by HPLC-RID. Surveyor HPLC system (Thermo Scientific Corporation, USA) consisted of a quadruple pump, an autosampler, a refractive index detector, a column of Hi-Plex Ca 7.7 x 300mm (Agilent, USA) with column oven equipped, was applied. Parameters: mobile phase was distilled water, the flow rate was 0.6 $mL.min^{-1}$, injected volume was 10 µl, the temperature of the column was maintained at 85 ºC, and running time was 20 min. The samples were diluted 100 times before HPLC analysis. Standard substances: lactulose, galactose, fructose, lactose, glucose, raffinose (considered as DP3), stachyose (considered as DP4) were prepared and injected to determine of individual sugar in the slurry.

3.11.2 Determination of protein content

The protein content of samples was determined by the Bradford dye-binding procedure (Bradford, 1976) using Bio-Rad Protein Assay Kit (Bio-Rad, USA). The amount of immobilized protein on activated CTS was calculated from the difference between the amount of protein loaded into the mixture and the amount of protein present in the filtrate solutions after immobilization.

3.11.3 Assay of *β*-galactosidase activities

Hydrolytic activity of free *β*-galactosidase was assayed using artificial *p*-NPGal as substrate at constant conditions. Test tubes contained 1 ml reaction mixture (McIlvaince buffer

pH 4.5 and substrate solutions) were incubated at 60˚C for 10 minutes. The enzyme reaction was started by adding 0.1 ml appropriately diluted enzyme solution and after 5 minutes, it was terminated with 5 ml of 0.1 M Na_2CO_3. After cooling to room temperature, the absorbance of the developed color was read at 405 nm of wavelength. One unit of hydrolytic activity of *β*-galactosidase was defined as the amount of enzyme required releasing 1 µmol of *p*-nitrophenol under one minute at 60 ºC and pH 4.5. In the case of IE, the experiment was done in a similar way with 0.2 mg of support and 10 minutes of reaction time.

Galactosyltransferase activity of *β*-galactosidase towards Lu substrate was assayed in 10 ml of a mixture of the substrate in a 0.1M buffer solution at optimal pH, and the enzyme preparations at optimum temperature. The samples were taken at the time periods, and placed into the boiling water for 10 minutes for deactivation of enzyme activity (if any). Then the sugars were analyzed by HPLC and TLC. One unit of galactosyltransferase activity of *β*-galactosidase was defined as the amount of enzyme required to syntheses 1 µmol of adjusted OS-Lu formation (calculated by adjusted-DP3, and 1 µmol of DP4 was equivalent 2 µmol of adjusted-DP3) under one minute at the relevant reaction conditions.

4 RESULTS AND DISCUSSION

4.1 Hydrolytic and trans-galactosyl activities of commercial enzyme preparation

The hydrolysis and trans-galactosyl activities of Pectinex Ultra SP-L were investigated on several carbohydrate substrates: mono-substrate (lactulose), and bi-substrates at a ratio of 1-1 mol/mol (lactulose - saccharose, lactulose - lactose, lactulose – fructose, lactulose galactose). In the case of mono-substrate, some new compounds were detected in the mixture after reaction based on the result of TLC (**Figure 4.1**- c). Smaller molecules (monosaccharides) should be galactose and fructose due to hydrolytic activity of enzyme preparation. This result was also confirmed by HPLC (**Figure 4.2**). Moreover, two sugar bands with high polymerization degree (correspondence to DP3 and DP4) also appeared on TLC picture. The results showed that the Pectinex Ultra SP-L preparation can catalyze the hydrolysis of lactulose, as well as trans-glycosylation reactions. This is in agreement with the results published by Cardelle-Cobas *et al*. (2008a).

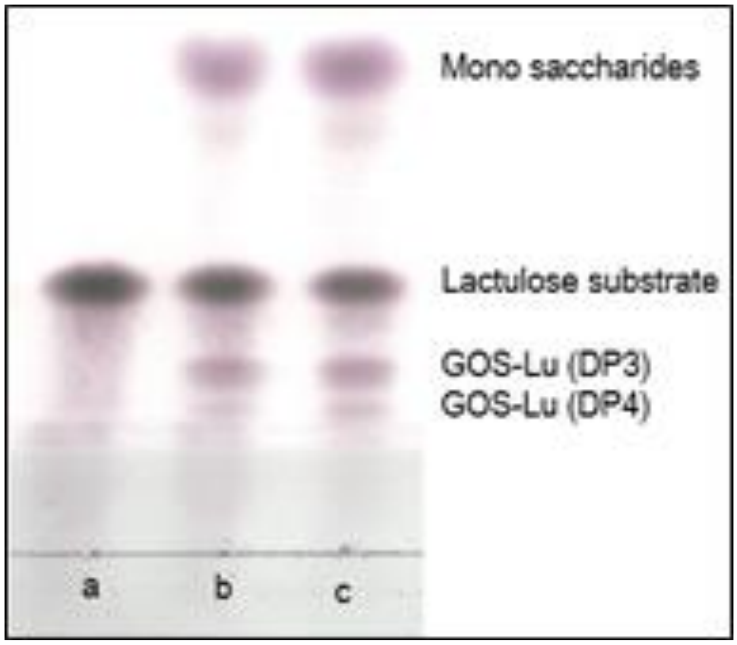

Figure 4.1 TLC zymogram of reaction mixture of lactulose without enzyme (a), lactulose with immobilized enzyme (b), and lactulose with native enzyme (c)

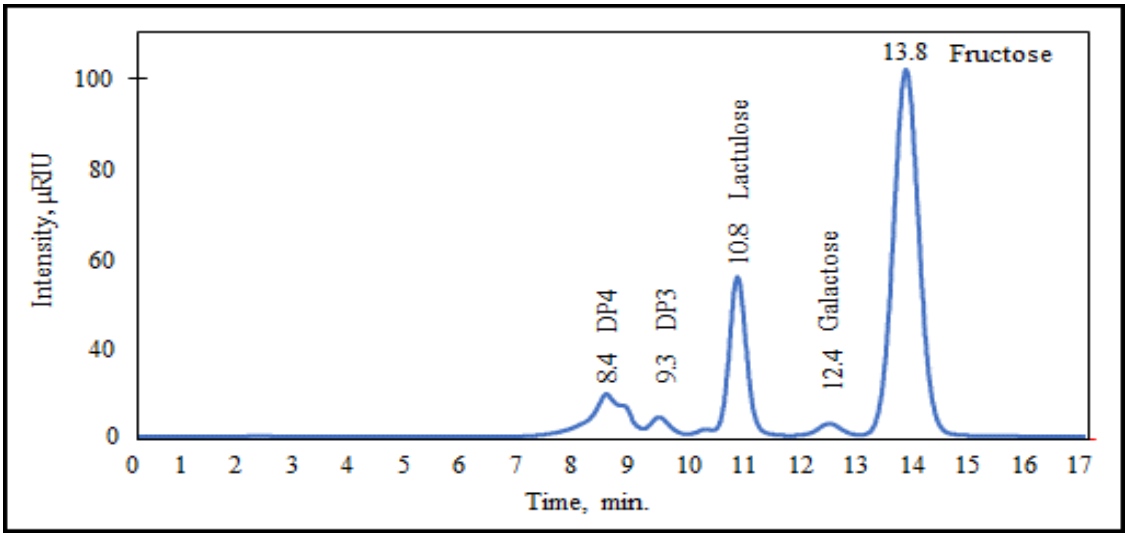

Figure 4.2 HPLC chromatogram of reaction mixture of lactulose with native enzyme

4.2 Immobilization of enzyme

4.2.1 Covalent immobilization of *β*-galactosidase on chitosan particles carrier

The qualitative results show that both trans-galactosylation and hydrolytic activities of *β*-galactosidase (Pectinex Ultra SP-L) on lactulose substrate remained after immobilization. Good bands of both DP3 and DP4 of OS-Lu obtained after 10 h of bioconversion appeared on TLC picture (**Figure 4.1** - b). The HPLC chromatogram (**Figure 4.3**) confirmed the presence of oligosaccharides (DP3 and DP4) also. These results demonstrated the success of the immobilization method and the Pectinex Ultra SP-L was immobilized onto chitosan particles carrier. The yield of immobilization in terms of expressed activity (YI) was as high as about 71% (**Table 4.1**).

Table 4.1 Immobilization yield and activity recovery of enzyme after immobilization

Enzyme load (mg/g)	Activity						YI (%)
	A_{ini} (U/mL)	A_{fin} (U/mL)	A_{imm} (U/g)	A_{off} (U/g)	A_{the} (U/g)	A_{rec} (%)	
13	994.0	288.2	111.9	437.3	310.5	36.1	71.0

A similar yield (70%) of immobilization was reported by Huerta and co-workers (2011) when *β*-galactosidase from *A. oryzae* was immobilized onto chitosan granules activated by glutaraldehyde. At condition of enzyme immobilization, about 36 % of hydrolysis activity was

recovered. This result is much higher than that of 12.4% in a report of Lima and co-workers (2013) when *β*-galactosidase from *K. lactis* was immobilized on activated CTS. Otherwise, higher results of enzyme activity recovery (from 55 to 95%) were reported by Pessela and co-workers (2007) when the *β*-galactosidase from *E. coli* was immobilized of on different supports (PEI-Glu, Eupergit, Glyoxyl, BrCN). The recovery of activity should depend on the properties of the carrier because it may be associated with the enzyme distortion at different levels caused by immobilization.

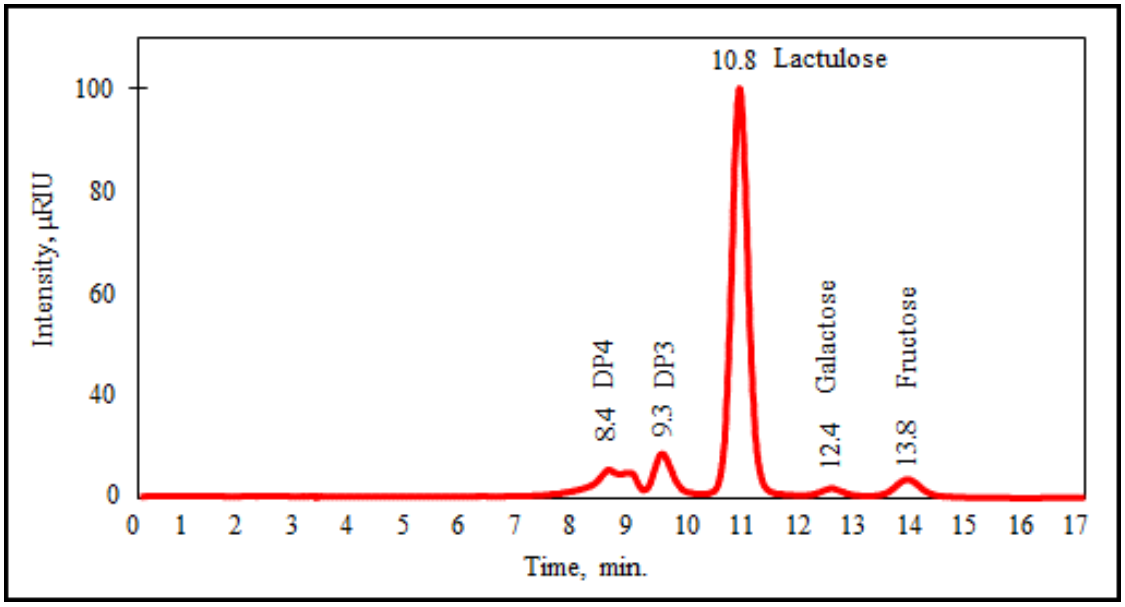

Figure 4.3 HPLC chromatogram of reaction mixture of lactulose substrate with immobilized enzyme

4.2.2 Covalent immobilization of *β*-galactosidase on magnetic particles carrier

Firstly, the response surface full second-order model (equation 4.1) was developed by employing a multiple regression technique onto data obtained by the 20 runs design (**Table 4.2**).

$$YI\ \% = -0.73-1.23*PC+15.11*pH+25.38*Time+4.44*PC*pH+1.86*PC*Time-0.49*pH*Time-31.37*PC^2-1.48*pH^2-2.43*Time^2 \quad (4.1)$$

Table 4.2 Experimental set of rotatable CCD and results

No	PC*, mg.mL^{-1}	pH	Time, h	YI, %
1	0,60 (+1)	3,50 (-1)	4,00 (-1)	98.807
2	0,60 (+1)	5,50 (+1)	6,00 (+1)	96.625
3	0,60 (+1)	3,50 (-1)	6,00 (+1)	92.011
4	0,20 (-1)	5,50(+1)	6,00 (+1)	91.829
5	0,20 (-1)	3,50 (-1)	6,00 (+1)	91.829
6	0,40 (0)	4,50 (0)	5,00 (0)	97.201
7	0,40 (0)	4,50 (0)	5,00 (0)	98.283
8	0,20 (-1)	3,50 (-1)	4,00 (-1)	90.053
9	0,40 (0)	4,50 (0)	5,00 (0)	98.973
10	0,20 (-1)	5,50 (+1)	4,00 (-1)	93.073
11	0,60 (+1)	5,50 (+1)	4,00 (-1)	95.317
12	0,40 (0)	4,50 (0)	6,68 (+1.68)	89.738
13	0,736 (+1.68)	4,50 (0)	5,00 (0)	96.395
14	0,40 (0)	4,50 (0)	3,32 (-1.68)	95.708
15	0,40 (0)	4,50 (0)	5,00 (0)	98.227
16	0,40 (0)	2,82 (-1.68)	5,00 (0)	94.776
17	0,40 (0)	6,18 (+1.68)	5,00 (0)	96.082
18	0,40 (0)	4,50 (0)	5,00 (0)	100.839
19	0,0636 (-1.68)	4,50 (0)	5,00 (0)	95.736
20	0,40 (0)	4,50 (0)	5,00 (0)	98.041

*Three factors [protein content (PC), pH and time of immobilization (Time)] and a response of immobilization yield (YI)

The R-square and adjust R-square were not high; it is 0.79 and 0.6, respectively. However, lack of fit is preferred to use for validating the fit of the model to the observed dataset. There is not enough evidence at the α level of 0.01 to conclude that there is a lack of fit in this regression model (model *(i))* due to a *p*-value of 0.02 (**Table 4.3**).

Table 4.3 Summary of results of stepwise Akaike Information Criterion (AIC)

Stepwise	**Model**	**AIC**	**R^2**	**Adjust R^2**	**LoF** [Pr (>F)]
Start (*Full model*)	%YI ~ [FO(PC, pH, Time) + TWI(PC, pH, Time) + PQ(PC, pH, Time)] *(i)*	34.41	0.79	0.6	0.02
	If TWI(PC, pH, Time) was eliminated	32.50			
	If FO(PC, pH, Time) was eliminated	48.78			
	If PQ(PC, pH, Time) was eliminated	55.70			
Step1	%YI ~ [FO(PC, pH, Time) + PQ(PC, pH, Time)] *(ii)*	32.50	0.74	0.62	0.03
	If PQ(PC, pH, Time) was eliminated	50.83			
	If FO(PC, pH, Time) was eliminated	51.28			
Stop (*Reduced model*)	%YI ~ [FO(PC, pH, Time) + PQ(PC, pH, Time)] *(iii)*	32.50	0.74	0.62	0.03

(Where FO, SO, TWI and PQ are first-order, second-order, two-way interaction and pure quadratic, respectively).

Secondly, the AIC was performed for stepwise selection of the model from the full second-order one. The **Table 4.3** showed that a reduced model (model (*iii*)) received the lowest AIC score (32.5), indicating that this model is the most parsimonious one for the given observed data. The table also shows the results of a comparison between the full model (*i*) and reduced model (*iii*). After reduction, AIC score was decreased, while R-square, adjust R-square and LoF were almost same. In addition, when validation the lack of fit of reduced model, there is not enough evidence at the α level of 0.01 to conclude that there is the lack of fit in reduced regression model due to the *p*-value of 0.03. Then, the reduced model (showing lowest AIC value) and full one were compared to each other using ANOVA, for checking whether the reduction is statistically significant or not. The result showed that the reduced model was not significantly different from the full one at the level α = 0.01 due to Pr (>F) = 0.54. So the reduced model (equation 4.2) was selected over the other, for determination of optimal point and making plots (**Figure 4.4**) as well.

$$YI\ \%= - 1.44 + 28.06*PC + 14.45*pH + 23.93*Time - 31.38*PC^2 - 1.48*pH^2 - 2.44*Time^2 \quad (4.2)$$

Thirdly, the stationary point of response surface (PC, pH, time) was determined at 0.45 mg/mL, pH 4.8 and 4.9 h, respectively. And the maximum response value was estimated to be

98.8%. These results are different than that published by Pan and co-workers (2009), where the enzyme was from *Aspergillus oryzae* and the effect of independent factors was investigated individually. In that the optimal PC and pH of enzyme solution were estimated to be 0.5 and 6, respectively; and time of immobilization had no significant influence on the immobilization efficiency and enzyme activity. These differences can be explained by the different source of enzyme preparation and/or the way of optimization: particularly (Pan *et al.*, 2009) versus together (this study) investigated the effect of three factors on the immobilization of enzyme.

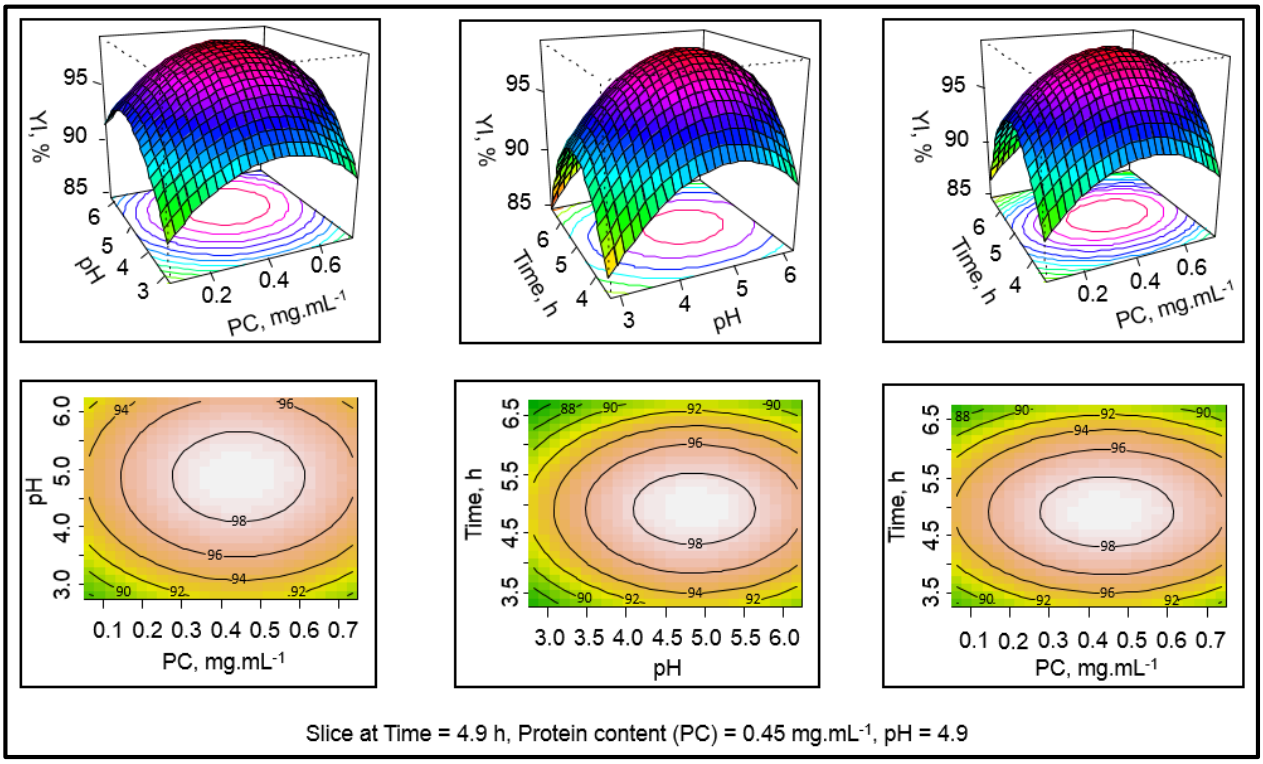

Figure 4.4 Surface and counterplot of fitted model describing the optimization of enzyme immobilization

It is interesting that the yield of enzyme immobilization was so high (98.8%), which was reached at optimal conditions of protein content, pH and time. It is much higher than that of CTS carrier case (71%). This could be explained by the difference in the size of MAG particles and CTS particles; hence, it makes a difference in surface area of solid carriers. **Figure 4.5** illustrates that the size of MAG is range from nm to less than around 5 µm (so-called nano-, micro-magnetic) compared to more than several dozen µm for size of CTS (so-called micro-chitosan)

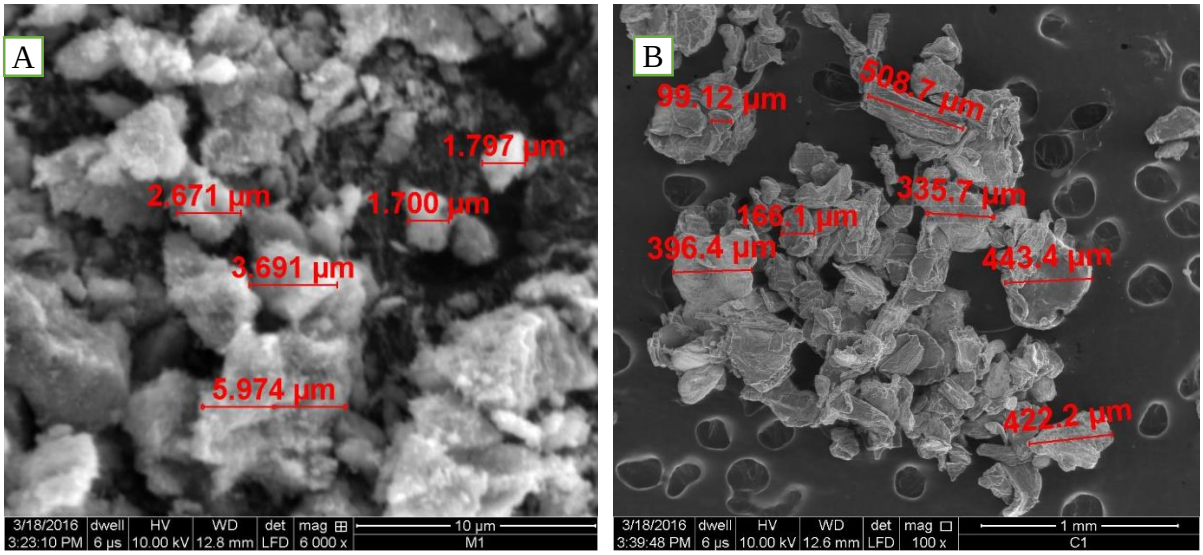

Figure 4.5 Scanning electron microscope picture of magnetic particles (A), and chitosan particles (B)

4.3 The characterization of immobilized enzymes

4.3.1 Effect of lactose substrate on activity of immobilized enzyme

For investigation of galactosyltransferase activity of immobilized enzyme onto CTS carrier, experimental sets were conducted. Substrate concentration was 20% w/v lactose in 0.1M McIlvaine buffer (pH 5.0), and the bioconversion was carried out at 60 ºC. The samples were taken at time intervals and analyzed by HPLC. The results were depicted in **Figure 4.6**.

In the first stage of bioconversion (0 h to 20 h), the concentration of lactose substrate dropped rapidly to about 55 %, while the concentration of glucose grew up fast. The concentration of OS total, as well as galactose, increased, but slower than glucose concentration. At the beginning, when the concentration of lactose was still high and concentration of glucose however low, both rates of hydrolytic and trans-galactosylation reactions were high. In the next stage, the bioconversion got a stationary phase where the concentration of all of the components was little changed. These findings are in agreement with previous studies. According to Iwasaki and co-workers (1996), the hydrolysis and trans-galactosylation reactions occur simultaneously, and the yield of GOS formation is much dependent on lactose concentration. The hydrolysis reaction dominates at low lactose concentration while GOS formation dominates at high lactose concentrations. *β*-Galactosyl groups should have a higher probability of attaching to lactose than water at increasing lactose concentrations. In addition, this can be attributed to the negative effect of monosaccharides

(Neri *et al.*, 2009), the product of hydrolysis process. Galactose is well known as a competitive inhibitor to the lactose hydrolysis reaction (Bakken *et al.*, 1991; Portaccio *et al.*, 1998; Shukla & Chaplin, 1993). In the study of glucose effect on yield of GOS formation from lactose by *Aspergillus oryzae β*-galactosidase, Neri and co-workers (2009) showed that the addition of glucose either alone or mixed with galactose, simultaneously decreased lactose hydrolysis and GOS formation.

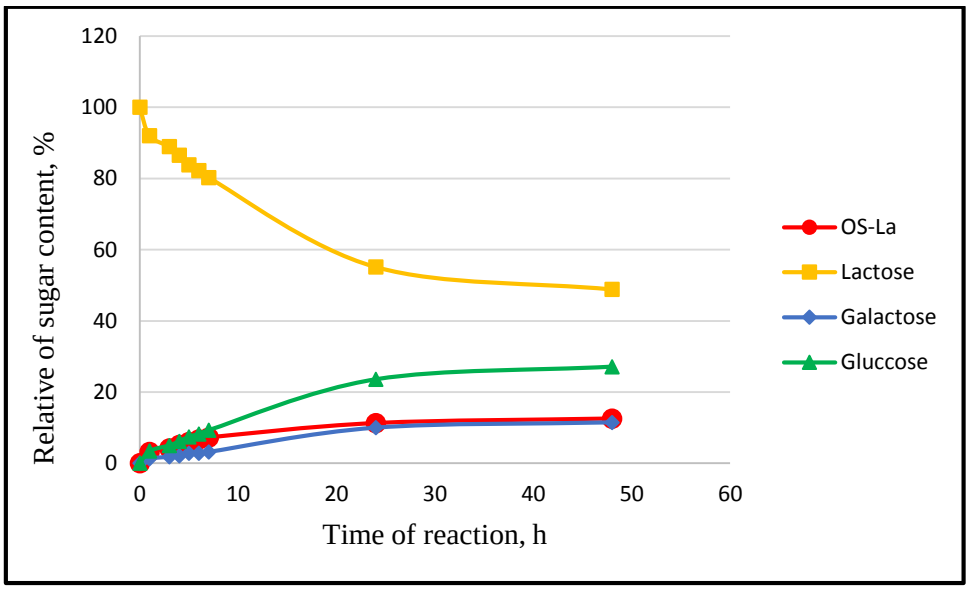

Figure 4.6 Enzymatic conversion of lactose by chitosan-immobilized enzyme

4.3.2 Effect of temperature on enzyme activity in lactulose substrate

The **Figure 4.7** presented the temperature effect on the activity of the enzyme and illustrated that the optimal temperature of the free enzyme is 60 °C, this finding is in agreement with the results published by Cardelle-Cobas and co-workers (2008a) when the authors investigated the OS-Lu synthesis with catalysis of Pectinex Ultra SP-L. This optimal temperature was not changed after immobilization of enzyme onto MAG and CTS carrier. This finding was also figured out by Tanriseven and Aslan (2005), and Aslan and Tanriseven (2007), where the author covalently immobilized the Pectinex Ultra SP-L onto Eupergit C for FOS, and OS-La synthesis.

It is interesting that the MAG-IE showed better operational stability including wider thermal range than that of the native enzyme. When the temperature was changing from 45 to 65 °C, the activity of the immobilized enzyme was remained well (relative ranges from 98 to 100 %) compared to relative ranges from 60 to 100% of free enzyme case. It is so interesting

that total 100 % of the relative activity of MAG – IE was observed when applying the MAG-IE at 50 °C instead of 60 or 65 °C. This could help us to save the energy, as well as extend the lifetime of MAG – IE preparation. Even at high temperature (75 °C), the activity of the immobilized enzyme was remained at above 50%, it was much higher than 10% for the case of free enzyme (**Figure 4.7**). This finding is agreement with Cardelle-Cobas and co-workers (2016) where they covalently immobilized *Aspergillus oryzae β*-galactosidase on a support of glutaraldehyde–agarose for lactulose-based galactooligosaccharides synthesis also. In the case of comparison between free enzyme and CTS-IE, there was no change of temperature effect on enzyme activity. When temperature increase from 40 °C, the activity of both native enzyme and CTS-IE was increased fast from 60 % to reach 100% of relative enzyme activity at 60 °C.

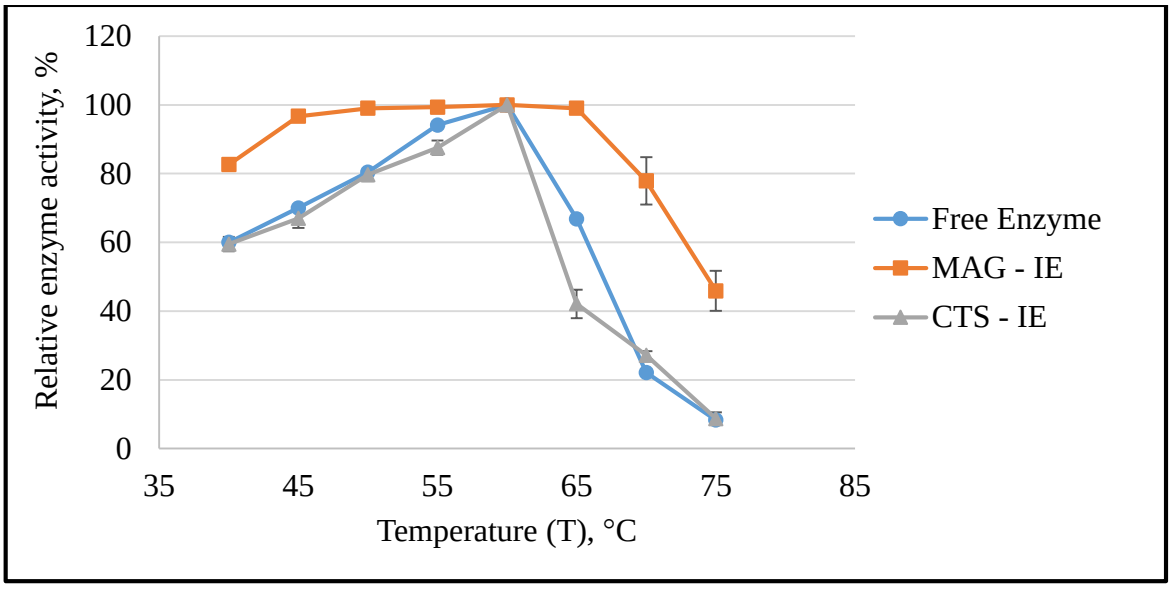

Figure 4.7 Effect of temperature on activity of free enzyme, immobilized enzyme onto magnetic particles carrier (MAG-IE), and immobilized enzyme onto chitosan particles carrier (CTS-IE)

4.3.3 Effect of pH on enzyme activity in lactulose substrate

In the case of MAG carrier, **Figure 4.8** illustrates that the optimal pH was not changed (pH 4.5) after immobilization, but the MAG–IE showed better stability in the wider pH range than that of FE. When pH was changing from pH 3.5 to pH 6.0, the activity of MAG–IE decreased only by about 95% compared to less than 70% of FE case. The line presented activity of MAG-IE was nearly vertical around the value of 100% relative activity when pH changing in this wide range. In other words, the MAG–IE show high activity in a range of pH (from pH 3.5 to pH 6.5) instead of a point (pH 4.5) of FE. This finding agrees with results of (Aslan &

Tanriseven, 2007) where Pectinex Ultra SP-L (*β*-galactosidase) was immobilized onto Eupergit C carrier, as well as of (Dwevedi & Kayastha, 2009) where *β*-galactosidase from Pea was immobilized onto the Sephadex carrier. Additionally (Cardelle-Cobas *et al.*, 2016) also reported that immobilized *β*-galactosidase (covalently immobilized on a support of glutaraldehyde–agarose) from *Aspergillus oryzae* exhibited wider pH stability range than free ones. This change revealed that MAG-IE might be applied at neutral pH 6.5 instead of acidic pH 3.5 – 4.0. The chitosan coating layer of this IE could be proposed more stable (less dissolved) in neutral pH environment, consequently, MAG-IE might be more safety. This change could be good for us due to improving IE stability is always aimed. The future experiment should be conducted for investigation.

The trend of CTS–IE activity line differs from that of MAG–IE. It goes up slowly from 40% (pH 3.0) to 100% of relative enzyme activity (pH 5.5). The optimal pH was shifted from 4.5 to 5.5 after immobilization of enzyme onto CTS carrier. This can be explained by covalent linkages between enzyme and glutaraldehyde, as well as charges of the surface and residual of the carrier (Nwagu *et al.*, 2013). The shift of the optimum pH was also observed by several publications (Du *et al.*, 2009; Nwagu *et al.*, 2013; Song *et al.*, 2010; Szczodrak, 2000; Tanriseven & Aslan, 2005; Tripathi *et al.*, 2007). After reaching 100%, the CTS–IE activity trend goes down slowly till reaching pH 6.0, then rapid felt to about 20% of enzyme activity relative when pH stepped forward 0.5 unit only (**Figure 4.8**).

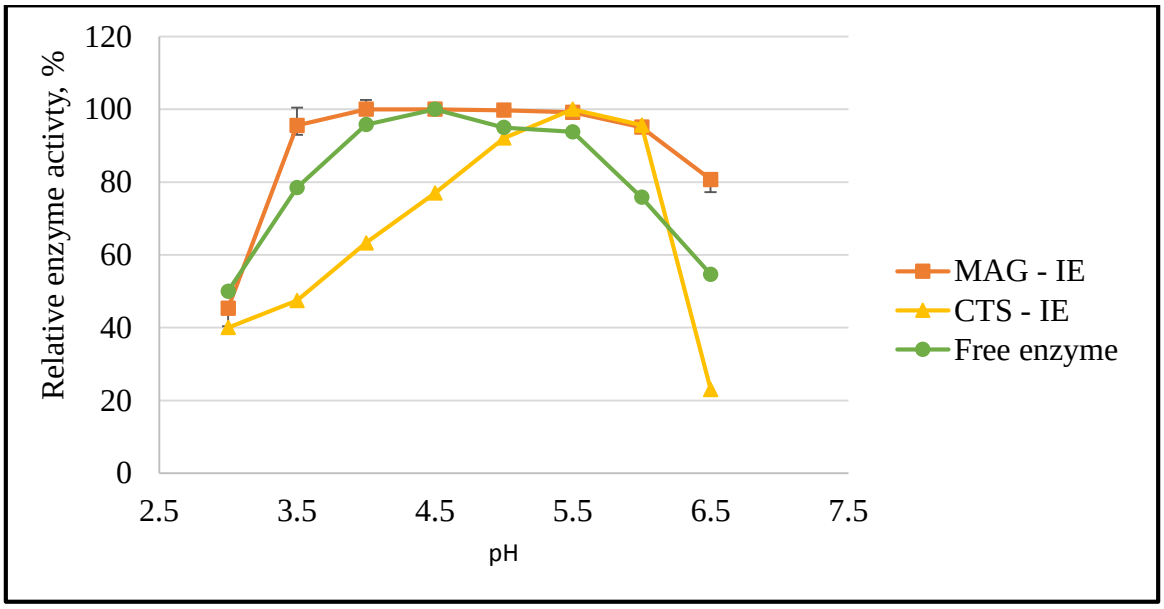

Figure 4.8 Effect of pH on activity of free enzyme, immobilized enzyme onto magnetic particles carrier, and immobilized enzyme onto chitosan particles carrier

4.3.4 Stability of enzyme

Stability is an important factor for the industrial processes because it affects the cost of the production (Klein *et al.*, 2013). As above mentioned, the operational stability of magnetic – immobilized enzyme was better than that of chitosan – immobilized enzyme and free enzyme because of a wider range of temperature and pH as well.

In addition to that, the enzyme stability was better demonstrated by keeping both FE and IE preparations at operational conditions (pH 4.5 and temperature 60 ºC) for a long term (so-called thermal stability of enzyme). The enzyme activity was checked day by day by protocol presented above. The higher stability of immobilized enzyme on CTS, MAG compared to the native enzyme is depicted in **Figure 4.9**. The half-life time of enzyme storing at optimum operational conditions (pH 4.5, 60 °C) increased from 2.5 days (in the case of FE) to over 6 and 14 days, in the case of MAG – IE and CTS-IE, respectively. The increased stability observed in the immobilized enzyme should be attributed to a reduction in the enzyme structure mobility, due to anchorage to the support promoted by the covalent bonds and subsequent translation of the rigidity at each anchorage point to the whole enzyme structure, thus shielding it from damaging effects of the environment (Taqieddin & Amiji, 2004). These finding are in agreement with several previous studies: Neri and co-workers (2008) reported that when *β*-Galactosidase from *Kluyveromyces lactis* was covalently immobilized onto an mPOS–PVA using glutaraldehyde as activating agent, the soluble enzyme lost all activity after 10 h of incubation at 35 º C whereas the immobilized one retained 47 % of its initial activity after 12 h. Palai and co-workers (2014) immobilized commercial grade *β*-galactosidase in Biolacta FN5 derived from *Bacillus circulans* by cross-linking through covalent bonding using glutaraldehyde on a microporous polyvinylidene fluoride membrane, and reported that native enzyme lost almost all of its activity after 21 days of storage at 20 ºC but the immobilized enzyme still retained ~ 50% of its activity even after 30 days.

Furthermore, **Figure 4.9** also illustrates that in term of storage, the stability of the immobilized enzyme was so high. Enzyme activity was not significantly different to initial one after 14 days of storage at 5 ºC.

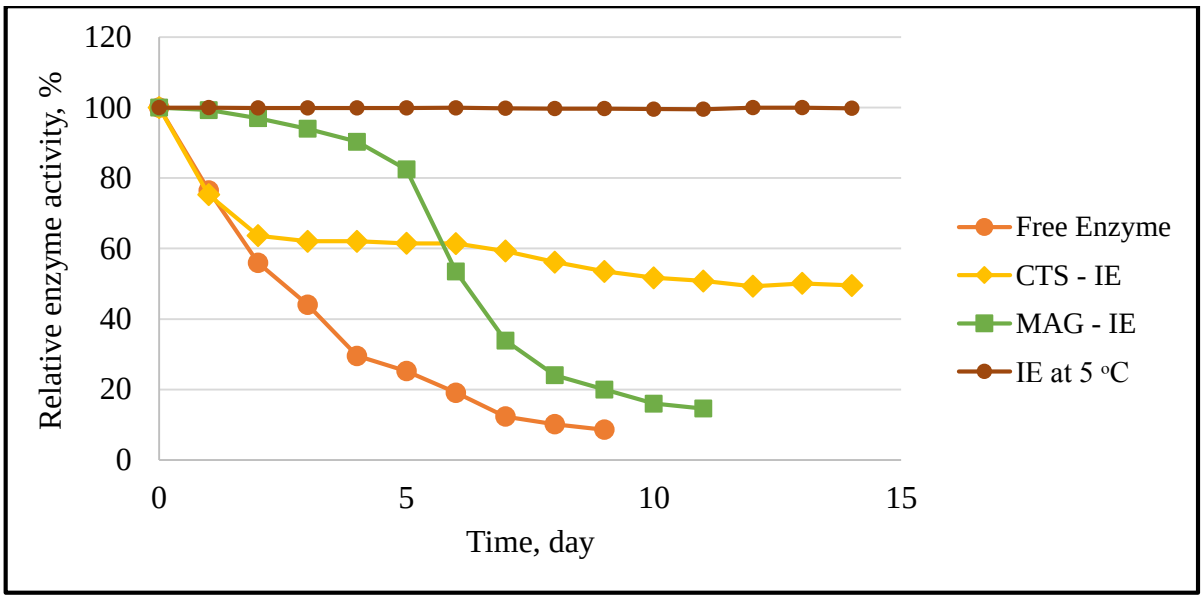

Figure 4.9 Stability of enzyme preparations at different conditions

4.3.5 Reuses of immobilized enzyme preparations

The reuse of IE preparations was investigated by assay of enzyme activity of in multi recycles. The **Figure 4.10** showed that the activity of MAG – IE remained at more than 85 % of origin after seven times of reuse at conditions of pH 4.5 and temperature 60 ºC for 4 h of each cycle. This performance of the magnetic-immobilized enzyme would be an additional advantage besides that from its easily separated from the reaction medium by a magnetic field. The **Figure 4.10** also illustrates the very good stability of CTS-IE when the activity was remained of about more than 50 % of its initial activity after thirty recycles at temperature 60 ºC and pH 5.0 for 12 h of each cycle. This is similar to result of immobilized *β*-galactosidase on the mPOS-PVA which was successively reused for twenty cycles at 25 ºC and IE retained approximately half of its initial activity (Neri *et al.*, 2008).

These results confirm that higher operational and thermal stability of IE preparations makes higher ability to be reused of them. This has been demonstrated to be one of the advantages of this system for immobilization of enzyme.

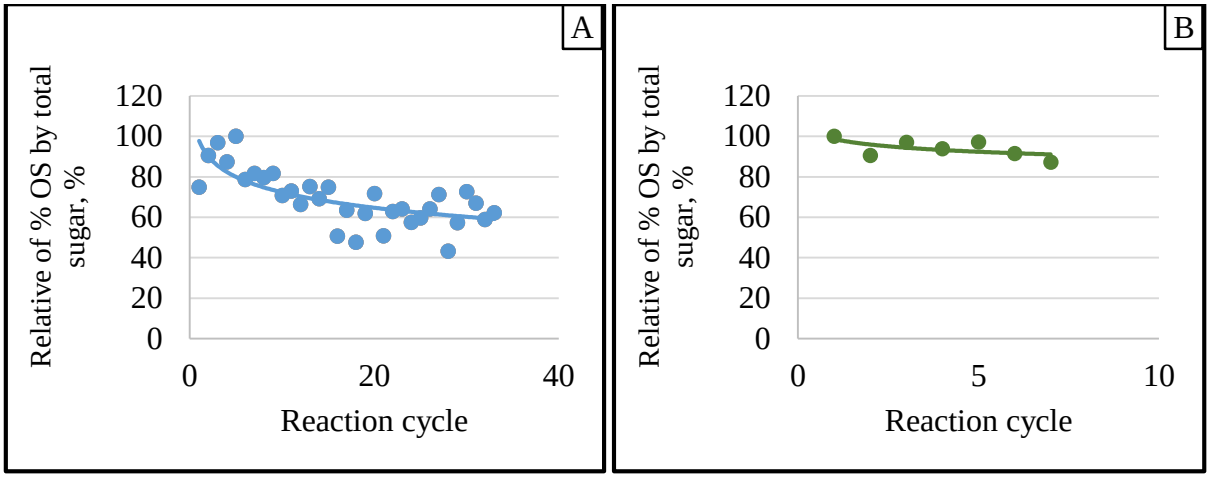

Figure 4.10 Reusability of immobilized enzyme preparations. A: Chitosan-immobilized enzyme, and B: Magnetic-immobilized enzyme

4.3.6 Effects of temperature and pH on activity of CTS-IE in lactose substrate

The activity of the free enzyme, as well as CTS-IE, were affected by pH and temperature (**Figure 4.11**). This figure illustrates, that after immobilization of enzyme, the optimal temperature was not changed. Both enzyme forms exhibited optimal temperature at 60 °C. This is similar to reported by Aslan and Tanriseven (2007) when they studied immobilization of Pectinex Ultra SP-L (from *A. aculeatus*) onto Eupergit C carrier, the optimal temperature of the enzyme after immobilization was not changed (55 – 60 ºC).

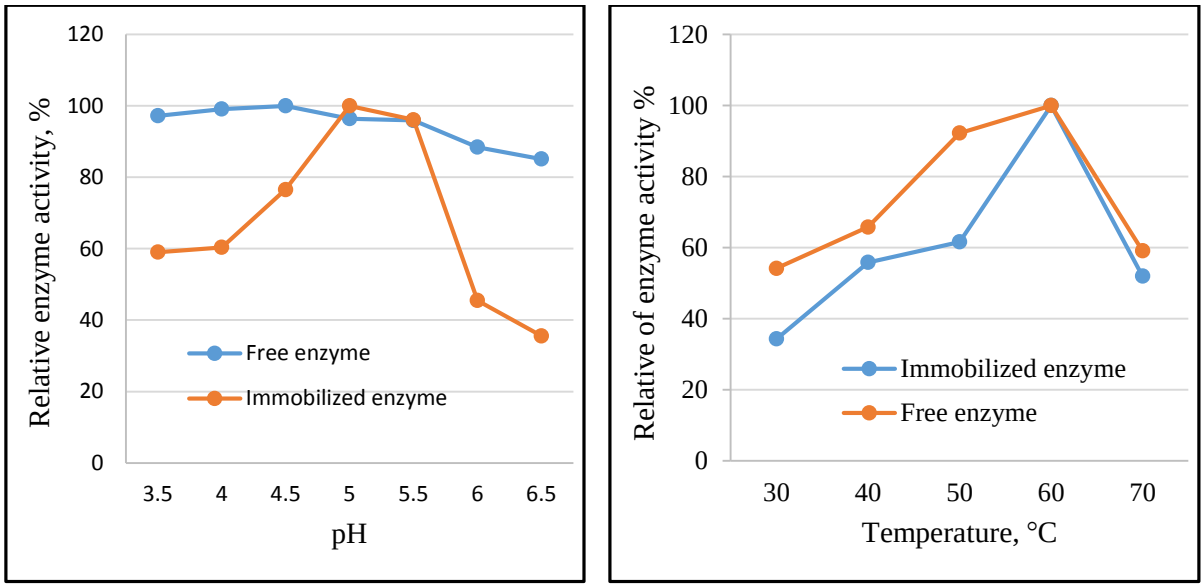

Figure 4.11 Effect of pH and temperature on activity of chitosan-immobilized and free enzyme preparations

The **Figure 4.11** also shows that the highest relative enzyme activity (largest yield of total oligosaccharides formation) was reached at pH 4.5. This finding is in an agreement with a study of GOS formation from lactose using Pectinex Ultra SP-L which produced by *A. aculeatus* (Cardelle-Cobas *et al.*, 2008b). After immobilization onto chitosan particles carrier, the optimal pH was increased from pH 4.5 to pH 5.0. This trend is similar to the result in the study of Pectinex Ultra SP-L (from *A. aculeatus*) immobilization onto Eupergit C to produce GOS (Aslan & Tanriseven, 2007), which showed that optimal pH was shifted from 4.0 to 5.0. Similarly, *β*-D-Galactosidase from *K. lactis* immobilized by covalent binding on glutaraldehyde-activated chitosan presented a shift in optimum pH from 6.5 to a broader range between 6.5 and 7.0 (Klein *et al.*, 2013)

4.3.7 Kinetic studies of immobilized enzymes

4.3.7.1 MAG-IE

The increase in initial lactulose concentration from 20 % (w/v) to 80 % (w/v) resulted the increase in yield of total GOS (DP3 and DP4) formation from 17 (w/w) % to 26.3 (w/w) % (**Figure 4.12**). This could be explained by the increase of competition to be an acceptor for the *β*-galactosyl groups of lactulose when initial concentration increases (Zhou & Chen, 2001).

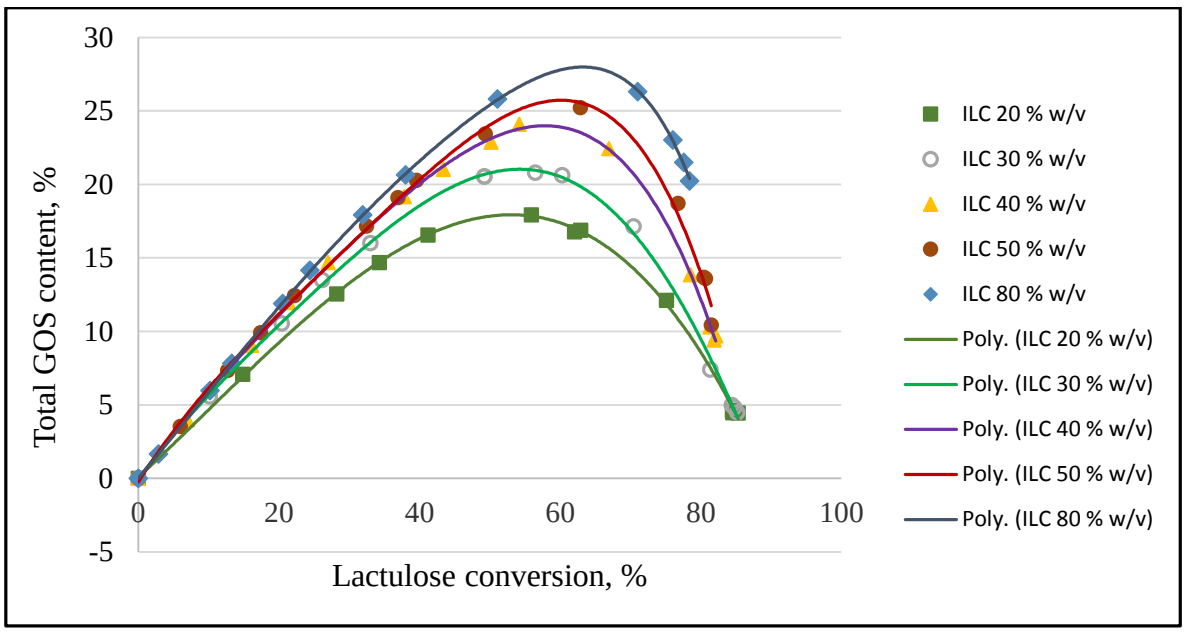

Figure 4.12 Effect of initial lactulose concentration and % converted lactulose substrate on the formation of lactulose-based oligosaccharides at pH 4.5 and 60 °C

The formation of oligo- and monosaccharides formation, which was a function of Lu conversion by catalysis of MAG-IE (at conditions of 80 % (w/v) ILC, pH 4.5 and temperature 60 °C) was presented in **Figure 4.13**. In the first stage – a range of the converted substrate was from 0 % to 50 % and time of reaction was respectively from 0 h to around 12 h, the total GOS and fructose almost linearly increased and reached about 25 % and above 15 %, respectively. It is interesting that the slope of GOS line is larger than that of fructose line. In that stage, from 0% to 30 % of converted lactulose (at around 7 h of reaction), the galactose concentration was not changed, and after that slightly increased. At the early stage of the reaction, trans-galactosylation predominates at the low substrate conversion, then total GOS content reached maximum, and the hydrolysis reaction predominates at the high lactulose conversion. This could be explained by a higher probability of attaching of the *β*-galactosyl group to water as an acceptor than to lactulose at low substrate concentration (Palai *et al.*, 2012). In the second stage – the converted lactulose ranged from 50 % to 70 % (time of reaction ranged from 12 h to 24 h), the line of total oligosaccharides was almost horizontal, while fructose and galactose lines have a larger slope than that of the first stage. In the last stage – the converted substrate was from above 70 % to 80% (time of bioconversion ranged from 36 h to 105 h), the total GOS formation decreased rapidly, and galactose and fructose formation increased faster than that of each (**Figure 4.13**). This means both hydrolysis and trans-galactosylation activities of enzyme well remained after immobilization.

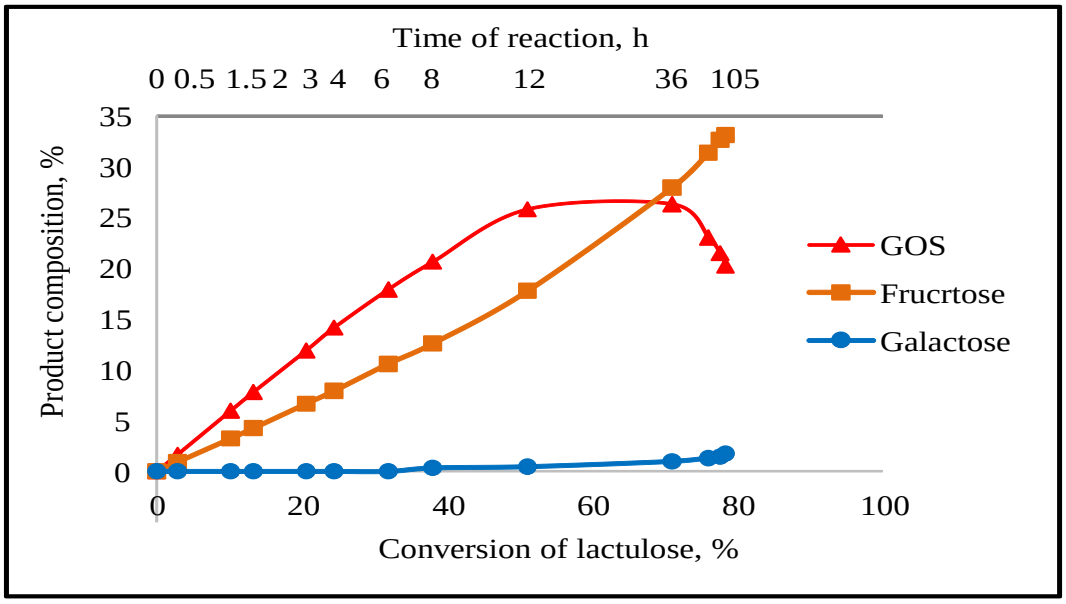

Figure 4.13 Product compositions as a function of % converted lactulose substrate by the reaction time, at initial lactulose concentration of 80 % w/v, pH 4.5 and 60 °C

Basically, the biochemical mechanism of Lu conversion to OS-Lu consisted two main processes including hydrolysis and trans-galactosylation, where Lu plays a role of a donor and acceptor of galactosylation, as well. There were several pathways of bio-reactions, with assuming negligible enzyme inhibition by fructose (Rodriguez-Fernandez *et al.*, 2011), were proposed (equation 4.3 to 4.10) for a simplistic understanding of the bio-reaction mechanism. In that, the components of mono-sugars (galactose and fructose) as well as of OS-Lu (DP3 and DP4) were either presented separately or incorporated into a single species (Palai *et al.*, 2012).

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} EGal+Fru\\
&EGal \underset{k2i}{\overset{k2}{\rightleftarrows}} Gal+E\\
&EGal+Lu \underset{k3i}{\overset{k3}{\rightleftarrows}} DP4+E\\
&EGal+DP3 \underset{k4i}{\overset{k4}{\rightleftarrows}} DP4+E
\end{aligned} \quad (4.3)$$

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} EGal+Fru\\
&EGal \underset{k2i}{\overset{k2}{\rightleftarrows}} Gal+E\\
&EGal+Lu \underset{k3i}{\overset{k3}{\rightleftarrows}} GOS+E\\
&EGal+GOS \underset{k4i}{\overset{k4}{\rightleftarrows}} GOS+E
\end{aligned} \quad (4.4)$$

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} EM+M\\
&EM \underset{k2i}{\overset{k2}{\rightleftarrows}} M+E\\
&EM+Lu \underset{k3i}{\overset{k3}{\rightleftarrows}} DP3+E\\
&EM+DP3 \underset{k4i}{\overset{k4}{\rightleftarrows}} DP4+E
\end{aligned} \quad (4.5)$$

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} EM+M\\
&EM \underset{k2i}{\overset{k2}{\rightleftarrows}} M+E\\
&EM+Lu \underset{k3i}{\overset{k3}{\rightleftarrows}} GOS+E\\
&EM+GOS \underset{k4i}{\overset{k4}{\rightleftarrows}} GOS+E
\end{aligned} \quad (4.6)$$

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} ELu\\
&ELu \xrightarrow{k2} EGal+Fru\\
&EGal \underset{k3i}{\overset{k3}{\rightleftarrows}} Gal+E\\
&EGal+Lu \underset{k4i}{\overset{k4}{\rightleftarrows}} DP3+E\\
&EGal+DP3 \underset{k5i}{\overset{k5}{\rightleftarrows}} DP4+E
\end{aligned} \quad (4.7)$$

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} ELu\\
&ELu \xrightarrow{k2} EGal+Fru\\
&EGal \underset{k3i}{\overset{k3}{\rightleftarrows}} Gal+E\\
&EGal+Lu \underset{k4i}{\overset{k4}{\rightleftarrows}} GOS+E\\
&EGal+GOS \underset{k5i}{\overset{k5}{\rightleftarrows}} GOS+E
\end{aligned} \quad (4.8)$$

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} ELu\\
&ELu \xrightarrow{k2} EM+M\\
&EM \underset{k3i}{\overset{k3}{\rightleftarrows}} M+E\\
&EM+Lu \underset{k4i}{\overset{k4}{\rightleftarrows}} DP3+E\\
&EM+DP3 \underset{k5i}{\overset{k5}{\rightleftarrows}} DP4+E
\end{aligned} \quad (4.9)$$

$$\begin{aligned}
&Lu+E \underset{k1i}{\overset{k1}{\rightleftarrows}} ELu\\
&ELu \xrightarrow{k2} EM+M\\
&EM \underset{k3i}{\overset{k3}{\rightleftarrows}} M+E\\
&EM+Lu \underset{k4i}{\overset{k4}{\rightleftarrows}} GOS+E\\
&EM+GOS \underset{k5i}{\overset{k5}{\rightleftarrows}} GOS+E
\end{aligned} \quad (4.10)$$

Where E, M, EM, EGal, Lu, and GOS denoted immobilized enzyme, monosaccharides (including fructose and galactose), a complex of IE and M, complex of IE and Gal, lactulose and total of GOS, respectively; *k* values are the rate constants for step reactions.

Consequently, eight candidates of mathematic models were given containing different equations of the nonlinear ordinary that define the rate of reactants change on time-course including the constant rates of reactions (such as equation 4.11 and 4.12), which were considered to the kinetic parameters of the model or the kinetic mechanism. Based on the observed data sets of experiments, the multiresponse nonlinear regression for the whole sets of experiments (at different ILC) was applied for estimation of the kinetic parameters (Bates & Watts, 1988), by using the LSODA solver of COPASI software (version 4.16) (Hoops *et al.*, 2006). Then, the best-fitted model, with the smallest root mean square error, was chosen from the eight ones.

By catalysis of MAG-IE, the biochemical model in equation 4.5 was the best choice to stimulate the trends and mechanism of bio-reactions by time-course. The rate each species formation involved in that four-step reactions were represented by a set of five mathematical ordinary equation 4.11.

In this case, the initial conditions including [EM], [M], [GOS] are of course equal zero. The best set of estimated values of the kinetic rate parameters was find out and presented in **Table 4.4.**

Table 4.4 Estimated kinetic constants of bioconversion

k_1 ($M^{-1}.h^{-1}$)	k_1i ($M^{-1}.h^{-1}$)	k_2 (h^{-1})	k_2i ($M^{-1}.h^{-1}$)	k_3 ($M^{-1}.h^{-1}$)	k_3i ($M^{-1}.h^{-1}$)	k_4 ($M^{-1}.h^{-1}$)	k_4i ($M^{-1}.h^{-1}$)
292.6	765030	52964.2	7.4	624822	248.8	$9.7x10^6$	947.4

With these kinetic parameters, the biochemical model 4.5, as well as mathematical model 4.11, was able to describe the bioconversion of Lu substrate for OS-Lu synthesis at any ILC among the considered range. **Figure 4.14** showed a good agreement between the predicted and experimental data.

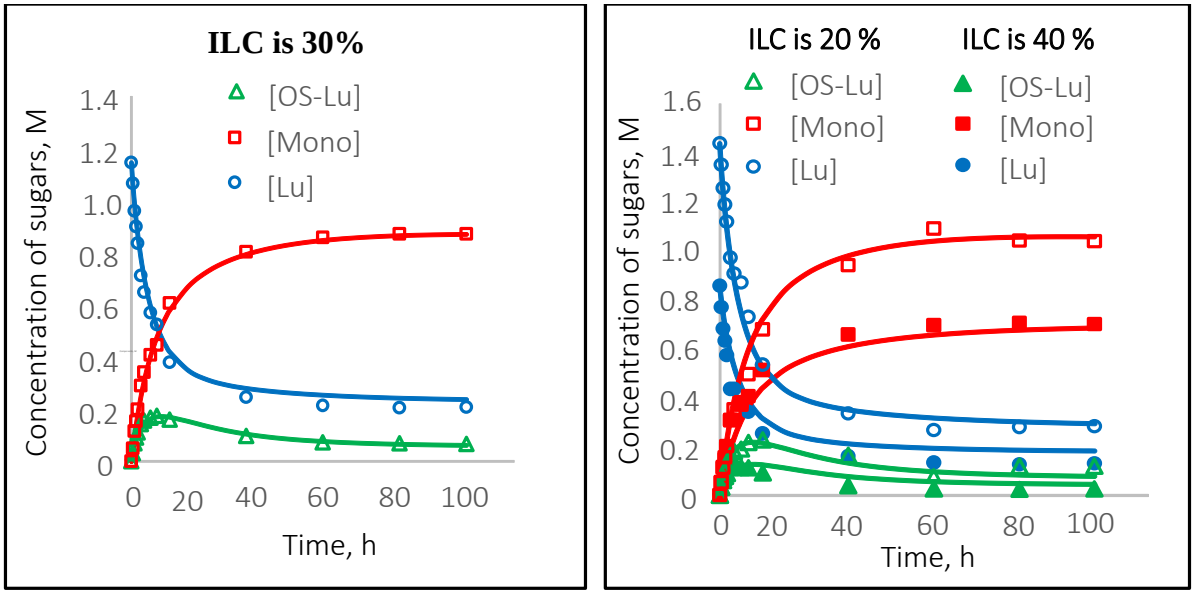

Figure 4.14 Comparison between predicted values (solid lines) versus experimental values (symbols) at three different initial lactulose concentrations (20, 30 and 40% w/v)

4.3.7.2 CTS-IE

Table 4.5 shows the kinetic parameters of hydrolytic activity of the immobilized and free enzyme which were calculated using the Hanes – Woolf plot method. The K_m values were 651.3 mM and 187.9 mM for FE and IE, respectively. The maximum velocity (V_{max}) of reaction increased from 121.9 µM Gal/min/µL to 227.3 µM Gal/min/g support, after immobilization. The similar result of the decrease in *K*m of *β*-galactosidase was found in some researchers such as Kishore and Kayastha (2012) when the enzyme was immobilized on alkylamine glass for lactose hydrolysis and Song and co-workers (Song *et al.*, 2012) when the enzyme was immobilized on nanotubes for lactulose synthesis. The decrease in K_m of enzyme after immobilization may be due to the affinity change of the enzyme to its substrate or conformational change in the immobilization of enzyme into carrier, which caused higher accessibility of the substrate to the IE (Du *et al.*, 2009) or increased the IE affinity to the substrate (Kishore & Kayastha, 2012). Otherwise, the Km of *β*-galactosidase was increased after immobilization (Lima *et al.*, 2013; Song *et al.*, 2012; Srivastava & Anand, 2014); but not always, *K*m of *β*-galactosidase was sometimes almost constant (Dwevedi & Kayastha, 2009).

Table 4.5 Kinetic parameters of free and chitosan-immobilized enzyme preparations towards hydrolytic activity

Free enzyme		Chitosan-immobilized enzyme	
*V*max (μM Gal/min/μL enzyme)	*K*m (mM)	*V*max (μM Gal/min/g support)	*K*m (mM)
122	651	227	187

A long-term (27 days) bioconversion of Lu for the production of OS-Lu using CTS-IE preparation was carried out at different ILC (0.101, 0.134, 0.172 and 0.249 mol.L^{-1}). By the similar way in case of kinetic study of MAG-IE, for the case of CTS-IS, the best biochemical model was chosen to be in equation 4.6. Consequently, the mathematical model was given in equation 4.12, including six non-linear ordinary differential equations for time-course simulation. Initial conditions herein consisting of: [E] = E_0, [Lu] = Lu_0, [M] = [EM] = [DP3] = [DP4] = 0 mol.L^{-1}

$$
\begin{aligned}
\frac{d[\mathrm{Lu}]}{d\mathrm{t}} &= k1i[\mathrm{EM}][\mathrm{M}]-k1[\mathrm{Lu}][\mathrm{E}]-k3[\mathrm{EM}][\mathrm{Lu}]+k3i[\mathrm{DP3}][\mathrm{IE}] \\
\frac{d[\mathrm{E}]}{d\mathrm{t}} &= k1i[\mathrm{EM}][\mathrm{M}]-k1[\mathrm{Lu}][\mathrm{E}]+k2[\mathrm{EM}]-k2i[\mathrm{M}][\mathrm{E}]+k3[\mathrm{EM}][\mathrm{Lu}]-k3i[\mathrm{DP3}][\mathrm{E}] \\
&\quad +k4[\mathrm{EM}][\mathrm{DP3}]-k4i[\mathrm{DP4}][\mathrm{E}] \\
\frac{d[\mathrm{M}]}{d\mathrm{t}} &= k1[\mathrm{E}][\mathrm{Lu}]-k1i[\mathrm{EM}][\mathrm{M}]+k2[\mathrm{EM}]-k2i[\mathrm{M}][\mathrm{E}] \\
\frac{d[\mathrm{EM}]}{d\mathrm{t}} &= k1[\mathrm{E}][\mathrm{Lu}]-k1i[\mathrm{EM}][\mathrm{Mo}]-k2[\mathrm{EM}]+k2i[\mathrm{E}][\mathrm{M}]+k3i[\mathrm{DP3}][\mathrm{E}]-k3[\mathrm{EM}][\mathrm{Lu}] \\
&\quad +k4i[\mathrm{DP4}][\mathrm{E}]-k4[\mathrm{EM}][\mathrm{DP3}] \\
\frac{d[\mathrm{DP3}]}{d\mathrm{t}} &= k3[\mathrm{EM}][\mathrm{Lu}]-k3i[\mathrm{DP3}][\mathrm{E}]-k4[\mathrm{EM}][\mathrm{DP3}]+k4i[\mathrm{DP4}][\mathrm{E}] \\
\frac{d[\mathrm{DP4}]}{d\mathrm{t}} &= k4[\mathrm{EM}][\mathrm{DP3}]-k4i[\mathrm{DP4}][\mathrm{E}]
\end{aligned}
\tag{4.12}
$$

The eight kinetic parameters of the model were estimated and presented in **Table 4.6**. With these estimated values, the simulated results show good agreement with the experimental values in several experimental sets (**Figure 4.15**). This fitted model simulates the process of Lu bioconversion forming OS-Lu by catalysis of CTS-IE by the time-course. By this, it helped us to understand the mechanism of GOS-Lu synthesis as well as to control the bioconversion.

Table 4.6 Estimated constant rates of multi-reactions with magnetic-immobilized enzyme catalysis

k_1 ($M^{-1}.h^{-1}$)	$k_1 i$ ($M^{-1}.h^{-1}$)	k_2 (h^{-1})	$k_2 i$ ($M^{-1}.h^{-1}$)	k_3 ($M^{-1}.h^{-1}$)	$k_3 i$ ($M^{-1}.h^{-1}$)	k_4 ($M^{-1}.h^{-1}$)	k_4i ($M^{-1}.h^{-1}$)
3943.48	0.015604	680.264	999999	427.348	97510.6	126524	$2.98x10^7$

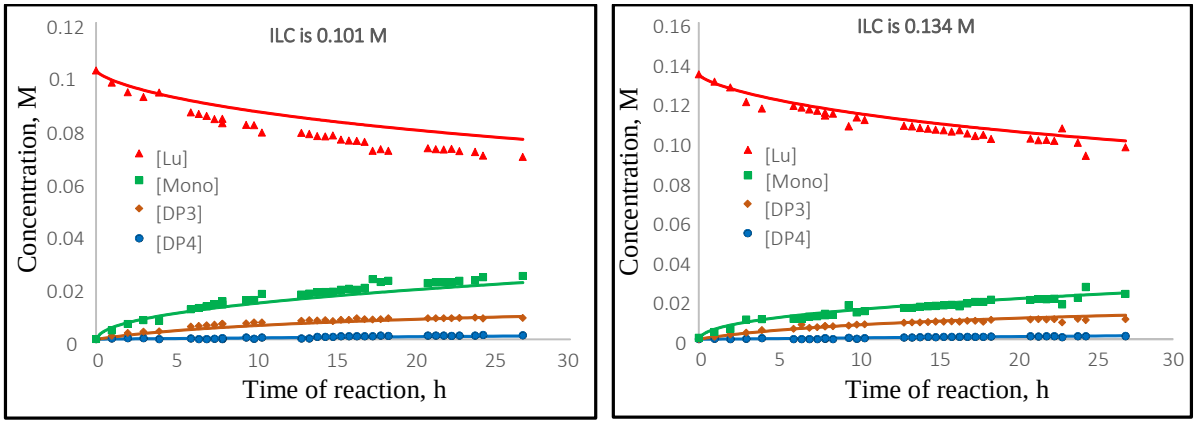

Figure 4.15 Plot of measured and fitted values of species in the mixture of reaction at different initial lactulose concentrations

4.4 Development of continuous bioconversion system

Figure 4.16 illustrates the resulting system of a bioreactor for continuous synthesis of galactooligosaccharides using the resulting chitosan-immobilized enzyme preparation. The packed-bed bioreactor with the dimensions of 40 cm height, 6 cm outer diameter and 1.9 cm inner diameter was applied. The pump was able to set the flow rate.

4.4.1 Effect of dilution rate on the yield of the continuous bioreactor

The experimental sets with three different flow rates 16, 27 and 60 $mL.h^{-1}$, consequently the dilution rate was 0.14, 0.24 and 0.53 h^{-1}, respectively, were build up and carried out. The bioconversion was done by the following parameters: initial lactose concentration of 20 % w/v, pH 4.5 and temperature of 60 °C. The results were plotted in **Figure 4.17**.

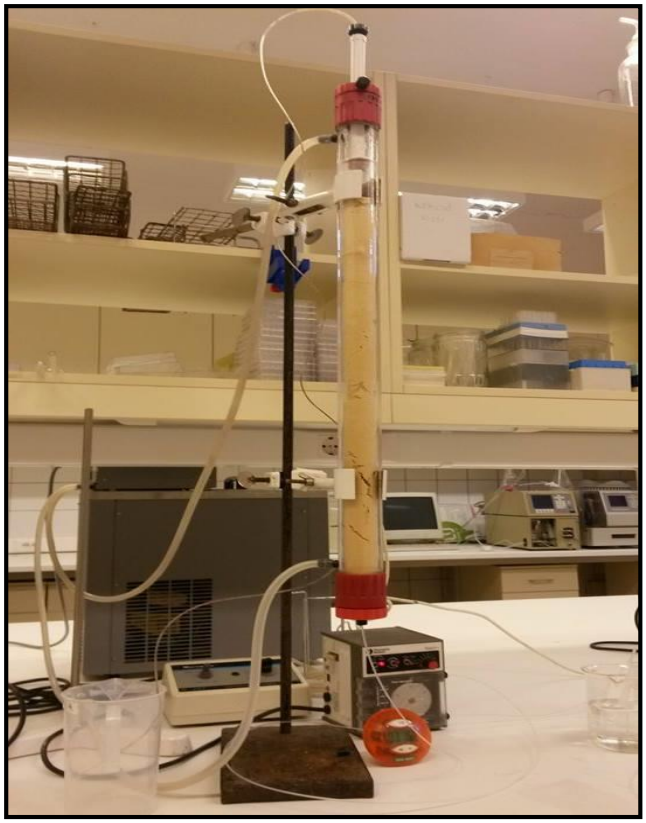

Figure 4.16 System of continuous bioconversion for oligosaccharides production

Figure 4.17 showed that the yield of bioconversion got maximum (10.5 %) at the dilution rate of 0.24 h^{-1}. When operating bioreactor at a lower dilution rate (0.14 h^{-1}), as well as at a higher dilution rate (0.53 h^{-1}), the efficiency of bioconversion dropped (the yields of GOS formation decreased). These results could be explained by the need of time for specific properties of multi-reaction of GOS synthesis with catalysis of *β*-galactosidase including hydrolysis, intra-molecular trans-galactosylation (making disaccharide of galactose-galactose), inter-molecular trans-galactosylation (making OS with DP ≥ 3). The retention time for both hydrolytic and trans-galactosylation reactions (in the case of higher dilution rate) may be not enough. It means the shorter retention time will result in a lower yield of GOS formation. Mammarella and Rubiolo (2006) have reported that higher lactose hydrolysis took place at a lower dilution rate when the authors studied to predict the packed-bed (with the immobilized micro lactase) reactor performance of lactose hydrolysis at different flow rates and with different initial lactose concentrations. This finding also reported later by Ansari and co-workers (2011) when *Aspergillus oryzae β*-galactosidase was immobilized on concanavalin A-layered calcium alginate-cellulose beads for continuous spiral bed hydrolysis of lactose.

These results indicated that the extent of lactose hydrolysis might be simply controlled in a way of adjusting the flow rate of the continuous operation mode. Similarly, Mayer and co-workers (2010), when *β*-glycosidase from *Pyrococcus furiosus* was covalently immobilized onto Eupergit C for the continuous production of oligosaccharide from lactose by a packed-bed reactor, stated that with increasing flow rate the apparent OS productivities increased, but the yield reached their maxima already at a lower flow-rate.

Otherwise, when the retention time of reaction was so long (in the case of lower flow rate), the synthesized GOS could also be hydrolysed by the enzyme, caused lower yield of GOS formation. This finding is in agreement with ones published by Klein and co-workers (2013) when they investigated the continuous synthesis of GOS with chitosan-immobilized *β*-D-galactosidase.

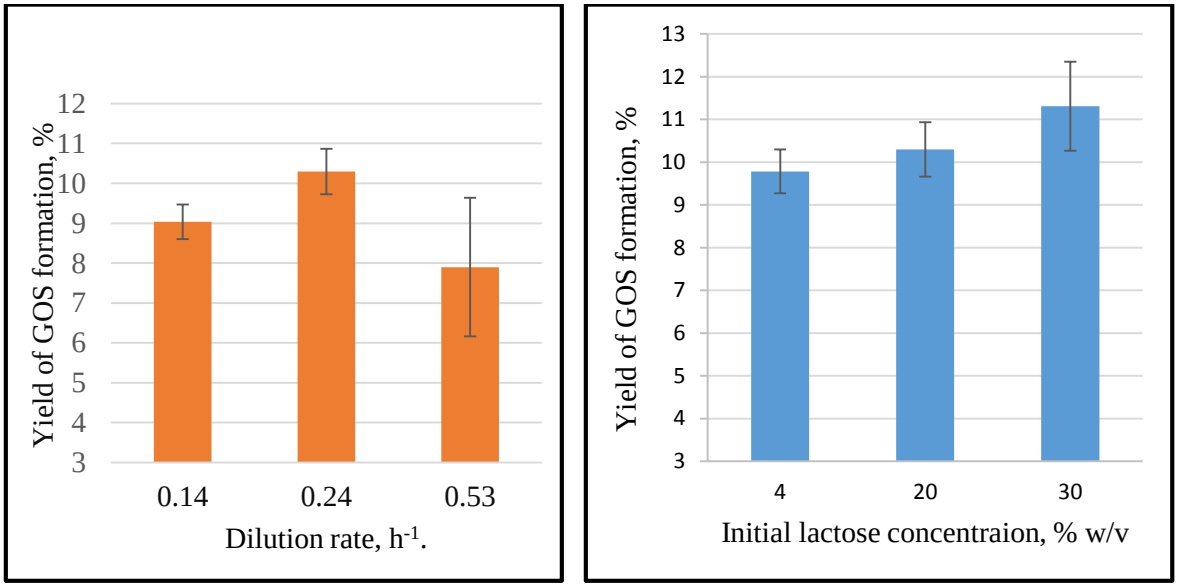

Figure 4.17 Effect of the dilution rate and initial lactose concentration on the yield of total lactose-based oligosaccharides formation by continuous bioconversion

4.4.2 Effect of initial lactose concentration on yield of continuous system

Three initial lactose concentrations solutions (4 % w/v, 20 % w/v and 30 % w/v), prepared in the 0.1 M McIlvaine buffer pH 4.5, were fed by the pump at optimal flow rate of 27 mL.h^{-1}, while the continuous system was kept at temperature of 60 °C. The results showed that the yield of GOS formation increased with the initial lactose concentration from 9.8 % to 11.3 % (**Figure 4.17**). The higher amount of GOS produced at higher initial lactose

concentration since *β*-galactosyl groups should have a higher probability of attaching to lactose (forming GOS) than water as an acceptor at increasing lactose concentration (Albayrak & Yang, 2002). In contrast, in a diluted lactose solution, water can be more competitive to be an acceptor for the binding with the enzyme-galactose complex forming GOS (Zhou & Chen, 2001). It reported an increase in GOS production from 2% to 32% as the initial lactose concentration increased from 14% to 40% (w/v). These are also finding agreement with the statement that more GOS can be produced with a higher lactose concentration feed (Albayrak & Yang, 2002), where *A. oryzae β*-galactosidase was immobilized on cotton cloth for GOS continuous production from lactose.

5 NOVEL SCIENTIFIC RESULTS

1) The Pectinex Ultra SP-L (*β*-Galactosidases) was successfully immobilized onto chitosan-coated magnetic nanoparticles, as well as chitosan microparticles by the covalent binding method. High yield of immobilization and recovery of activity were obtained. In the case of magnetic nanoparticles, protein content, pH and time were optimized using response surface methodology for enhancement of enzyme immobilization yield. The optimal factors were determined to be 0.45 $mg.mL^{-1}$, pH 4.9 and 4.9 h, respectively. The yields of enzyme immobilization in the cases of magnetic nanoparticles and chitosan microparticles were calculated to be 98.8% and 71%, respectively.

2) Effects of pH and temperature on catalytic and stability of the immobilized enzyme preparations were determined. In the case of chitosan microparticle carrier, the optimal temperature of the immobilized enzyme was 60 ºC and was similar to that of a free enzyme; while in the case of magnetic nanoparticles, the optimal temperature was in the range from 45 ºC to 65 ºC and much broader than that of the free one. Both immobilization methods did not affect significantly optimal pH values. The immobilized enzymes exhibited extremely stable in storage at 5 ºC and half-life times of magnetic-immobilized enzyme and chitosan-immobilized enzyme were 6 days and 14 days, respectively at pH 4.5 and 60 °C. The reuses of immobilized enzymes were investigated and more than 50 % of their activities were retained after 30 cycles of use.

3) Kinetic constants of bioconversion with magnetic-immobilized enzyme were estimated by the four-step pathway of trans-galactosylation using different initial lactulose concentrations (20, 30, 40 and 50 w/v %). In the case of the chitosan-immobilized enzyme, kinetic constants of both hydrolytic and galactosyltransferase activities were determined. The *K*m and *V*max of hydrolytic activity of the chitosan-immobilized enzyme in lactulose substrate were 187 mM and 227 µM Gal/min/g support, respectively. The kinetic constants of bioconversion with chitosan-immobilized enzyme were estimated by the four-step pathway of reaction using 0.101, 0.134, 0.172 and 0.249 $mol.L^{-1}$ of initial lactulose concentrations.

4) System using chitosan-immobilized enzyme preparation was set up for continuous bioconversion to produce the galactooligosaccharides. The working dimensions of the packed-bed column were 40 cm height, 6 cm outer diameter, and 1.9 cm inner diameter. Maximum yield of GOS on lactose substrate was obtained at 27 $mL.h^{-1}$ of flow rate and 30 % of initial substrate concentration.

6 SUMMARY

Two solid carriers, including chitosan-coated magnetic nanoparticles and chitosan microparticles, were prepared, then measured their particles size. After activated by glutaraldehyde, they were applied successfully for covalent immobilization of Pectinex Ultra SP-L contained *β*-galactosidase with high immobilization yield, respectively to be 98.8 % and 71 % in term of enzyme activity. The optimization of enzyme immobilization was conducted on three main factors (protein content, pH and time of immobilization) with the response of immobilization yield by response surface methodology combined with Akaike Information Criterion. The optimal factors were evaluated to be 0.45 mg.mL-1, 4.9 and 4.9 h, respectively.

Both two immobilized enzyme preparations were characterized for the synthesis of oligosaccharides from lactulose and lactose as well. Several interesting obtained results, which contributed to the advantages of covalent enzyme immobilization method, were presented below.

The optimal temperature was constant at 60 °C after enzyme immobilization onto both two carriers. However, it is so interesting that the magnetic-immobilized enzyme showed much better operational stability referred to a broader thermal range than that of the other immobilized enzyme, as well as compared to free enzyme. In the range of temperature from 45 to 65 ^{o}C (approximately 20 units), the activity of the immobilized enzyme was remained well at over 98 % of original, compared to a temperature range from 55 to a bit over 60 ^{o}C (approximately 5 units) of free enzyme case. It was considered to decide that we can totally get 100 % relative activity of the magnetic-immobilized enzyme when applying at around 50 ^{o}C instead of 65 ^{o}C. This is an amazing thing because helping us to save money such as for energy, as well as extend the lifetime of enzyme preparation. In addition, even at high temperature (70 ^{o}C), the activity of the immobilized enzyme remained at 80%, it was much higher than 20%, approximately, for the case of free enzyme and chitosan-immobilized enzyme as well. Otherwise, this advantage of the magnetic-immobilized enzyme could be useful to control the structure of oligosaccharides synthesis because of temperature effect on the direction of the enzymatic reaction.

The optimal pH of the magnetic-immobilized enzyme was kept at around 4.5, while shifted from 4.5 to 5.5 for the case of the chitosan-immobilized enzyme. Similarly to effect of temperature on enzyme activity, the magnetic-immobilized enzyme showed higher stability than that of the native enzyme referred to the broader range of operational pH. When pH was changing from 3.5 to 6.0, the relative activity of this immobilized enzyme was retained above

95 % of original, compared to below 70 % for free enzyme case. In other words, the magnetic-immobilized enzyme showed high activity in a wide range of pH (from pH 3.5 to pH 6.5) instead of a point (pH 4.5) for the case of the native enzyme. This changing after immobilization was considered to be good when using this immobilized enzyme at neutral pH 6.5 instead of acidic pH 3.5 – 4.0, because chitosan coating layer of this immobilized enzyme could be proposed more stable (less dissolved) in neutral pH environment. The future experiment should be conducted.

Besides the higher operational stability, immobilized enzyme showed greater thermal stability after immobilization. The half-life time of enzyme, when they were stored at optimum operational conditions (pH and temperature 60 ºC), increased from 2.5 days (in case of free enzyme) to over 6 days, appropriate 3 folds higher (in case of magnetic-immobilized enzyme), and 14 days, appropriate 6 folds greater (in case of chitosan-immobilized enzyme). These finding revealed, one more time, that immobilization of enzymes makes them greater stability.

Higher stability making better reusability. In the case of the magnetic-immobilized enzyme, a batch conduction at optimal pH and temperature (60 ºC) for 4 h was considered to one time of enzyme use. The relative of lactulose-based oligosaccharides formation by the total of carbohydrates was remained at around 85 % after eight times of reuses. In the case of the chitosan-immobilized enzyme, a cycle was finished after 12 h of applying immobilized enzyme at optimal conditions pH and temperature (60 ºC). After thirty-three recycles, the relative of lactulose-based oligosaccharide by the total of carbohydrates was remained at around 60%. These results of immobilized enzyme improved the advantages of enzyme immobilization.

The batches bioconversion of lactulose to lactulose-based oligosaccharides using immobilized enzyme preparations were successfully carried out at optimal condition pH and temperature for a long term. The resulted lactulose-based oligosaccharides were evaluated to be DP3 and DP4. The effect of initial lactulose concentration, as well as converted lactulose on the yield of oligosaccharide formation was investigated. The higher yield of oligosaccharides formation took place at the highest initial lactulose concentration. In the case of the magnetic-immobilized enzyme, at initial lactulose concentration of 80% w/v (the saturated concentration at 60 ºC), the oligosaccharide formation reached over 26% when lactulose substrate was converted at 60%. Also, the kinetic model was estimated using the method of multiresponse nonlinear regression for the whole set of experimental data. The kinetic models for two cases including magnetic-immobilized and chitosan-immobilized enzyme were not same and were both well validated.

A packed-bed continuous system using chitosan-immobilized enzyme preparation for the synthesis of lactose-based oligosaccharide was successfully developed. The effect of two main factors including flow rates and initial lactose concentrations on the yield of oligosaccharide formation were investigated. In term of effect of flow rates, the yield of continuous bioconversion of 20 % w/w lactulose in McIlvaine buffer got maximum (10.5 %) at the flow rate of 27 mL.h-1. Lower (16 mL.h-1) or higher (60 mL.h-1) flow rate made a decrease of oligosaccharides formation yield. For investigation of effect of initial lactose concentration on bioconversion yield, three different initial concentrations (4 % w/v, 20 % w/v and 30 % w/v) of lactose in the McIlvaine buffer solution were conducted at constant conditions of optimal pH and temperature, the flow rate at 27 $mL.h^{-1}$ with immobilized enzyme. The results showed that higher yield of lactose bioconversion forming oligosaccharides took place at larger initial lactose concentration.

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